

CLONING OF GS1 GENE ENCODES GLUTAMINE SYNTHETASE 1 AND CONSTRUCTION OF TRANSFORMATION VECTOR

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SUMMARY

Glutamine synthetase (EC 6.3.1.2) is an enzyme which assimilates ammonium into amino acids. Therefore, GS plays an essential role in the metabolism of nitrogen. In vascular plants, GS is divided into two groups: cytosol - located isoform (*GS1*) and plastid - located isoform (*GS2*). Surveys have shown that *GS1* gene was used widely to generate transgenic plants. Effects on the growth characteristics of *GS1* transgenic plants have been reported in many researches. In this research, in order to prepare for generating *GS1* transgenic plants, the 1074 bp - length cDNA of *GS1* gene is successfully cloned from *Pinus sylvestris*. Compared the sequence of the isolated cDNA of *GS1* with other sequences on Genbank (Accession number X69822.1 and X74429.1), the similarities are both higher than 99%. This result indicates a high sequence conservation of *GS1* gene. The gene was then ligated into vector pBI121 to construct transformation vector pBI121 – *GS1* which can be used in next steps to generate transgenic plants which have high - growth rate.

Keywords: Glutamine synthetase 1, *GS1* gene, *Pinus sylvestris*, transformation, vector construction.

I. INTRODUCTION

Glutamine synthetase (GS, E.C. 6.3.1.2) assimilates ammonium into amino acids, thus it is a key enzyme in efficient use of nitrogen sources and nitrogen metabolism in organisms. In vascular plants, GS is an octameric enzyme of 320 to 380 kb (Stewart et al., 1980) that occurs as a number of isoenzymes, the subunits of which are encoded by members of a small multigene family (Bennett et al., 1989; Peterman and Goodman, 1991; Roche et al., 1993; Temple et al., 1995; Dubois et al., 1996). In the photosynthetic tissues of many angiosperms, GS2, a plastid - located isoform of GS, is responsible for the assimilation of ammonium derived from nitrate reduction and photorespiration (Ireland and Lear, 1999). Whereas GS1, a cytosolic isoform, is the predominant enzyme in roots and non - photosynthetic tissues and much less abundant in green tissues (Ireland and Lea, 1999). GS1 is particularly important for assimilating ammonium from different sources for both primary nitrogen assimilation and recycling.

GS1 are derived from primary nitrogen uptake and from various internal nitrogen recycling pathways. In this way, cytosolic glutamine synthetase is crucial for the remobilization of protein – derived nitrogen (Stéphanie and Habash, 2009).

The GS1 genes in all plants studied are members of small gene families, and the different members are differentially regulated (Bennett et al., 1989; Peterman and Goodman, 1991; Roche et al., 1993; Temple et al., 1995; Dubois et al., 1996). Analysis of *Arabidopsis thaliana* genome has identified five GS1 genes, but only four of which appear to be expressed (Ishiyama et al., 2004). Each of the GS genes appears to participate in different metabolic processes, based on where and how they are expressed. According to Martin et al. (2006), in *Zea mays*, GS1-1 is expressed in root cortex, GS1-2 in phloem, GS1-3, GS1-4, GS1-5 in leaf.

Surveys have shown that GS1 gene was used widely to generate transgenic plants. Ribarits et al. (2007) has generated transgenic

tobacco with mutated GS genes driven by tapetum and microspore – specific promoters. Consequently, the plants were male sterile, but this could be reversed by spraying with glutamine. This result demonstrated the importance of GS1 in anthers and pollen. There is also evidence of compensation between GS1 and GS2 enzyme levels in transgenic plants. For example, wheat lines transformed with the GS1 gene PvGln- α under the control of the Rubisco small subunit promoter had more GS1 and less GS2 enzyme activity in the flag leaves during grain filling (Habash et al., 2001).

Effects on the growth characteristics of the transgenic plants have been reported. Fuentes et al. (2001) found that T1 generation tobacco plants, transformed with CaMV 35S: GS1 constructs, were similar to the wild type under high N nutrition but were greener and had higher shoot and root dry weight than the controls in low N. In contrast, Oliveira et al. (2002) found that similar tobacco transformants grew better under both N – sufficient and N – limiting conditions. In part, this might be because transformed plants appeared to mature earlier (Vincent et al., 1997). Martin et al. (2006) transformed maize with GS1 GLN1-3 gene, driven by a constitutive CsVMV promoter, and the resulting plants had higher activities of GS1 and an increase in grain yield and number. Some wheat lines transformed with the GS1 gene PvGln- α , under the control of the Rubisco small subunit promoter, also showed increased grain yield, although this was due to increased grain weight (Habash et al., 2001). In many of the studies, the effects varied between different transformed lines in the same experiment (Fei et al., 2003, 2006).

Some researches of generating of GS1-transformation in forest plants were also

reported. More consistent results have been obtained with transgenic clones of hybrid poplar. Clones expressing a GS1a gene from the conifer *Populus sylvestris*, under the control of the CaMV 35S promoter, have been shown to have higher vegetative growth with enhanced nitrogen assimilation efficiency, higher net photosynthetic rates, higher rates of photorespiration and enhanced resistance to water stress (Kirby et al., 2006). Many of these benefits have been sustained in a 3 – year field test (Jing et al., 2004).

In this research, we isolated GS1 gene and then constructed transformation vector pBI121 – GS1 which can be used in next steps to generate transgenic plants which have high-growth rate.

II. MATERIALS AND METHODS

2.1. Materials

Pinus sylvestris (leaf, stem and root); pBT T/A cloning vector (Institue of Biotechnology); pBI121-*GUS* with nptII selection gene (which resists kanamycin) transformation vector; *E. coli* strain DH5 α from Invitrogen.

Two specific DNA primers for amplifying *GS1* were designed based on the *GS1* gene sequence of *Pinus sylvestris* (Accession number X69822.1). Forward primer (*GS1F*): 5'-ATG TCG AGC GTA TTA ACA GAC C-3'; Reverse primer (*GS1R*): 5'-TTA AGG TTT CCA TAG AAT GGT CG-3' (the two primers were conjugated with restriction sites of *Xba*I and *Sac*I).

Chemicals: restriction enzyme (*Xba*I, *Sac*I), T4 DNA ligase, *Taq* polymerase, Gel purification Kit from Fermentas, QIAquick Gel Extraction Kit, QIAprep Spin Miniprep Kit (QIAGEN), PureLink™ Plant RNA Reagent Kit, FastTrack® MAG mRNA Isolation Kit (Invitrogen), ReverAid™ H Minus First Strand Synthesis Kit.

2.2. Methods

2.2.1. Gene cloning of *GS1* gene

Firstly, total RNA was isolated from the mixture of leaf, stem and root of *Pinus sylvestris*: using the protocol of PureLink™ Plant RNA Reagent Kit from Invitrogen (USA). In the next step, mRNA was purified from total RNA by using FastTrack® MAG mRNA Isolation Kit (Invitrogen), according to the manufacturer's instruction. mRNA was then used as the template for cDNA synthesis, and first – strand cDNA was reverse – transcribed using reverse transcriptase which was supplied with ReverAid™ H Minus First Strand Synthesis Kit, according to the manufacturer's instruction.

Subsequently, the first strand of cDNA was used as template for polymerase chain reaction amplification of the *GS1* gene using two primers *GS1F* and *GS1R*. The mixture of PCR reaction included 15.7 µl deionized H₂O, 2.5 µl PCR buffer (10X), 2.5 µl MgCl₂ (50mM), 1 µl dNTP (10mM), 1 µl each of primers (*GS1F/GS1R*, 10 µM), 1 µl DNA template (50 ng/µl) and 0.3 µl Taq DNA polymerase (5 U/µl).

The PCR reaction was performed at 94°C at 5 min, then 35 cycles of PCR as follows: [denaturation at 94°C for 1 min, annealing at 55°C for 45 second, extension at 72°C for 1.5 min], 72°C for 8 min, store at 4°C. PCR products were separated on a 1% (w/v) of agarose gel using electrophoresis, stained with ethidium bromide and photographed under UV light.

GS1 gene was then cloned into pBT T/A cloning vector. The mixture of ligation reaction included 9.0 µl deionized H₂O, 2.0 µl T4 DNA ligase buffer (10X), 1.0 µl pBT cloning vector, 7.0 µl *GS1*, 1.0 µl T4 DNA ligase (1 U/µl). The mixture was incubated at 22°C for 2h.

The ligation mixture was then used for

transforming the recombinant vector pBT – *GS1* into *E.coli* strain DH5α using heat – shock (42°C for 90 seconds). The recombinant *E.coli* was screened on LB medium (added 100 mg/l Ampicillin, 0.004% X-gal, 100µM IPTG) and then extracted plasmid, tested using PCR method and *XbaI/SacI* double digestion.

GS1 gene which was cloned successfully is sequenced by automatic DNA sequencer (ABI PRISM 377, Applied Biosystems, USA) and compared with those of the *GS1* genes on Genebank.

2.2.2. Construction of transformation vector pBI121–*GS1*

Firstly, pBI121 – *GUS* and pBT – *GS1* were double digested by the same restriction enzymes (*XbaI/SacI*). The mixture of double digestion reaction included 18.0 µl deionized H₂O, 4.0 µl Tango buffer (10X), 10.0 µl vector (pBI121 – *GUS* or pBT – *GS1*), 4.0 µl *XbaI* (1 U/µl), 4.0 µl *SacI* (1 U/µl). The mixture was incubated at 37°C for 3h.

Next, vector pBI121 and *GS1* gene which were double digested were purified using gel purification kit (Bioneer, Korea) and then ligated together with the catalysation of T4 DNA ligase. The mixture of ligation reaction included 6.0 µl deionized H₂O, 2.0 µl T4 DNA ligase buffer (10X), 5.0 µl pBI121 vector, 5.0 µl *GS1*, 2.0 µl T4 DNA ligase (1 U/µl). The mixture was incubated at 22°C for 2h.

Subsequently, the recombinant vector pBI121 – *GS1* is transformed into *E.coli* strain DH5α using heat - shock (42°C for 90 second). The recombinant *E.coli* was screened on LB medium (added 50 mg/l kanamycin) and then extracted plasmid, tested using PCR method and *XbaI/SacI* double digestion.

III. RESULTS AND DISCUSSION

3.1. Cloning of *GS1* gene

The mRNA which was isolated from mixture of leaf, stem and root of *Pinus*

sylvestris was used for first – strand cDNA synthesis. The first – strand cDNA was then amplified by using PCR reaction with the specific primers GS1F/GS1R. The PCR product of *GS1* gene was separated by 1%

(w/v) agarose gel electrophoresis. The result in figure 1 shows that, only one clear band of approximately 1.1 kb was amplified, which was consistent with predictions.

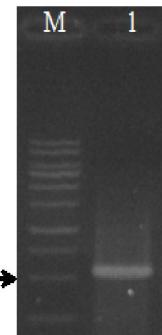


Figure 1. Amplifying of GS1 from mRNA of *P. sylvestris*

M: Marker 1kb; lane 1: PCR product amplified of GS1

GS1 gene was ligated into pBT T/A cloning vector to create recombination vector pBT – *GS1* which was transformed into *E.coli* strain DH5 α using heat – shock. *E. coli* was then cultured on selection medium (LB + 100 mg/l ampicillin, 0.004% X-gal + 100 μ M IPTG) for 12 hours. 5 white and 1 blue colonies of *E. coli*

were selected for plasmid extraction (the blue one was for negative control). The plasmids were also used for PCR reaction to amplify *GS1* gene using specific primers GS1F/GS1R. The result in figure 2A and 2B shows that *GS1* gene was successfully cloned to vector pBT and transformed into *E.coli* DH5 α .

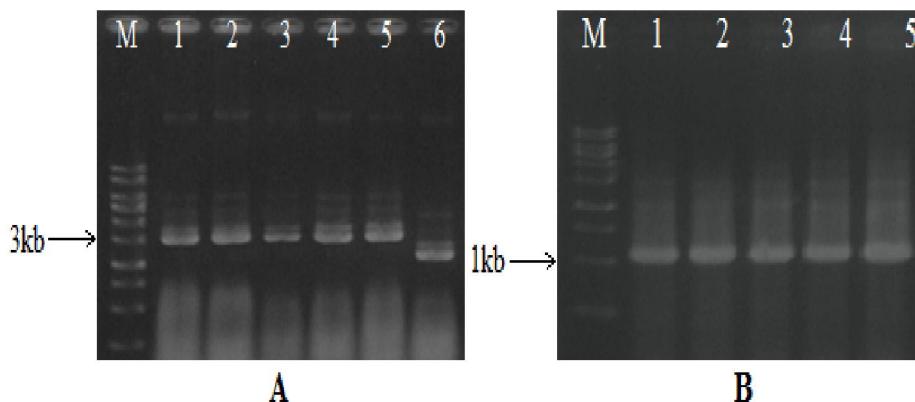


Figure 2. Plasmid pBT – GS1 extracted from *E.coli* (A) and PCR reaction of GS1 (B)

(A: M: Marker 1kb; lane 1 - 5: white clones of *E.coli*; lane 6: blue clone; B: M: Marker 1kb; lane 1 – 5: PCR product amplified of *GS1* from plasmid pBT – GS1)

The plasmids were then used for next step of sequencing. Sequencing results indicated that the sequence of full – length cDNA of *GS1* contained an entire open reading frame (ORF) of 1074 bp, from start codon ATG to stop codon TAA. Compared the sequence of the isolated cDNA of *GS1* with other

sequences on Genebank (Accession number X69822.1 and X74429.1), the similarities are both higher than 99%. This result indicates a high sequence conservation of *GS1* gene.

3.2. Construction of transformation vector pBI121–*GS1*

The binary vector pBI121 was used for vector construction. The T-DNA region of standard binary vector pBI121 contains the cauliflower mosaic virus 35S promoter (CaMV 35S promoter) and NOS terminator which regulate the expression of beta – glucuronidase reporter gene (*gus* gene). After ligating into vector pBI121, the target gene (*GS1*) replaces *gus* gene. Besides, the vector also contains kanamycin - resistant gene (*nptII* gene) which can be used for selection.

The two primers which is used for amplifying *GS1* is conjugated with *Xba*I (in *GS1F*) and *Sac*I (in *GS1R*) restriction enzyme sites. Therefore, the recombinant vector pBT – *GS1* contains *Xba*I and *Sac*I sites in the two ends of *GS1* gene. As the same, vector pBI121 contains *Xba*I and *Sac*I sites in the two ends of *gus* gene. As the consequence, after double

digestion of pBT – *GS1* and pBI121 using *Xba*I/*Sac*I, the result is showed in Figure 3A. Vector pBT – *GS1* was digested into 2 bands, the first band (about 2.7 kb) is the linear backbone of vector pBT, and the another band (about 1.1 kb) is *GS1* gene. Similarly, vector pBI121- *Gus* was also digested into 2 bands, the first one (about 12 kb) is the linear backbone of vector pBI121, and the another one (about 1.8 kb) is *Gus* gene.

We used Gel purification Kit (Fermentas, Korea) to purify the linear vector pBI121 (without *Gus* gene) and the target gene (*GS1*). The result in figure 3B shows that the bands of linear vector pBI121 and target gene *GS1* are very clear, which means that we are successful in purification of vector pBI121 (without *Gus* gene) and *GS1* gene for next steps.

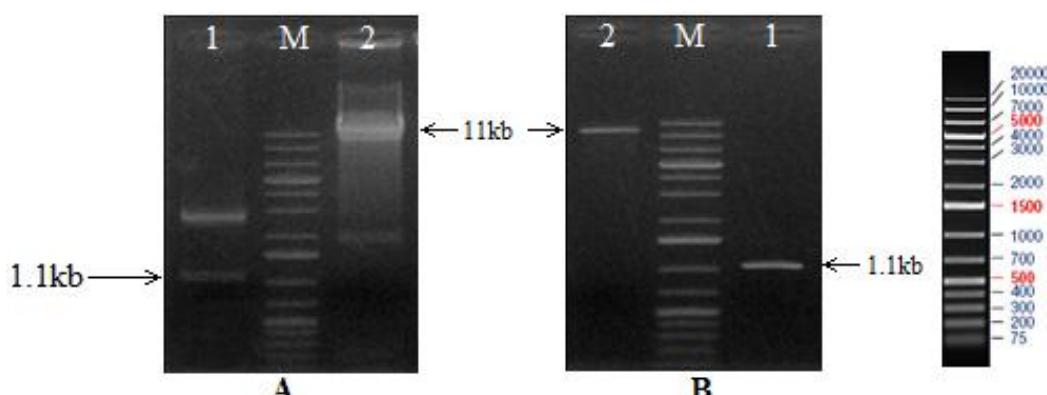


Figure 3. pBI121-Gus and pBT – GS1 after *Xba*I/*Sac*I double digestion (A) and gel purification (B)

(A: M: Marker 1kb; lane 1: pBT - *GS1*; lane 2: pBI121-*Gus*; B: M: Marker 1kb; lane 1: *GS1* gene; lane 2: pBI121- without *Gus* gene)

In the next step, *GS1* gene is ligated into vector pBI121 with the catalysis of T4 DNA ligase. The recombinant vector (pBI121 – *GS1*) was transformed into *E.coli* strain DH5α by heat-shock (42°C for 90 seconds). *E.coli* was then cultured on LB medium which contained 50 mg/l kanamycin. The plasmids which were extracted from survival clones of

E.coli were used for *GS1*- amplified PCR reaction and *Xba*I/*Sac*I double digestion.

The result of PCR reaction in figure 4A shows that we amplified successfully *GS1* gene from plasmid of 11 *E.coli* clones. The *Xba*I/*Sac*I double digestion of plasmid from clone 1 also confirmed the existence of *GS1* gene (figure 4B).

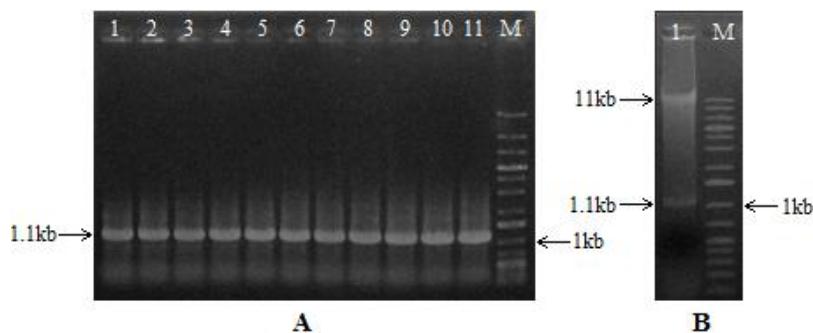


Figure 4. Confirmation of GS1 by PCR reaction (A) and pBI121-GS1 after XbaI/SacI double digestion (B)

(A: M: Marker 1kb; lane 1 – 11: *GS1* amplification from 11 *E.coli* clones; B: M: Marker 1kb; lane 1: *pBI121-GS1* after *Xba*I/*Sac*I double digestion)

IV. CONCLUSION

GS1 gene from mRNA of *Pinus sylvestris* is successfully cloned. The gene has 1074 bp in length from start codon ATG to stop codon TAA. Compared the sequence of the isolated *GS1* with the other sequences on Genebank (Accession number X69822.1 and X74429.1), the similarities are both higher than 99%. This result indicates a high sequence conservation of *GS1* gene.

The transformation vector pBI121 – *GS1* used for next steps of generating of transgenic plants via *Agrobacterium* – mediated transformation is successfully constructed.

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TÁCH DÒNG GEN GS1 MÃ HÓA GLUTAMINE SYNTHETASE 1 VÀ THIẾT KẾ CẤU TRÚC VECTOR CHUYỂN GEN THỰC VẬT

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TÓM TẮT

Glutamine synthetase (GS, EC 6.3.1.2) là enzyme xúc tác cho phản ứng đồng hóa ammonium thành các axit amin. Do đó, GS giữ vai trò thiết yếu trong cơ chế trao đổi nitơ ở thực vật. Ở thực vật bậc cao, GS được chia thành 2 nhóm: GS1 (tồn tại trong tế bào chất) và GS2 (tồn tại trong thê hạt). Gen *GS1* đã được sử dụng trong tạo giống cây trồng biến đổi gen cải thiện khả năng sinh trưởng. Trong nghiên cứu này, để tạo tiền đề cho việc tạo giống cây trồng biến đổi gen có khả năng sinh trưởng nhanh, chúng tôi đã tách dòng thành công gen *GS1* từ mRNA của cây Thông *Pinus sylvestris*. Gen *GS1* đã được tách dòng có kích thước 1.074 bp, có trình tự giống với các trình tự gen *GS1* đã công bố trên Ngân hàng gen quốc tế -NCBI (mức độ tương đồng cao hơn 99%). Gen *GS1* sau đó được gắn vào vector chuyển gen thực vật pBI121 để tạo cấu trúc vector pBI121 - *GS1* (trong đó, gen *GS1* được điều khiển bởi promoter 35S). Cấu trúc vector này sẽ được sử dụng để chuyển gen vào cây thông lâm nghiệp nhằm cải thiện khả năng sinh trưởng.

Từ khóa: Gen *GS1*, glutamine synthetase 1, *Pinus sylvestris*, tách dòng gen, thiết kế vector chuyển gen.

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