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Research Report

Cloning and Expression Analysis of *GmCYP78A5* Promoter

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Abstract: *CYP78A5* promoter was isolated from soybean (*Glycine max* L. Merrill) plant by using PCR technology. DNA sequence alignment indicated that the amplified fragment (1650bp) was 99.21% homologous to the correspondent regions of the reported sequences. Bioinformatics analysis showed that the *GmCYP78A5* promoter contains a lot of inducible or tissue-specific expression elements. RT-PCR results indicated that the gene *GmCYP78A5* highly expressed in immature seed, weekly expressed in stem of soybean, but no expressed in root, leaf and flower. To further study the tissue expression patterns of *GmCYP78A5* gene, the promoter of the gene *GmCYP78A5* was fused with *GUS* reporter gene to construct a plant expression vector and the vector was transformed into tobacco (*Nicotiana tabacum*) by *Agrobacterium*-meditated method. The expression of the *GUS* gene in the transgenic tobacco plants indicated that the *GmCYP78A5* promoter could drive the *GUS* reporter gene to express highly in the leaf, stem, sepal, pedicel, seeds of the transgenic tobacco plants, demonstrating that the expression patterns of the *GmCYP78A5* promoters in soybean and tobacco were inconsistent.

Keywords: Soybean, *GmCYP78A5* Promoter, Tissue Specific Expression, GUS Assay

1. Introduction

The size of plant organs is regulated by genes. The *CYP78A5* is a member of the *CYP78* family encoding cytochrome P450 monooxygenase [1-3]. The expression patterns of the *CYP78A5* was varied at different stages of growth and development in *Arabidopsis*. The *CYP78A5* was mainly expressed in the apical meristem region at vegetative growth stage, but the expression of the *CYP78A5* was unstable at reproductive growth stage [4]. The stem of *Arabidopsis* was distorted and the floral organs were defected when the *CYP78A5* was overexpressed in transgenic *Arabidopsis* [4], but the cell division terminated earlier that resulted in smaller floral, leaf, and stem when the gene was knocked out [5]. Adamski et al. (2009) also found that the overexpression of the *CYP78A5* in *Arabidopsis* caused organ enlargement, otherwise, the organ became smaller when the gene expression was inhibited, thus determining the yield of *Arabidopsis thaliana* [6]. It is obvious that the *CYP78A5* encodes a class of transcription regulators, which is an important gene regulating the size of plant organs and plays a very important role in plant growth and development. However, the upstream regulatory sequence of the *CYP78A5* is seldom studied.

In the previous study, we cloned a *CYP78A5* from soybean. To clarify the tissue expression patterns of the gene, the upstream regulatory sequence of *GmCYP78A5* was taken from GenBank, and the promoter sequence of the gene was amplified by PCR. The expression patterns of the promoter in soybean tissues were analyzed by RT-PCR technology. The promoter fragment was linked with *Gus* reporter gene to construct the recombinant expression vector. The constructer

was transformed into tobacco by *Agrobacterium tumefaciens*-mediated method [7]. The tissue expression patterns of the *CYP78A5* in tobacco were analyzed by GUS assay [8]. The aim was to clarify the expression pattern of promoter of *GmCYP78A5* and provide valuable regulatory elements for crop molecular breeding.

2. Materials and Methods

2.1. Materials

Soybean varieties, plant expression vector pCAMBIA1301S, *E.coli* DH5a and *Agrobacterium* EHA105 strains; clone vector pMD19-T purchased from TaKaRa Company (Dalian); PCR Product Recovery Kit purchased from Omega Company; PCR primers were synthesized by Beijing Sunbiotech co., Ltd. and sequenced in TaKaRa Company (Dalian).

2.2. Analysis of RT-PCR Expression Patterns

RNA was extracted from soybean root, epicotyl, hypocotyl, flower and immature seed by Trizol method [9, 10]. The extracted RNA was used as template to synthesize the cDNA by TaKaRa PrimeScriptTM 1st Strand cDNA Synthesis Kit. RT-PCR primers were designed based on known cDNA sequences, named Rp-F and Rp-R respectively. Actin gene of soybean was used as internal reference in this study, the primers were named Actin-F and Actin-R. The sequences of all primers were listed in Table 1.

PCR reaction conditions were as follows: firstly, pre-denaturation at 94 C for 5 min, denaturation at 94 C for 50 s, annealing at 50 C for 50 s, extension at 72 C for 1.5 min, 30 cycles, and extension at 72 C for 10 min. The PCR product runned electrophoresis in 1% agarose gel, and the expected size of the product was 675bp.

2.3. Extraction of Soybean Genomic DNA and Cloning of Promoter

CYP78A5 (ID: AT1G13710) gene sequence was retrieved from NCBI database, and a pair of specific primers named CYPPF and CYPPR were designed with Primer Premier 5.0 software. The sequence is shown in Table 1.

Table 1. *Number and sequence of primers.*

Name	Sequence
Rp-F	5' AgCATAgggTgAAgAgggA 3'
Rp-R	5' gAAACTCATCCAACTCCACAg 3'
Actin-F	5' ATTggACTCTggTgATggTg3'
Actin-R	5'CTCCTTgCTCATACggTCTg3'
CYPPF	5' TTACCCAAgACACTCggTC 3'
CYPPR	5'gTTgTgCTggAACTAAgAAgAg 3'
Hyg-F	5'TACTTCTACACAgCCATCggTC3'
Hyg-R	5'gCAAggAATCggTCAATACACT3'

Genomic DNA was extracted from young leaves of soybean by CTAB method [11, 12]. The promoter fragment was amplified from soybean genome DNA by PCR and its expected size was 1650 bp. PCR reaction procedure: pre-denaturation at 94 C for 5 min, denaturation at 94 C for 50 s, annealing at 50 C for 50 s, extension at 72 C for 1.5 min, 30 cycles, and extension at 72 C for 10 min. The PCR products isolated from agarose gel were recovered and cloned into 19-T vector by TA clone and sent to TaKaRa Company (Dalian) for sequencing.

2.4. Construction of Plant Expression Vector

The promoter cloned by TA method was digested with *Eco*RⅠand *Pst*Ⅰ, then linked to the upstream of *GUS* gene on the plant expression vector pCAMBIA1301Z, transformed into *E.coli* DH5a competent cells. Colonies were picked up, plasmids were extracted and identified by double digestion with *Eco*RⅠand *Pst*Ⅰ.

2.5. Transformation of Tobacco and Analysis of Transgenic Plants

2.5.1. Agrobacterium Mediated Transformation of Tobacco

The constructed plant expression vector was introduced into Agrobacterium tumefaciens EHA105 by liquid nitrogen freeze-thaw [13]and transformed into tobacco by Agrobacterium-mediated method. Adventitious buds were induced from tobacco leaves treated with Agrobacterium tumefaciens on MS screening medium containing 50mg/L hygromycin (1mg/L 6-BA, 0.1mg/L NAA, 100mg/L carbenicillin). The root inducing medium was 1/2MS containing 50mg/L hygromycin and 80mg/L carbenicillin.

2.5.2. PCR Detection of Transgenic Tobacco Plants

Resistant tobacco plants were detected by PCR using nontransformed tobacco plants as control. The primers were named Hyg-F and Hyg-R. The reaction procedure: pre-denaturation at 94 C for 5 min; denaturation at 94 C for 40 s; annealing at 57.6 C for 40 s; extension at 72 C for 1.5 min; extension at 72 C for 10 min. The expected size of PCR product is 750bp.

2.5.3. GUS Staining

The tobacco tissues were assayed by GUS staining according to Jefferson (1987) method [14].

3. Results

3.1. The Expression Patterns of Different Organs in Soybean

The expression patterns of the *GmCYP78A5* gene in soybean root, epicotyl, hypocotyl, flower, immature seeds and leaves were analyzed by RT-PCR using actin gene as internal reference. The amplified products were assayed by 1% agarose gel electrophoresis. The results showed that the *GmCYP78A5* was expressed differently in different tissues of soybean. The expression level of *GmCYP78A5* gene was higher in immature seed, weakly expressed in epicotyl and hypocotyl, but not in root, leaf and flower (Figure 1).

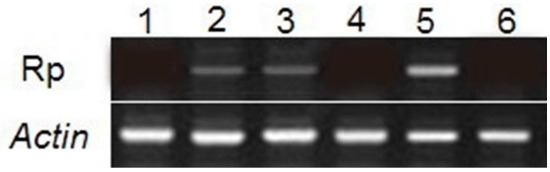


Figure 1. *The expression of GmCYP78A5 gene at the transcriptional level.*

Note: Rp: RT-PCR is performed by using Rp primers; Actin: The actin gene is used as control; 1: root; 2: epicotyl; 3: hypocotyl; 4: flower; 5: Immature seed; 6: leaf

3.2. Cloning and Sequence Analysis of Promoter of the GmCYP78A5

The soybean genome DNA was used as template for PCR amplification, and the promoter fragment about 1.6kb was amplified (Figure 2A). The PCR products were linked to pMD®19-T Vector and the recombinant plasmid, named Rp-P, was identified by enzyme digestion with *Eco*RI and *Pst*I (Figure 2B).

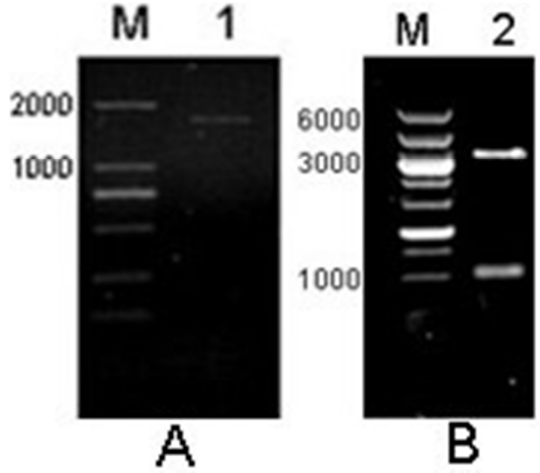


Figure 2. *Cloning of the GmCYP78A5 promoter.*

Note: M: DNA Marker; A: PCR amplification of promoter; B: The recombinant vector is digested with EcoRI and PstI

The positive clone plasmid identified by enzyme digestion was sent to Takara company for sequencing. The result showed that the gene fragment was about 1650 bp, which had 99.21% homology with the promoter sequence of *CYP78A5* on GenBank. The promoter sequence was then analyzed on the promoter cis-element prediction website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). In addition to the basic promoter elements and a large number of CAAT-boxes (related to enhanced transcription efficiency), *GmCYP78A5* promoter also contains TCA-elements responded to salicylic acid, and TA-rich repeat sequences, TGACG-motif, BoxI, Box4, ARE, ERE, Skn-1_motif, MBS, as-2-box, etc. (Table 2, Figure 3), in which as-2-box participated in the specific expression and photoresponse of buds, and Skn-1_motif participated in the endosperm expression of seeds, indicated that the promoter was inducible or tissue-specific expression promoter [15, 16].

Table 2. *Analysis of putative cis-acting elements of GmCYP78A5 promoter.*

***cis*-acting elements**	**Numbers**	**Core sequence**	**Function**
TGACG-motif	2	TGACG	Response for jasmonic acid
as-2-box	1	GATAATGATG	Response for stem-specific expression and light
TCA-element	1	CCATATTTTT	Response for salicylic acid
Box 4	1	ATTAAT	Response for light
Box I	1	TTTCAAA	Response for light
ERE	1	ATTTCAAA	Response for ethylene
Skn-1-motif	1	GTCAT	Response for endosperm expression

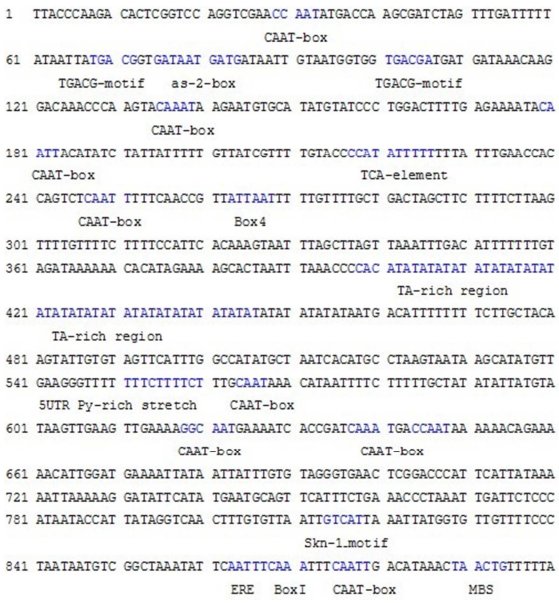
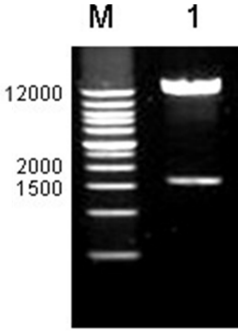


Figure 3. *Bioinformatics analysis of the GmCYP78A5 promoter.*

3.3. Construction of Plant Expression Vector

Recombinant plasmid Rp-P and plant expression vector pCAMBIA1391Z were double digested with *Eco*RIand *Pst*Ienzymes, respectively. The promoter fragment from the Rp-P was linked to the upstream of GUS gene on pCAMBIA1391Z vector by T4 DNA ligase. The constructed vector was identified by double digestion and a specific band of 1.6 kb that was consistent with the expected fragment was obtained (Figure 4), indicated that the plant expression vector that *GUS* gene was driven by the promoter was constructed.

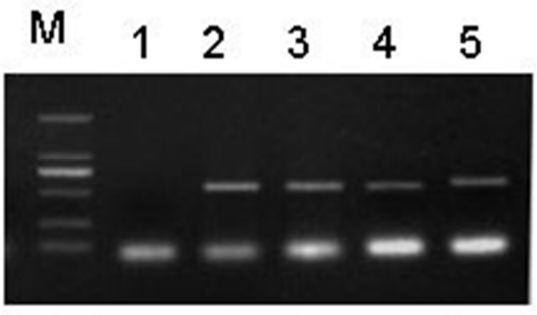


Note: M: DNA Marker; 1: The construct is digested with EcoRI and PstI

Figure 4. *Construction of the expression vector.*

3.4. Identification of Transgenic Tobacco

In order to verify the transgenic tobacco plants, genomic DNA was extracted from the young leaves of hygromycin-resistant tobacco plants, and hygromycin gene was amplified by PCR. The results showed that there was no target band in the untransformed control tobacco plant, but the target band of hygromycin gene was amplified in the hygromycin resistant tobacco plants (Figure 5), proved that the exogenous gene had been integrated into the tobacco genome.



Note: M: DL2000 Marker; 1: control; 2-5: The resistant tobacco plants

Figure 5. *PCR identification of transgenic tobacco plants.*

3.5. Analysis of GUS Gene Expression in Transgenic Tobacco

Three transgenic tobacco lines were selected and the expression patterns of the promoter were analyzed by GUS activity assay. The results showed that GUS gene was highly expressed in the root of transgenic tobacco seedlings (Figure 6A), sepal and stalk (Figure 6B) and seeds (Figure 6C), but no *GUS* gene was expressed in other tissues and organs, indicating that the target promoter had tissue-specific expression characteristics.

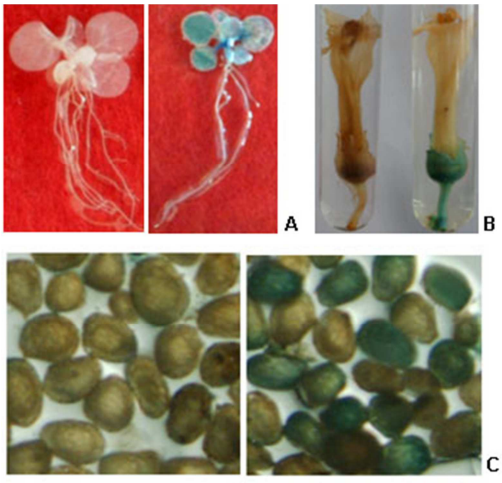


Figure 6. *GUS expression of the transgenic tobacco plants.*

Note: A: Non-transgenic tobacco plantlet（left）and transgenic tobacco plantlet (right); B: Non-transgenic tobacco flower (left) and transgenic tobacco flower (right); C: Non-transgenic tobacco seeds (left) and transgenic tobacco T0 seeds (right)

4. Discussion

In order to clarify the expression patterns of *GmCYP78A5* gene, the promoter fragment of *GmCYP78A5* gene was cloned and its regulatory elements and expression patterns were analyzed. Analysis of promoter cis-element showed that the 5'-site upstream promoter sequence of *GmCYP78A5* gene contained not only basic regulatory elements, but also light and salicylic acid related regulatory elements, as well as bud-specific expression and endosperm-specific expression regulatory elements, suggesting that the promoter may be inducible and tissue-specific promoter. Inducible or tissue-specific promoters can activate the expression of foreign genes under specific conditions, thus overcoming the waste caused by the continuous and efficient expression of constitutive promoters [17, 18].

In order to verify the function of promoter, the gene expression was analyzed by RT-PCR technology. The results showed that *GmCYP78A5* gene was expressed differently in different tissues of soybean. The expression level of *GmCYP78A5* gene was higher in immature seeds, weakly in hypocotyl and epicotyl, but not in root and flower tissues, which was consistent with the results reported by Zondlo and Irish (1999). To further study the tissue expression patterns of *GmCYP78A5* gene, the promoter of *GmCYP78A5* gene was linked to *GUS* gene, constructed a plant expression vector and transformed to tobacco. The results showed that the promoter of *GmCYP78A5* could activate the high expression of *GUS* gene in roots, sepal and flower stalk and seeds of transgenic tobacco seedlings, while the GUS activity was not detected in other parts of transgenic tobacco seedlings, indicating that the promoter had certain tissue expression characteristics. It can be concluded that the expression patterns of the *GmCYP78A5* promoter in tobacco and soybean has the same way, but also has a different way. The same is that it can be highly expressed in seeds, indicating that the promoter has seed-specific high-level expression; the difference is that the promoter can be expressed in tobacco flowers but not in soybean flowers, showing differences in expression, which may be due to different plant materials.

5. Conclusion

A promoter of *CYP78A5* was cloned from soybean (*Glycine max* L. Merrill). RT-PCR results showed that the *GmCYP78A5* was expressed highly in soybean immature seed and weakly in epicotyl and hypocotyl, but not in root, leaf and flower. The expression of the *GUS* in the transgenic tobacco indicated that the *GmCYP78A5* promoter could drive the *GUS* to express highly in the leaf, stem, sepal, pedicel, seeds of the transgenic tobacco plants.

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