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Full Length Research Paper

Cloning and expression analysis of a nitrate reductase gene related to somatic embryogenesis in *Gossypium hirsutum*

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Nitrate reductase is a key enzyme in the overall process of nitrate assimilation by plants. A nitrate reductase (NR) gene related to somatic embryogenesis was firstly cloned from *Gossypium hirsutum*. Sequence alignment shows that the GhNR is closest with Tilia platyphyllos NR gene. The expression of GhNR was investigated using semi-quantitative RT-PCR in nonembryogenic calli, embryogenic calli and somatic embryoids which showed that the expression level of GhNR was higher in embryogenic calli and somatic embryoids than in nonembryogenic calli. Besides, the expression of GhNR gene was verified by transformation in *E. coli* BL21 (DE3) strain with the recombinant expression vector pET-32a. NR activity assay showed that the crude GhNR protein had obvious activity to KNO_3 substrate. The results implied that NR has a critical function in the assimilation of nitrogen in cotton somatic embryo germination.

Key words: Cotton, somatic embryogenesis, nitrate reductase, gene expression.

INTRODUCTION

Nitrate is the major compound for nitrogen acquisition of plant in nature. In most organisms, nitrate assimilation is carried out by the sequential reduction of nitrate to nitrite and then to ammonium. Nitrate reductase (NR) which converts nitrate to nitrite catalyzes the first and rate-limiting step in this pathway (Campbell, 2001). Also, nitrate is commonly used as the source of nitrogen in plant tissue culture (Hellens et al., 2000). So the study on NR role in the course of plant somatic embryogenesis and regeneration would be of importance for plant regeneration. To date, some NR open reading frame (ORF) has been cloned from several higher plant resources (Guillaume et al., 2004; Hua et al., 2007; Hideyuki et al., 2007; Sun et al., 2008; Li et al., 2011). But none NR gene was obtained related to cotton somatic embryogenesis and regeneration. According to previous studies, NR is homomultimeric protein, each subunit containing three cofactors: flavin adenine dinucleotide (FAD), heme (heme) and a molybdenum-containing factor (Moco), each cofactor is a redox center. In higher plants, NR is homodimer, each subunit's molecular weight is 100 ~ 200 kD and each subunit contains a complete cofactors (Solomonson et al., 1972).

Somatic embryogenesis involves a number of molecular events encompassing not only differential gene expression, but various signal transduction pathways for activating/repressing numerous gene sets, many of which have been identified and characterized (Archana and Paramjit, 2002). These include genes encoding auxin-induced genes—SAUR (small auxin up-regulated) genes, pJCW1 and pJCW2 (Hagen et al., 1984; Padmanabhan

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Abbreviations: RT-PCR, reverse transcriptase-PCR; **NR,** nitrate reductase; **GhNR,** nitrate reductase gene cloned from *Gossypium hirsutum*; **ORF,** open reading frame; **IPTG,** isopropylthiogalactoside; **SDS-PAGE,** sodium dodesyl sulfate-polyacrylamide gel electrophoresis.

Table 1. Primers used for cloning full-length cDNA and genomic sequence of GhNR.

Primer	Sequence	Description
X1f	5'-ATGGCGGCTTACGTCGATAATC-3'	ORF forward primer, outer
X1r	5'-GGATCTCTTCATGACAAGGCGT-3'	ORF reverse primer, outer
X2f	5'-GTGCTCAAACGATGTGGGATCT-3'	ORF forward primer, nested
X2r	5'-CCTTCTCTCAAACTACAACACCCTTG-3'	ORF reverse primer, nested
3'GSP1	5'-TCTGGTCGCTTGAGGTCGAAGTGCT -3'	3'-RACE forward primer, outer
3'GSP2	5'-ATAGCGGTTCGAGCCTGGGATGAGA-3'	3'-RACE forward primer, nested
5'GSP1	5'-TGGTAAGAGGGCGGCTACTTGGGAC-3'	5'-RACE forward primer, outer
5'GSP2	5'-GGTACCAGGCCCCAATTTTCAAGGG-3'	5'-RACE forward primer, nested

et al., 2001), somatic embryogenesis receptor kinase (SERK) gene (Torii et al., 1996; Schmidt et al., 1997; Shah et al., 2001) and 244 EMB genes were also identified during somatic embryogenesis in *Arabidopsis* system (Iris et al., 2004). Nevertheless, the previous studies on cotton somatic embryogenesis and plant regeneration mainly focused on exoteric effect factors, genetic backgrounds and cytology, little of the molecular mechanism of somatic embryogenesis was known so far (Archana and Paramjit, 2002; Zeng et al., 2006). To this day, reports of high-frequency regeneration of cotton through somatic embryogenesis were still limited due to a genotype-dependent response (Sakhanokho et al., 2000; Kumria et al., 2003; Mishra et al., 2003; Ganesan and Jayabalan, 2004; Wang et al., 2006; Xie et al., 2007). Consequently, the identification and isolation of the vital genes of somatic embryogenesis are of great significance for improving regeneration ability of a wider range of cultivars.

In this study, we firstly cloned a NR gene, GhNR from *Gossypium hirsutum* (upland cotton) combining in silico cloning with RT-PCR according to the reported NR of *Nicotiana benthamiana* (PIR accession no. NM_103364).

MATERIALS AND METHODS

Total RNA extraction and purification

Total RNA was extracted from embryogenic calli of cv. Zhong6331 with RNAPlant Kit (Tiangen) according to the manufacture's instruction. The isolated RNA was treated by RNase-free DNaseI according to manufacture's protocol to remove any potential gDNA contamination. The purity and concentration of the total RNA were determined using gel electrophoresis and Beckman spectrophotometer.

Splicing and ORF cloning of GhNR sequence

According to the sequence of *Arabidopsis thaliana* NR, sequence splicing was processed using the EST data with high homology through NCBI blast and the ORF was analyzed on line (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Two primer pairs, X1f, X1r and X2f, X2r were designed according to ORF sequence (Table 1). Each RT-PCR reaction was carried out in 20 µl solution containing 1 µL of cDNA, 1.6 µL of 2.5 mM dNTP, 2 µL of 10×PCR buffer, 1 µL of 10 µM each primer, 0.2 µL Taq DNA polymerase and 13.2 µL of ddH_2O. The mixture was covered with mineral oil, denatured at 94°C (5 min) and subjected to 30 cycles of amplification (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) with a final elongation cycle of 10 min at 72°C. The products from RT-PCR of cDNA were cloned into the pGEM-T (Tiangen, China), and six clones were sequenced by Sangon, China.

The sequenced result of RT-PCR products and phylogenetic tree were analyzed with DNAStar. Homological comparison was made using ClustalW software.

3'- and 5'-RACE (rapid amplification of cDNA ends) of GhNR

According to the sequence of the cloned internal contig, specific primer 3'GSP1 and the nested specific primer 3'GSP2 (Table 1) were designed and synthesized for 3'-RACE according to the kit manual (SMART™ RACE cDNA synthesis Kit, Clontech). The amplified fragment was cloned into the pGEM-T (Tiangen) and then sequenced. For the 5'-end of cDNA cloning, GeneRacerTM RACE Ready cDNA Kit (Invitrogen) was used. Primer 5'GSP1 and the nested specific primer 5'GSP2 (Table 1) were designed based on the sequenced contig. The products were cloned into the pGEM-T (Tiangen) and then sequenced.

Bioinformatics analysis

The DNA sequences were analyzed with DNAstar software and the BLAST program (hppt://ncbi.nlm.nih.gov). Open reading frames (ORFs) were identified with an ORF finder (NCBI) and the protein sequences were deduced. Homology comparisons were conducted through BLASTN and BLASTP (http://www.ncbi.nlm.nih.gov/Blast.cgi). Preliminary properties of the encoded protein were predicted by NCBI Blast. Sub-cellular localization was predicted by Psort (Horton and Nakai, 1997).

Semi-quantitative RT-PCR analysis

Total RNA samples (3 µg per reaction) from nonembryogenic calli, embryogenic calli and somatic embryoids were reversely transcribed into cDNA using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, China). Primer pairs NRf: 5'-GGCGGCGGATCCAAGTATG GAAC-3'; NRr: 5'-GGCAGTGTCGGGTGCT CGAAAACA-3' were used for GhNR mRNA expression level assay by semi-quantitative RT-PCR. The cotton polyubiquitin gene (Ubiquitinf: 5'-CTGAATCTTCGCTTTCACGTTAT C-3'; Ubiquitinr: 5'-GGGATGCAAAT CTTCGTGAAAAC-3') was used as internal

control to normalize the differences of template concentrations. Thermal cycling was performed under the following conditions: one cycle at 95°C (5 min), then 30 cycles at 95°C (30 s), 58°C (30 s), and 72°C (1 min) followed by 72°C (10 min) for the final extension.

PCR products were detected on 1.0% agarose gel and viewed under the BIO-RAD Gel Doc imaging apparatus.

Sub-cellular localization of GhNR proteins

The coding sequence of GhNR was first amplified by PCR and subsequently cloned into destination vector pCamE. The resulting construct pCamE-GhNR-GFP which includes the CaMV 35S promoter was biolistically co-transformed into onion epidermis cells together with a control GFP vector which served as a maker of cytomembrane and nuclear proteins (Zhang et al., 2011). Transformation was achieved with the PDS 1000/He device (Bio-Rad) utilizing the following parameters: target distance 6 cm and rupture disk pressure 1,100 psi. Localization of GFP was monitored with a fluorescence microscope.

GhNR prokaryotic expression

The cDNA fragment of GhNR full ORF was amplified from pGEM T-GhNR cloning plasmid with primer pairs of 5'-GGTACCATGGCGGCTTACGTCGATAAT-3' and 5'-CGGGTCGACTTAAAATACCAACAAAGAATC-3', and the target product digested with KpnI and SalI was ligated into the expression vector pET-32a with the same restriction enzyme digestion to yield pET-GhNR. The recombinant plasmid pET-GhNR and pET-32a were expressed in the *E. coli* BL21 (DE3). Protein expression was induced by using IPTG with the final concentration of 1.0 mM for 3, 6, 9 and 12 h according to the pET system manual and the protein extracts were analyzed using SDS-PAGE electrophoresis.

GhNR enzyme activity assay

The GhNR activity was assayed based on the release amount of NO_2 in the hydrolysis course of the potassium nitrate (KNO_3) as substrate and the produces were detected at A520 using spectrophotometer. 1 ml of culture bacteria was collected by centrifugation containing GhNR-fusion protein and the protein of pET-32A without GhNR gene, respectively. The collected bacteria were centrifuged at 7,000 rpm for 2 min. The depositions were resuspended by phosphate buffer and then centrifuged at 7,000 rpm for 2 min. The depositions were put in 500 µL phosphate buffer and broken in ice. Then the mixture was centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was transferred into another tube and centrifuged at 12,000 rpm for 15 min at 4°C. The obtained supernatant was namely enzyme solution and used for the GhNR activity assay. Then 100 µL of enzyme solution, 800 µL of reaction solution (5 ml of 0.2M phosphate buffer and 5 ml of 0.2M KNO_3) and 200 µL of 0.2 mg/ml NADH react for 1 h at 28°C. Afterwards, 100 µL of 30% acetocaustin was added to terminate reaction. Take 1 ml of the solution in the test tube by adding 2 ml sulfanilic acid, 2 ml of α-Naphthylamine, shaking and bathing at 30°C water for 20 min. At last, the absorbance was read once every minute at 520 nm.

RESULTS

Isolation and characterization of GbNR gene

A 2,995 bp cDNA sequence of GhNR was obtained from *G. hirsutum*. The nucleotide sequence and deduced amino-acid sequence of this gene were shown in Figure 1. A 2,670 bp ORF encoded a protein of 899 amino acids with molecular mass of 100.28 kDa including 24.41% polar amino acids, 12.04% acidic amino acids, 11.25% basic amino acids and 31.83% hydrophobic amino aids; the pI was 6.82. A 93 and 232 bp fragment of 5' end and 3' end were obtained by using gene-specific primers, respectively. And a putative polyadenylation signal AATAAA was present at the 2,894th base pairs.

Sequence analysis of the deduced GbRL1 protein

The amino acid sequence, Motif and protein domain of GhNR was analyzed with NCBI cds Blast (http://www.ncbi.nlm.nih.gov/structure/), Motif search (http://au.expasy.org/tools/scanprosite/) and protein domain analysis (http://www.ebi.ac.uk/InterProScan/), respectively. The results show that the deduced GhNR amino acid sequences have a complete NR structure which includes Eukaryotes oxidoreductase signal area (144 to 179) and three cofactors. The 515 ~ 590 amino acid sequence is highly homologous with the CGTb5 family's protein and the CGTb5 protein binding with heme, and GhNR simultaneously with the Oxidoreductase FAD-binding region (633 to 745) and the Mo-co oxidoreductase dimerisation domain (332 to 467) (Figure 1). In addition, the CGPPP-M (861 to 866) motif was found at C 'terminal FAD domains. It indicates that GhNR is NADH-type NR. The GhNR polypeptide was 86, 80, 78, 78 and 77% sequence homology to those of *Tilia platyphyllos*, *Vitis vinifera*, *Nicotiana benthamiana*, *Ricinus communis* and *Populus trichocarpa*, respectively (Figure 2).

For phylogenetic analysis, the GhNR sequence and *T. platyphyllos* NR were classified into the same group (Figure 3).

Expression analysis of GhNR in different tissues

To determine the expression pattern of GhNR, semi-quantitative RT-PCR analysis was performed. The results showed that the expression level of GhNR was higher in embryogenic calli and somatic embryoids with the transcript abundance was more than then-fold compared to nonembryogenic calli, whereas, there was no remarkable difference between embryogenic calli and somatic embryoids for the expression level of GhNR (Figure 4). The results suggested that GhNR may have an important role in the assimilation of nitrogen in cotton somatic embryo germination.

Sub-cellular localization of GhNR protein

To study the sub-cellular localization of GhNR protein, an

1 GGCATCGCAGTGATTGTATACGACTCACTATAGGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGC

76 GGCCGCGGGAATTCGATTATGGCGGCTTACGTCGATAATCGACAATTCAGGGGGCTTGAACCGACTTTGAACGGC

1 M A A Y V D N R Q F R G L E P T L N G

151 GTGTACACCCCCTTGAAGCCCGGTCCAACCCCAACCCACCGTCATGTTCACCAGGTTTTCCTCGACCATGATTAT

20 V Y T P L K P G P T P T H R H V H Q V F L D H D Y

226 GAATCCTCCAGCGATGATGATGATAACGAGATCGAATATTACAAAGCCATGGTCGTCAAATCCAACAACGAGGTT

45 E S S S D D D D N E I E Y Y K A M V V K S N N E V

301 GAATCGTCAATCTTGGACCCTCGAGACGAAGCAACCGCTGATAACTGGATCGAGCGGAACCCTTCCCTAGTCCGT

70 E S S I L D P R D E A T A D N W I E R N P S L V R

376 CTTACAGGGAAACACCCTTTCAACTCTGAACCCCCTTTGAACCGCCTCATGTACCACGGTTTCATCACCCCGGTT

95 L T G K H P F N S E P P L N R L M Y H G F I T P V

451 CCTCTCCACTACGTTCGTAACCACGGTGCGGTCCCCAAAGCTTCGTGGGATGACTGGACCGTTGAAATAACCGGT

120 P L H Y V R N H G A V P K A S W D D W T V E I T G

526

TTAGTTAAAAGGCCAATGAAGTTGACGATGGATCAGCTCGTGAAAGAGTTTCAAAGCCGTAAGTTCCCGGTTACC

145 L V K R P M K L T M D Q L V K E F Q S R K F P V T

601 TTGGTTTGCGCCGGGAACCGTCGTAAAGAACAAAACATGATCAAACCAACGGTGGGTTTCAACTGGGGAGCGTCG

170 L V C A G N R R K E Q N M I K P T V G F N W G A S

676 GGGATTTCCACGTCGATATGGAGGGGTGTACCATTGCGTGACTTGCTCAAACGATGTGGGATCTACAGCAAGAAA

195 G I S T S I W R G V P L R D L L K R C G I Y S K K

751 CATGGCGCCCTCAATGTGTGTTTCGAAGGAGCTGAGCATTTGCCCGGCGGCGGCGGATCCAAGTATGGAACTAGC

220 H G A L N V C F E G A E H L P G G G G S K Y G T S

826 ATCAAGAAGGAATTCGCCATGGACCCAGCTCGGGATATTATATTAGCTTACATGCAAAACGGGGAACTTTTAACC

245 I K K E F A M D P A R D I I L A Y M Q N G E L L T

901 CCGGACCATGGCTTCCCTGTTCGAATCATCATACCAGGGTTCATCGGCGGGAGAATGGTGAAATGGTTGAAACGA

270 P D H G F P V R I I I P G F I G G R M V K W L K R

976 ATCATTGTTACAACGAAAGAATCTGATAATTTTTATCACTATAGAGATAATAAGGTCCTTCCTTCTCATGTTGAT

295 I I V T T K E S D N F Y H Y R D N K V L P S H V D

1051 TCAGAGCTAGCCAACGCTGAAGCTTGGTGGTATAAGCCGGAATACGTCATCAATGAGCTAAACATCAGCTCGGTG

320 S E L A N A E A W W Y K P E Y V I N E L N I S S V

1126 ATAACGACGCCTTGTCATGAAGAGATCCTACCAATCAACTCGTGGACAACTCAGAGGCCGTACACTCTGAGGGGC

345 I T T P C H E E I L P I N S W T T Q R P Y T L R G

1201 TATTCATATTCGGGCGGTGGGAAAAAGGTAACACGTGTAGAAGTAACAATGGACGGTGGTGAAACTTGGCAAGTG

370 Y S Y S G G G K K V T R V E V T M D G G E T W Q V

1276 TGCACGTTGGACCACCCCGAAAAGCCTAATAAATACGGTAAATTCTGGTGCTGGTGCTTCTGGTCGCTTGAGGTC

395 C T L D H P E K P N K Y G K F W C W C F W S L E V

1351 GAAGTGCTTGACCTTTTGGGTGCCAAAGAGATAGCGGTTCGAGCCTGGGATGAGACCAATAACACCCAGCCCGAA

420 E V L D L L G A K E I A V R A W D E T N N T Q P E

1426 AAGCTCATTTGGAATCTTATGGGAATGATGAACAACCGCTGGTTCCGTGTAAAAACAAACGTGTGCAGGCCACAC

445 K L I W N L M G M M N N R W F R V K T N V C R P H

1501 AAAGGTGAAATCGGGATTGTTTTCGAGCACCCGACACTGCCGGGCAACCAATCCGGTGGATGGATGGCTAAGGAA

470 K G E I G I V F E H P T L P G N Q S G G W M A K E

1576 CGACACCCCGAAAACCCAACCATAAAGAAGAGTGTCTCTTCTCCTTTCATGAACACTGCCTCCAAAACATTTTCA

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495  R H P E N P T I K K S V S S P F M N T A S K T F S
1651 ATGTCCGAGGTCAATAAACACAACTCTGCTGATTCGGCTTGGATCGTTGTCCATGGTAACATCTATGATTGCACT
520  M S E V N K H N S A D S A W I V V H G N I Y D C T
1726 CGGTTCCTCAAGGATCACCCTGGTGGCACCGACAGTATTCTTATCAACGCCGGAACCGACTGCACCGAGGAGTTC
545  R F L K D H P G G T D S I L I N A G T D C T E E F
1801 GACGCCATTCACTCCGACAAAGCCAAGAAAATGCTAGAAGATTATCGAATCGGTGAGTTGTTAACGTCGGGATAT
570  D A I H S D K A K K M L E D Y R I G E L L T S G Y
1876 GTTTCCGACTCGGCGGGGTCCTCGCCTAATACTTCCGTGCATGGAGCGTCCAACATGAGCTTTTTAGCTCCTATT
595  V S D S A G S S P N T S V H G A S N M S F L A P I
1951 AAAGAAGTTGCGCCCACAAGACCTGTGGCGCTTATTCCACGTGAGAAAATCCCATGCAAACTCGTCGAAAAAACA
620  K E V A P T R P V A L I P R E K I P C K L V E K T
2026 TCCATCTCCCATGACGTTCGTCGGTTCCGATTCGCGTTGCCGTCGGAAGATCAAGTGCTCGGGTTACCTGTAGGG
645  S I S H D V R R F R F A L P S E D Q V L G L P V G
2101 AAGCACATATTTTTGTGTGCCACCATTGGTGATAAGCTTTGCATGCGAGCCTACACCCCAACTAGTAGCATTGAT
670  K H I F L C A T I G D K L C M R A Y T P T S S I D
2176 GAAGTTGGCCACTTTGATCTAGTCGTCAAGATTTACTTCAAAGGGGTGCACCCGAAATTCCCTAACGGTGGCCTC
695  E V G H F D L V V K I Y F K G V H P K F P N G G L
2251 ATGTCACAATATTTGGACTCTCTCCCCCTCGGTTCGTCACTAGACGTGAAGGGTCCTTTGGGTCACATCGAATAC
720  M S Q Y L D S L P L G S S L D V K G P L G H I E Y
2326 ACCGGTCAGGGCAACTTTTTGGTTCACGGCAAACCCAAGTTTGCCAAGAAACTAGCAATGTTAGCCGGTGGGACG
745  T G Q G N F L V H G K P K F A K K L A M L A G G T
2401 GGGATCACCCCCATTTATCAACTCATCCAAGCCATTTTAAAAGACCCCAAGGACGAGACCGAGATGTATGTCGTG
770  G I T P I Y Q L I Q A I L K D P K D E T E M Y V V
2476 TACGCAAACCGAACCGAGGACGACATTTTGCTGAAAGAGGAGCTCGACGACTGGGCTAAGAAGCATGATCGGTTG
795  Y A N R T E D D I L L K E E L D D W A K K H D R L
2551 AAAGTATGGTACGTCGTACAAGAATCTATAAGGGAAGGATGGCAATATAGCACAGGTTTCATCACGGAAAGTGTA
820  K V W Y V V Q E S I R E G W Q Y S T G F I T E S V
2626 ATGAGGGATCACATCCCGGAGGGCTCCAGCGACACCCTTGCGTTGGCTTGTGGGCCGCCGCCGATGATCCAGTTC
845  M R D H I P E G S S D T L A L A C G P P P M I Q F
2701 GCCGTGCAGCCTAACATAGAGAAGATGAAGTATTACGTCAAGGATTCTTTGTTGGTATTTTAAGGAAAATTCGGA
870  A V Q P N I E K M K Y Y V K D S L L V F *
2776 GATGTTTAAATGTTGTCTTGTATTCTTATTAATTTTTCCCATGGCGTAGCAAAGTTGTTGGGAAAACGATGTACA
2851 TAGAGAAATCAAGGGTGTTGTAGTTTGAGAGAAGGAAAATAAAAATAAAAAAGTCTTCTTGTAAAAAAAAAAAAA
2926 AAAAAAAAAAAAAAAGTACTCTGCGTTGATACCACTGCTTGCCCTATAGTGAGTCGTATTAGAATCGAATT
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Figure 1. The full-length cDNA sequence and the deductive amino acids sequence of GhNR. The stop codon was represented by an asterisk. The putative polyadenylation signal was shown as boxed sequence in the 3'UTR. The three regions which were identical to the amino acid sequences of heme-binding domain profile, Mo-co oxidoreductase dimerisation domain and Oxidoreductase FAD-binding region are showed in the boxes; underline is eukaryotic molybdopterin oxidoreductases signature; double underline is GPPP-M motif.

expression vector containing green fluorescence protein-GhNR (pCamE-GhNR-GFP) fusion gene driven by 35S promoter was constructed and introduced into onion epidermis cells by biolistic transformation. As shown in Figure 5, pCamE-GhNR-GFP was clearly localized to the nucleus (Figure 5). By contrast, free GFP was found in the nucleus, cytoplasm and peripheral regions of the onion cells (Figure 5).

Prokaryotic expression of the GhNR

The *E. coli* cell with pET-*GhNR* could be induced by IPTG and a novel polypeptide with the molecular mass of about 124.744 kDa was expressed in *E. coli* by SDS-PAGE test. The expression of GhNR had achieved the highest level after being induced by IPTG for 6 h and the expression level had not obvious alteration with the increase of induction time. But under the same culture conditions, the novel polypeptide could not be expressed in the positive control of pET-*GhNR* with no IPTG induction (Figure 6).

GhNR activity analysis

GhNR activity assay showed that the crude extract with pET-GhNR gene had high activity to KNO_3 as substrate and the activity values of three unattached clones with pET-GhNR induced by IPTG were 6 times of the pET-32a (Figure 7).

DISCUSSION

In the present study, we firstly cloned the GhNR gene related with somatic embryogenesis in *G. hirsutum* by RT-PCR and RACE. The ORF of the GhNR was 2,670 bp in length which encoded 899 amino acids polypeptide. Prokaryotic synthesized GhNR displayed the NR activity with KNO_3 as substrate. Somatic embryogenesis and plant regeneration ability are influenced by many exterior factors such as the period of illumination, hormone and the time of subculture, but they depend mainly on a few key genes (Takeuchi et al., 2000; Kwon et al., 2001;

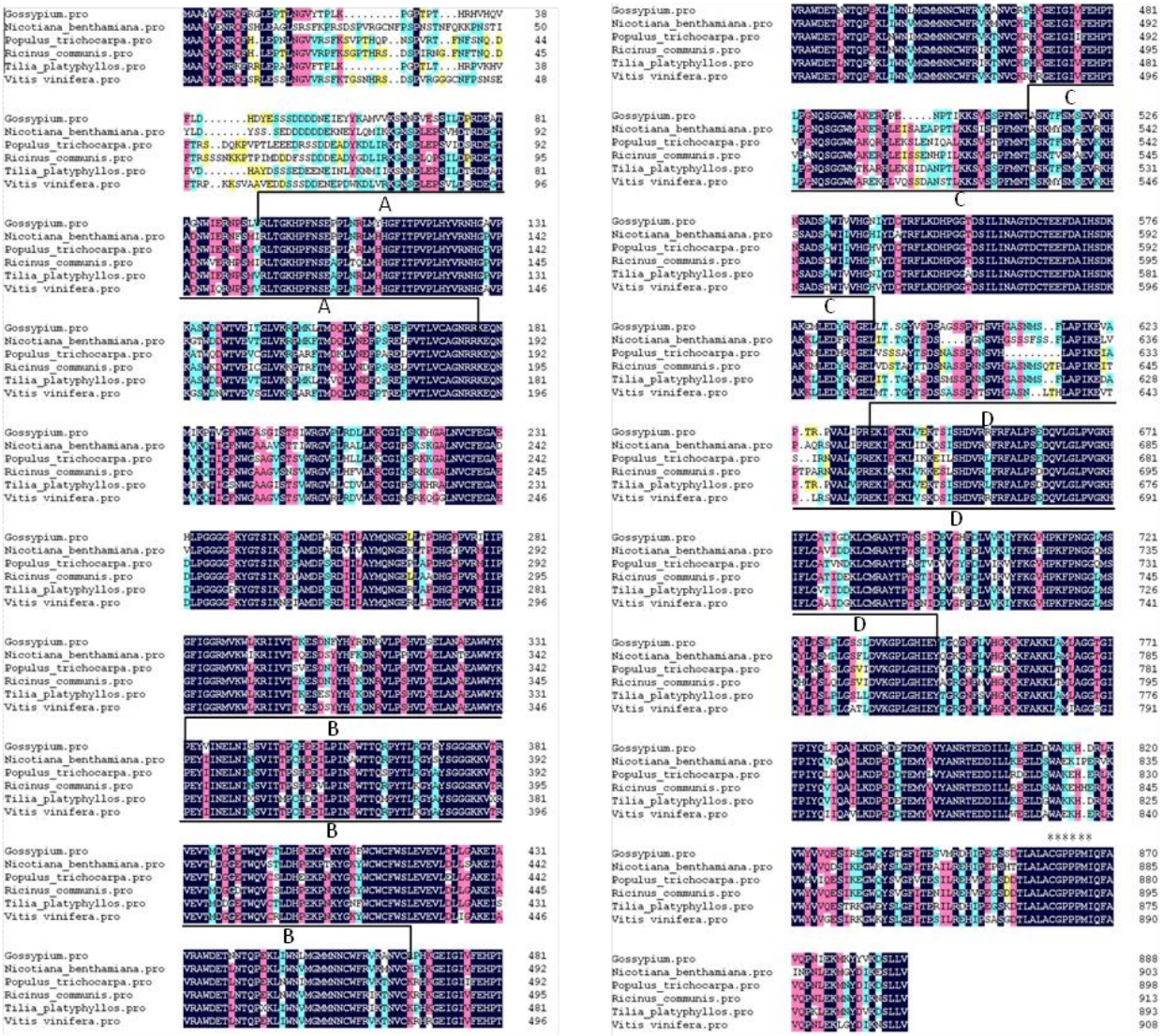

Figure 2. Multiple sequence alignment of GhNR conserved domain. A) Eukaryotic molybdopterin oxidoreductases signature, B) Heme-binding domain profile, C) Mo-co oxidoreductase dimerisation domain, D) Oxidoreductase FAD-binding region and *) shows the CGPPP-M motif.

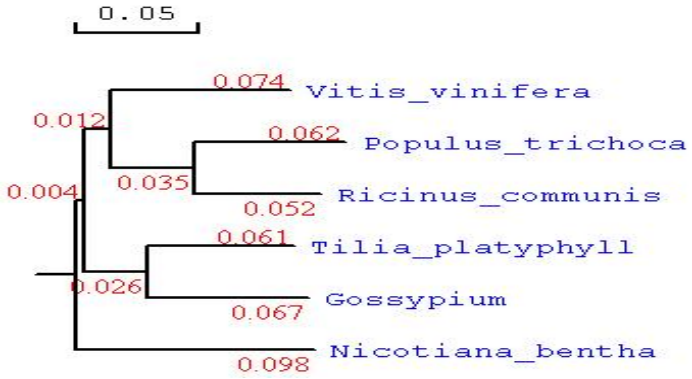

Figure 3. The phylogenetic tree drawn using the program MegAlign of DNAstar.

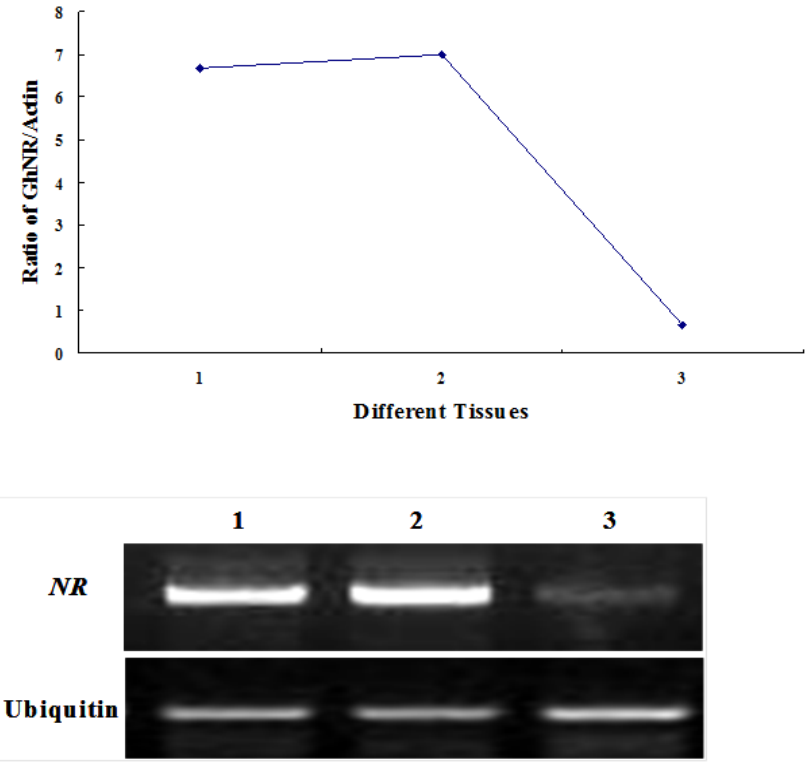

Figure 4. Semi-quantitative RT-PCR analysis of GhNR expression in different tissues. somatic embryoids (Lane 1), embryogenic calli (Lane 2), nonembryogenic calli (Lane 3).

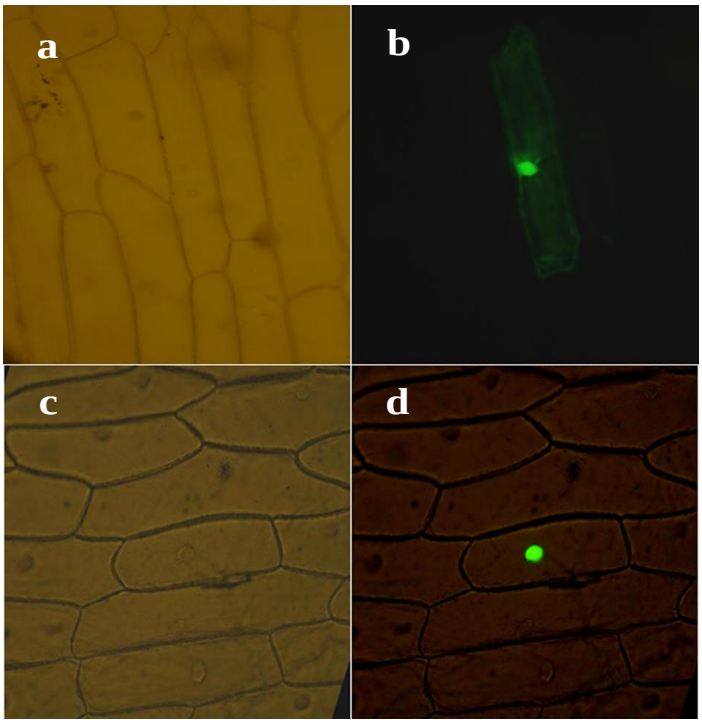

Figure 5. Subcellular localization of GhNR–GFP fusion protein. Onion epidermal cells containing free GFP (a, b) or GhNR–GFP fusion box (c, d). Fluorescence images (b, d). Bright-field images (a, c).

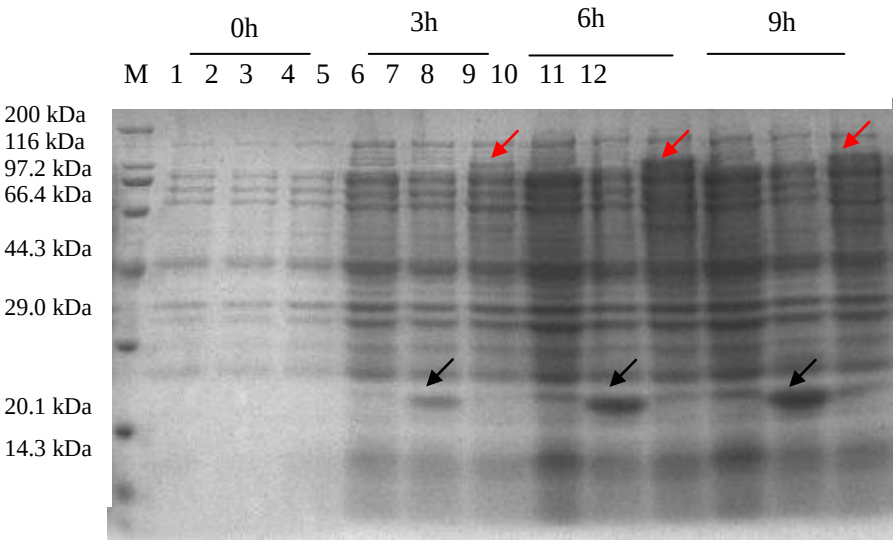

Figure 6. Expression of the recombinant GhNR in E. coli. The red arrow indicated the position of the GhNR recombinant protein. Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. 0 h meant the non-induced cell. 3 h, 6 h and 9 h meant that the cells were produced with IPTG for 3 h, 6 h and 9 h, respectively. Ladder marker (M); control E. coli cell harboring no plasmid (1, 4, 7 and 10); E. coli cell harboring pET-32A (2, 5, 8 and 11); E. coli cell harboring pET-GhNR (3, 6, 9 and 12).

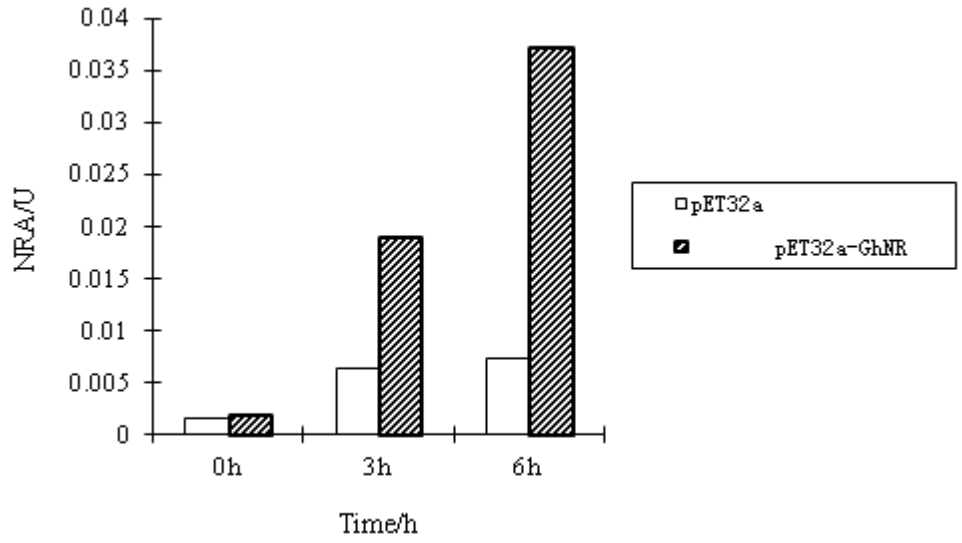

Figure 7. NRA analysis of GhNR protein.

Rode et al., 2011). To this day, some genes that are expressed during the induction and development of somatic embryogenesis have been cloned such as housekeeping genes, hormone-responsive genes and homeobox genes (Archana and Paramjit, 2002). As well known, nitrate is the major source of nitrogen in tissue culture of most plants; and nitrate assimilation is carried out by the sequential reduction of nitrate to nitrite and then to ammonium (Campbell, 2001). Therefore, it is important to clone the NR gene. NR gene had been cloned in many plants (Kuhlemeier et al., 1984; Hua et al., 2007; Hideyuki et al., 2007; Sun et al., 2008), but they are not related with somatic embryogenesis. In this paper, GhNR gene was firstly cloned which will be helpful

to widen gene resources of cotton regeneration.

In higher plants, studies on *Arabidopsis* NR were much deeper. Its separated strategy was to build a cDNA library inducted by nitrate and then selected positive clones used winter squash NR cDNA as a probe. The *Arabidopsis* NR is 3.2 kb containing 917 amino acid residues. Its 540 ~ 620 site is homology to CGTb5 family. The GhNR gene related with somatic embryogenesis in *G. hirsutum* cloned in the present study has the same structure as that of the *Arabidopsis*. According to the different electron donor, NR can be divided into three types, namely, NADH:NR (EC1.6.6.1), NAD(P)H:NR (EC1.6.6.2) and NADPH:NR (EC1.6.6.3). NADPH:NR presents in the fungus, while NADH:NR is most common in algae and higher plants, but some species also contain NAD (P) H:NR. The FAD domain of the C 'terminal has (Cys-Gly) conserved motif, the amino acid residues in its downstream decided to use for the hydrogen donor, NADH-NR CGPPP-M (*A. thaliana*), when P is replaced by A or S, then the NADH specific can be turned into NAD (P) H-NR, that is CGAPS-M (birch) or CGPPA-M (barley) (Schnorr et al., 1991). It have been reported that two NR genes, NADH:NR (Schnorr et al., 1991) and NAD(P)H:NR (Jensen et al., 1994) was cloned from barley. This study shows that the motif of CGPPP-M (881 to 886) was found in the downstream of FAD domain of C 'terminal from *G. hirsutum* indicating that this NR belongs to NADH type. Semi-quantitative RT-PCR analysis showed that GhNR had higher expression level in embryogenic calli and somatic embryoids than in nonembryogenic among different somatic embryogenesis stages. The results implied that NR has a critical function in the assimilation of nitrogen in cotton somatic embryo germination.

The conversion of nitrate into organic carbon compound proceeds through nitrite and ammonium by nitrate reductase (Hattori and Myers, 1967) and the latter compound is incorporated into glutamate to form glutamine (Kuhlemeier et al., 1984). Furthermore, glutamine is efficient for somatic embryo germination (Han et al., 2009).

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