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Full Length Research Paper

Cloning and characterization of a nitrite reductase gene related to somatic embryogenesis in *Gossypium hirsutum*

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A nitrite reductase gene related to somatic embryogenesis was first cloned from *Gossypium hirsutum*. The cDNA sequence of the gene, named *GhNiR*, is 2,257 bp in length, with 254 bp of the 5' untranslated region and 236 bp of the 3' untranslated region. The open reading frame is 1,767 bp in length, encoding a deduced amino acid sequence of 588 residues with a molecular weight of 65.722 kDa and an isoelectric point of 7.07. Semi-quantitative RT-PCR analysis showed that the expression level of *GhNiR* was higher in embryogenic calli and somatic embryoids than in nonembryogenic calli among different somatic embryogenesis stages, and that the level of *GhNiR* mRNA was also higher in the cultivar with higher somatic embryogenesis ability. The catalytic *GhNiR* was verified by transformation in *E. coli* BL21 (DE3) strain with the recombinant expression vector pET-28A-*GhNiR*. NiR activity assay showed that the crude *GhNiR* protein had obvious activity to NaNO₂ substrate.

Key words: Cotton, nitrite reductase, prokaryotic expression, semi-quantitative RT-PCR, GenBank Accession No: GQ389691.

INTRODUCTION

Nitrate is the major source of nitrogen for photosynthetic organisms such as plants, algae and cyanobacteria. It is transported into the cells by an active transport system and reduced to nitrite by nitrate reductase (NR). Nitrite is further reduced to ammonium by nitrite reductase (NiR)

and the resulting ammonium is fixed as the amide group of glutamine by glutamine synthetase (Iwane et al., 1995; Antonio et al., 2005). Also, nitrate is commonly used as the source of nitrogen in plant tissue culture. But its metabolite, nitrite, has a toxic effect on plant cell growth (Hellens et al., 2000). Thus, rapid metabolism of nitrite is important for plant cell growth and regeneration. Study has shown the importance of N compounds, particularly glutamine, for the proliferation and maturation of somatic embryos in cotton (Price and Smith, 1979). More studies on nitrate and nitrite assimilation were focused in rice (Asuka et al., 2005; Ogawa, 2000). The gene related with somatic embryogenesis, nitrite reductase gene (*NiR*), was cloned by map-based cloning method (Asuka et al., 2005). Enzymatic analyses revealed that the varieties with high regeneration ability always had high NiR activity, whereas the low regenerative varieties showed low activity. This indicated that NiR activity is a decisive factor for regeneration ability of rice varieties. So the study on nitrite reductase role in the course of plant somatic embryogenesis and regeneration would be of importance for plant tissue culture. To date, some *NiR* ORF has been cloned from several higher plant sources

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Abbreviations: cDNA, Complementary deoxyribonucleic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; bp, base pair; NR, nitrate reductase; NiR, nitrite reductase enzyme; *NiR*, nitrite reductase gene; *GhNiR*, a nitrite reductase gene cloned from *Gossypium hirsutum*; IBA, indolebutyric acid; KT, kinetin; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, murashige and skoog medium; CTAB, cetyltrimethyl ammonium bromide; ORF, open reading frame; EST, expressed sequence tag; EtBr, ethidium bromide; IPTG, isopropylthiogalactoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSH, suppression subtractive hybridization.

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which are normally classified into three types: ferredoxin-nitrite reductase (Iwane et al., 1995; Matsumura et al., 1997; Chiharu et al., 2004), copper-type nitrite reductase genes (Braker et al., 2000; Yamaguchi et al., 2004; Alexander et al., 2005) and heme-type nitrite reductase gene (Smith and Tiedje, 1992).

Somatic embryogenesis has been regarded as a potential model system for the study of the regulation of gene expression required for the earliest developmental events in the life of higher plants (Zimmerman, 1993). It involves a plethora of molecular events including not only differential gene expression but also various signal transduction pathways for activating/repressing numerous gene sets, many of which have been identified and characterized in carrot; some different genes related with somatic embryogenesis have been isolated (Choi et al., 1987; Wilde et al., 1988; Kiyosue et al., 1993; Wurtele et al., 1993; Aleith and Richter, 1992; Rojas et al., 2002; Iris et al., 2004; Kiminori, 2008). 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 is known so far (Zimmerman, 1993; Chugh and Khurana, 2002; Zeng et al., 2006). Since 1983, reports of high-frequency regeneration of cotton via somatic embryogenesis were still limited, owing to a genotype-dependent response (Zhang et al., 1991; Sakhanokho et al., 2000; Kumria et al., 2003; Mishra et al., 2003; Ganesan and Jayabalan, 2004; Wang et al., 2006; Xie et al., 2007). But none of nitrite reductase genes was cloned related to cotton somatic embryogenesis and regeneration. The identification and isolation of the vital genes of somatic embryogenesis are of great importance for improving the embryogenic competence and regeneration ability of a wider range of cultivars.

In this study, we first cloned a nitrite reductase gene from *Gossypium hirsutum* (Upland cotton) (*GhNiR*) combining *in silico* cloning with RT-PCR according to the reported NiR of rice (PIR accession no. JC4395).

MATERIALS AND METHODS

Material preparation

Callus initiation of upland cotton cultivars with verticillium wilt resistance, Shann724, Luwu401, Coker201 and Coker312, was carried out in MSB medium (MS medium plus B5 vitamins) containing 0.1 mg.L⁻¹ indolebutyric acid (IBA), 0.1 mg.L⁻¹ kinetin (KT) and 0.1 mg.L⁻¹ 2,4-dichloro-phenoxyacetic acid (2,4-D). Following callus initiation, the callus was subsequently isolated and subcultured in MSB medium containing 0.3 mg.L⁻¹ IBA and 0.05 mg.L⁻¹ KT for the induction of embryogenic callus. Then the embryogenic callus was transferred on hormone-free MSB medium supplemented with 2.0 g.L⁻¹ glutamine and 0.5 g.L⁻¹ asparagine for somatic embryoids induction.

Total RNA extraction and purification

Total RNA was extracted from nonembryogenic calli, embryogenic

calli and somatic embryoids of Shann724 and from embryogenic calli of cotton cultivars, Luwu401, Coker201 and Coker312, using a modified CTAB method (Ambion, USA), respectively. The isolated RNA was treated by RNase-free DNase I according to manufacturer's protocol to remove any potential gDNA contamination. The purity and concentration of the total RNA were determined using gel electrophoresis and Beckman spectrophotometer (DU800, USA).

Splicing and open reading frame cloning of *GhNiR* sequence

According to the sequence of rice NiR (PIR: JC4395), sequence splicing was processed using the EST data with high homology through NCBI Blast, and the open reading frame (ORF) was analyzed on line (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). A pair of gene specific primers with restriction enzyme site was designed according to the two ends of ORF for the cloning of *GhNiR*. The primer sequence was 5'-CATGGATCCCACATGTCTTCCCTTTCCG GTCC-3' (sense primer with *Bam*H) and 5'-TGCGAGCTC TGAATCAATCTCCCTTC-3' (antisense with *Sac* I), respectively. Each RT-PCR reaction was carried out in 20 µL solution containing 1 µL of cDNA, 0.5 µL of 10 mM dNTP, 2 µL of 10×PCR buffer, 1 µL of 10 µM each primer, 0.2 µL *Taq* DNA polymerase and 14.3 µL of ddH₂O. The mixture was covered with mineral oil, denatured at 94°C (4 min) and subjected to 33 cycles of amplification (94°C for 40 s, 65°C for 40 s, 72°C for 2 min) with a final elongation cycle of 10 min at 72°C. The products from RT-PCR of cDNA were cloned into the pMD18-T (TaKaRa, China), and 6 clones were sequenced by Sangon, China. The DNA sequences and phylogenetic tree were analyzed with DNASTar software and the BLAST program (<http://ncbi.nlm.nih.gov>). Homological comparison was made using ClustalW software.

Cloning of 5' and 3' end of *GhNiR*

A pair of primers was designed for the 5' and 3' end reactions by Primer Premier 5.0 software for 5' -UTR and 3' -UTR cloning. The 5' -UTR primer sequences were 5' -GACAAACCCAAACATTG TCAGTTTT- 3' (sense) and 5' -AGCAAAGAAACGGACCGAAAA GGAA- 3' (antisense), and the 3' -UTR primer sequences were 5' -GGGAAGAAGGGGAAGATTGATTCAA- 3' (sense) and 5' -CATGA TCATCTCTAAATTTTAACTG - 3' (antisense). The PCR products were analyzed on a 1.0% agarose EtBr gel. The purified DNA was cloned using pGEM-T Easy Vector and *E. coli* Top10 strain according to the Kit manual instruction (Tiangen, China). Selected positive clones were analyzed using PCR, then sequenced by Sangon, China.

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 5'-CACATGTCTTCCT TTTCGGTCC-3' and 5'-TGAATCAATCTCCCTTC-3' were used for *GhNiR* mRNA expression level assay by semi-quantitative RT-PCR. The cotton polyubiquitin gene (*GhUBI*): 5'-CTGAATCTTCGCT TTCACGTTATC-3', 5'-GGGATGCAAATCTTCGTGAAAAC-3' (Li et al., 2005) 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 25 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.

GhNiR prokaryotic expression

The cDNA fragment of *GhNiR* full ORF was amplified from pGEM T-Easy-GhNiR cloning plasmid with primer pairs of 5' -CATGGATCCCACATGTCTTCCCTTTTCGGTCC- 3' and 5' -TGCGA GCTCTCAATCTTCCGCTTCTCC- 3', and the target product digested with *Bam*HI and *Sac* I was ligated into the expression vector pET-28A with the same restriction enzymes digestion to yield pET-*GhNiR*. The recombinant plasmid pET-*GhNiR* and pET-28A 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 and 6 h according to the pET system manual, and the protein extracts were analyzed using SDS-PAGE electrophoresis.

GhNiR enzyme activity assay

Recombinant proteins were induced by IPTG with the final concentration of 1.0 mM 25°C culture for 3 h and extracted by sonication. NiR activity was assayed by the addition of NH_3^+ in the assay mixture. For *in vitro* assays of NiR, 50 μL of crude extracts of recombinant proteins and the protein of pET-28A without *GhNiR* gene, respectively, was added to an 850 μL assay mixture (50 mM Tris-HCl, pH 7.5/0.5 mM NaNO_2 /1 mM methyl viologen), and the enzyme reaction was initiated by the addition of 100 μL of 0.12 M $\text{Na}_2\text{S}_2\text{O}_4$ dissolved in 100 μL 0.2 M NaHCO_3 . After incubation at 30°C for 60 min, the reaction was terminated by vigorous vortexing until the color of methyl viologen disappeared completely. Then 100 μL 1 M $\text{Zn}(\text{CH}_3\text{COO})_2$ was added and centrifuged at 10,000 g for 10 min at room temperature. The NO_2^- content was determined by a spectrophotometer at 595 nm. The NiR activity was expressed as increased NH_3^+ per hour. One unit of NiR activity was defined as the dosage of NiR protein for releasing 1 mM NH_3^+ per hour at pH 7.5 at 25°C. We analyzed recombinant proteins pET-28A-GhNiR enzyme activity in nonembryogenic calli, embryogenic calli and somatic embryoids, respectively.

RESULTS

Sequence characterization and gene structure of GhNiR

A 2,257 bp cDNA sequence of *GhNiR* was obtained from *G. hirsutum*. The nucleotide sequence and deduced amino-acid sequence of this gene were shown in Figure 1. A 1,767 bp ORF encoded a protein of 588 amino acids with molecular mass of 65.722 kDa, including 20.53% polar amino acids, 14.73% acidic amino acids, 16.96% basic amino acids and 32.79% hydrophobic amino acids. The pI was 7.07. A 254 bp and 236 bp fragments of 5' end and 3' end were obtained by using gene-specific primers, respectively. And a putative polyadenylation signal AATAAA was present at the 2,208 th base pairs.

Homology analysis of GhNiR

The GhNiR polypeptide was 86, 80, 75 and 77% sequence homology to those of *Nicotiana tabacum*, *Arabidopsis thaliana*, *Zea mays* and *Oryza sativa*, respectively (Figure 2). For phylogenetic analysis, the GhNiR sequence and *Nicotiana tabacum* NiR were divided into the same group

(Figure 3) and this result indicated that the differentiation of *GhNiR* occurred after the differentiation of monocotyledonous and dicotyledonous plants, that is, *NiR* could differentiate monocotyledonous and dicotyledonous plants.

Expression profiles of GhNiR in different tissues

To determine the expression pattern of *GhNiR*, semi-quantitative RT-PCR analysis was performed. The results showed that the expression level of *GhNiR* was higher in embryogenic calli and somatic embryoids than in nonembryogenic calli, whereas, there was no remarkable difference between embryogenic calli and somatic embryoids for the expression level of *GhNiR* (Figure 4). And among different cotton cultivars (the regenerated period of Shann724 and Luwu401 was about 4-5 months, and that of Coker201 and Coker312 was about 3 months), the higher expression level of *GhNiR* was detected in cultivars with higher somatic embryogenesis ability (Figure 5).

Prokaryotic expression of the GhNiR

The *E. coli* cell with pET-*GhNiR* could be induced by IPTG, and a novel polypeptide with the molecular mass of about 66.0 kDa was expressed in *E. coli* by SDS-PAGE test (Figure 6). The expression of GhNiR had achieved the highest level after being induced by IPTG for 3 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-*GhNiR* with no IPTG induction (Figure 6).

GhNiR activity analysis

GhNiR activity assay showed that GhNiR activity in non-embryogenic calli, embryogenic calli and somatic embryoids crude extracts of *E. coli* BL21(DE3) with pET-*GhNiR* gene induced by IPTG were 0.1305, 0.5078 and 0.4956 U/h, respectively, and GhNiR activity in extracts of non-embryogenic calli was about 4 times lower than that of embryogenic calli and somatic embryoids (Figure 7). And the activity of pET-28A without *GhNiR* gene induced by IPTG was 0.1274 U/h.

DISCUSSION

In the present study, we firstly cloned and analyzed the *GhNiR* gene (GenBank Accession No: GQ389691) related with somatic embryogenesis in *G. hirsutum*. The ORF of the *GhNiR* was 1,767 bp in length, which encoded 588 amino acids polypeptide. Semi-quantitative RT-PCR indicated that the expression level of *GhNiR*

GACAAACCCAAACATTGTCTAG TTTTCTCGGTTCTT TCTTACAAACCAAGCTGACCCCTTAACAT CG
 TCGAAGAGTCCCCCCCATCGA AAGAAAAAACC AGTAAAAAACA CAAAAAACCCCTC CC
 CTTTCTCTTAAAGTCCCTT CATTCCATTACAAA AATGTCCGAACTCT GATATCCCTTCACT TA
 TCTTTCCACCACCAATTCACCAAGTTCCAAGCTTCTTTTCAAAACAACAAAACCCCATGTCT
M S
 TCCTTTTCGGTCCGTTTCTTTGCTCCACAACAGCCGTTACTGCGGTCACAGCTTCCCTCTTTCAA
 S F S V R F F A F Q Q F L L P S T A S S F K
 GCCCAAAACATGGGTTATGGCAGTCCACAGCGGGCGCGGCGGACTTCGGTGGATGTGACGGGG
 P K T W V M A A P T T A F A T S V D V D G G
 GGAGTTGGAACCCCGAGTTGAAGAACGAGAGGGGTTACTTTCGTGTTGAAGAGAAGTTCAGAGAT
 R L E P R V E R E R E G Y F V L K E K F R D
 GGCATCACCCTCAGGAGAAAATAAAGATCGAGAAAGACCCCTTGAAGCTTTTCATGGAAAGCT GG
 G I N P Q E K I K I E K D P L K L F M E A G
 GATTGATAAACTCGGTANGATGTGTTTCGAGGATCTTGATAAAGCTAAGGCTACAAAGCAGACA
 I D K L A K M S F E D L D K A K A T K D D I
 TTGATGTTAGACTTAAATGGCTCGGCTTGTTCATAGGAGAAAACATCAATATGGGAGATTTATG
 D V R L K W L G L F H R R K H Q Y G R F M
 ATGAGACTAAAATACCAATGGTGTAAACAACAGTGCACAAACACGGTACTTAGCCAGTGTGAT
 M R L K L P N G V F E T S A Q T R Y L A S V I
 AAGGAATACGGCAAGAAGGGTGTGCCGATGTTACGACAGGCAAAACTGGCAAAATCCGTGGAG
 R K Y G K E G C A D V T T R Q N W Q I R G A
 CGGTGTTGCTGATGTGCTGAAATACTTAAAGGCTCTCGACCAAGTAGGCTTGAACGAGCCCTACG
 V L P D V P E I L K G L D E V G L T S L Q
 ACTGGCATGGACAATGTGAGCAACCTGTCCGTAATCTCTTCCCGGCATCGACCCCGAAGAGAT
 S G M D N V R N P V G N P L A G I D P E E I
 TGTGATATCGACCTTATACCACTTGTATCTCAGTTCATERACCCCAATTCGCGGGCAATC
 V D T R P Y T N L L S Q F I T A N S R G N P
 CGGCTGTTGCCAATTCGCTAGGAATGGAATGTCTGTGTCTGCGGGTCTCATGATCTTTATGAA
 A V A N L P R K W N V C V V G S H D L Y E
 CATCCCATATCAATGATCTCGCTTACATGCCGGGCAAGAAAACCGACGATTTGGCTTTAATTT
 H P H I N D L A Y M P A T K N G R F G F N L
 GCTGGTTGGTGGGTTCTTTAGTGGCAAGAGATGTGATGAGGCCATTCTCTTGATGCTTGGGCT
 L V G G F S A K R C D E A I P L D A W V S
 CAGCTGATGATGTGATCCATTGTGCAAGCTGTGTTAGAAGCCTATAGGGATCTTGGATACCGG
 A D D V I P L C K A V L E A Y R D L G Y R
 GCCAATAGCCAAAAGACTAGAATGATGGCTGATTGATGAACCTGGCGATTGAAGTGTTCAGATC
 G N R Q K T R M M W L I D E L G I E V R S
 AGAGTAGCCAAAAGAAATGCCCTCAGAAAGAGTTGGAGAGGCATCTGATGAAGGTTTGGTTCAA
 E V A K R M P Q K E L E R A S D E G L V Q K
 AGCAATGGCAAGAGAGACTACCTCGGCTGCCATCCGCAAAAGCAAGAGGTTTCAGCTACATT
 Q W E R R D Y L G V H P Q K Q E G F S Y I
 GCAATCACATCCCACTCGGTCGCGTCCAGCTGACGACATGGACGAACTAGCCCGGTTAGCCGA
 G I H I P V G R V Q A D D M D E L A R L A D
 CATGTATGGCTCCGCGAAGCTCAGACTCACTGTGAGCCAAACATCATAATCCCAACGTTGACA
 M Y G S G E L R L T V E Q N I I I P N V E N
 ACTCGAACTAGAACATTACCCCAAGAGCCTCTATTGAAGATCGGTTTTTCACCCCAACCAAGT
 S K L E A L P N E P L L K D R F S P Q P S
 ATTCTCATGAAAGCCCTAGTAGCTTGTACTGCTAACCAAGTTTTGCGGACCAAGCCATTATTGAAAC
 I L M K G L V A C T G N Q P C G Q A I I E T
 AAAAGCTAGAGCCCTTGAAGCTGACCGAGAGGCTTGAAGGCTAGTGTCCGTCAGCCCGCGGCTGA
 K A R A L K V T E E V E R L V S V S R P V R
 GGATGCCATTGGCCCGTTCCCGAACACGCTGTGCTCAAGTCCCAAGTGGCGCATATAGGTTTCATG
 M H W A C C P N T C G Q V Q V A D I G F M
 CGCTCCATGCCAAGCCATGCAATCCGAAACCATGTGAGCGCGCAGACATATTCTTGGAGCCGAG
 C C M A R D E N G K P C E G A D I F L G G R
 AATTGGCAGTCACTCCCATTTAGGAGAGCTTTATAAGAGGCTGTCCCTTGTAGAAGCTTGGTAC
 I G S D S H L C E L Y K K G V P C K N L V P
 CCCTAGTTGCTGACATTTTCTGGAACACTTTGGAGCTGTCCCTAGCCAAAGCGAAGAGCCGAA
 V V A D I L V E H F G A V P R Q R E E C E
 GATTGATTCAAATCAACTTCAATTTCAATTCATTCTATATTTGTTTTATTTTTTTTTTCTA
 D *
 ATAACCAAGAAAATCAAGCC TTTCAAACATACTC CAGACCATTAATTTCCAGCAATATTGAT CA
 ATGCCATGATGATCAAGCCCT TTCTATTATAAAT ATCTAACATTTTCA GCATATGTATTAC **CAAT**
ATACTTACTGCTAATATATTTTCACTTAAATTTAGAGATGATCATC

Figure 1. The full-length cDNA sequence and the deductive amino acids sequence of GhNiR. The stop codon was represented by an asterisk. The putative polyadenylation signal was shown as boxed sequence in the 3'-UTR.

was much higher in embryogenic calli and somatic embryoids than in nonembryogenic calli, and the higher expression level of *GhNiR* was detected in cultivars with

higher somatic embryogenesis ability. Prokaryotic synthesized GhNiR displayed the NiR activity with NaNO₂ as substrate.

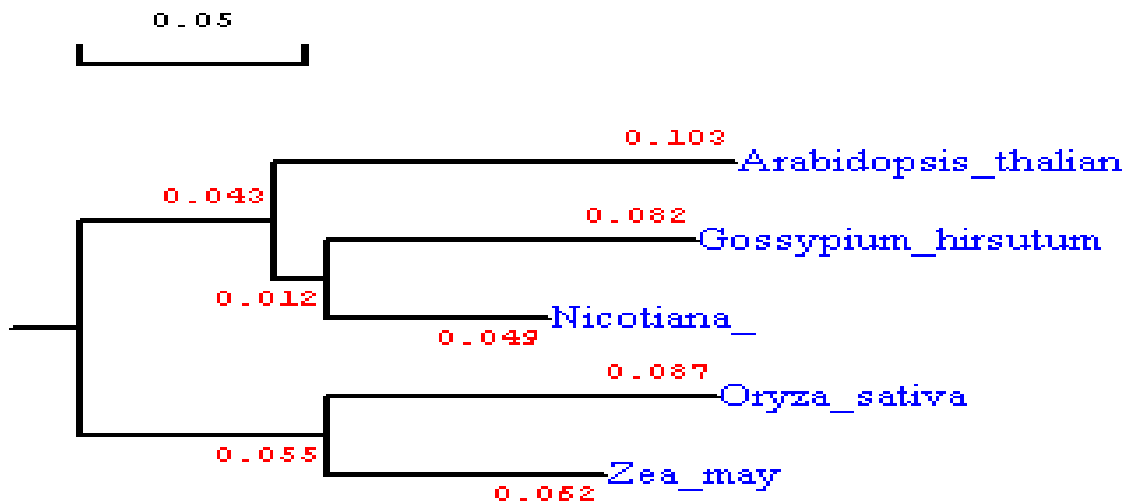


Figure 3. The phylogenetic tree drawn using the program MegAlign of DNASTar.

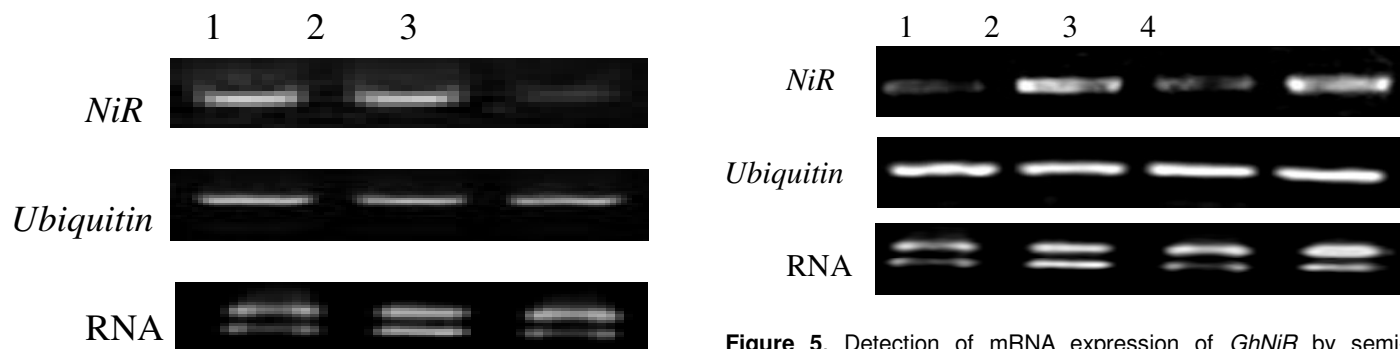


Figure 4. Detection of mRNA expression of *GhNiR* by semi-quantitative RT-PCR in different tissues. (Lane 1) somatic embryoids; (Lane 2) embryogenic calli; and (Lane 3) nonembryogenic calli.

Figure 5. Detection of mRNA expression of *GhNiR* by semi-quantitative RT-PCR in different cotton cultivars. (Lanes 1 and 3) Shann724 and Luwu401 with lower somatic embryogenesis ability; and (Lanes 2 and 4) Coker201 and Coker312 with higher somatic embryogenesis ability.

putative beta-galactosidase (Zeng et al., 2006). And several other studies indicated that nitrate is the major source of nitrogen in tissue culture of most plants. But its metabolite, nitrite, has a toxic effect on plant cell growth, thus, rapid metabolism of nitrite is important for plant cell growth and regeneration (Hellens et al., 2000). Therefore, it is important to clone the *NiR* gene. *NiR* gene had been cloned in many plants (Iwane et al., 1995; Matsumura et al., 1997; Chiharu et al., 2004; Asuka et al., 2005), but only the *NiR* gene in rice was related to somatic embryogenesis (Asuka et al., 2005) among these cloned *NiR* genes. The *GhNiR* gene was not reported to this day, therefore this study would play an important role in studies of cotton somatic embryogenesis and transformation.

Semi-quantitative RT-PCR analysis showed that *GhNiR* had higher expression level in embryogenic calli and somatic embryoids than in nonembryogenic among

different somatic embryogenesis stages. As for different cotton cultivars, the cultivar with higher regeneration capability had higher expression level of *GhNiR*. And the *GhNiR* activity assay showed that the *GhNiR* activity in extracts of embryogenic calli and somatic embryoids was about 4 times higher than that of nonembryogenic calli. These results implied that *NiR* has a critical function in the assimilation of nitrogen in cotton somatic embryogenesis. The result was similar to that of the previous study in rice (Asuka et al., 2005).

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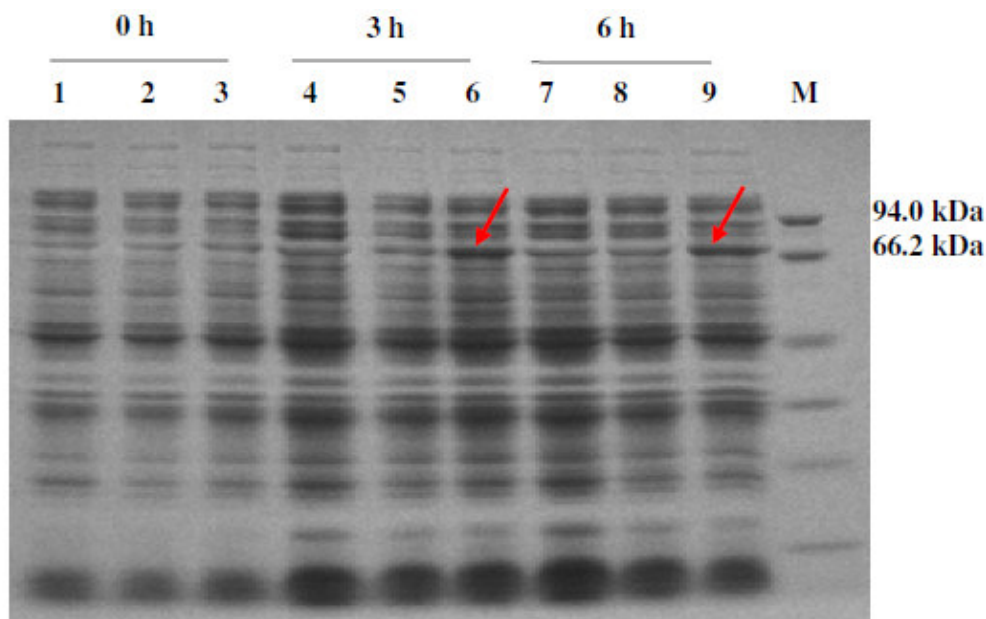


Figure 6. Expression of the recombinant GhNiR in *E. coli*. The red arrow indicated the position of the GhNiR recombinant protein. Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. 0 h meant the non-induced cells. 3 h and 6 h meant that the cells were produced with IPTG for 3 h and 6 h, respectively. (M) ladder marker; (1, 4 and 7) control *E. coli* strain harboring no plasmid; (2, 5 and 8) *E. coli* strain harboring pET-28A; (3, 6 and 9) *E. coli* strain harboring pET-GhNiR.

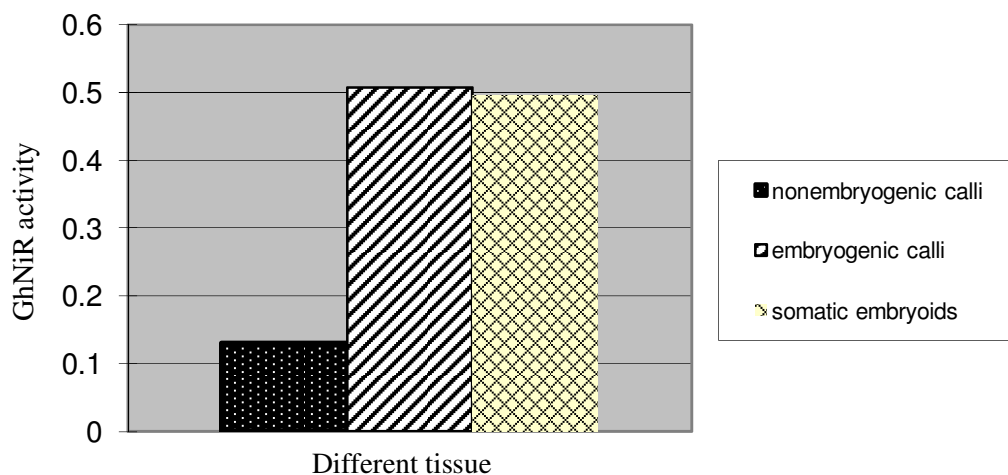


Figure 7. GhNiR activity in different tissue.

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