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Molecular cloning and activity analysis of a seed-specific *FAD2-1B* gene promoter from *Glycine max*

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Abstract

Microsomal omega-6 fatty acid desaturase (*FAD2-1B*) is an enzyme that regulates the polyunsaturated fatty acid content in soybeans (*Glycine max*). In this study, the *FAD2-1B* gene was determined to be highly expressed in soybean seeds using quantitative real-time PCR(qRT-PCR). To investigate the expression pattern and activity of the *FAD2-1B* promoter, a 1929 bp 5'-upstream genomic DNA fragment, named PF, was isolated according to the soybean genomic sequence. Sequence analysis revealed the presence of many motifs related to seed-specific promoters in the PF fragment, such as E-box, SEF4, Skn-1 motif, AACACA, AATAAA and so on. Tobacco transgenics carrying the *gus* reporter gene driven by the PF and/or 35S promoters were confirmed by PCR and RT-PCR. qRT-PCR and histochemical GUS assays showed that the PF promoter could regulate *gus* gene accumulation in seeds and the expression level was higher than in other organs. In the meantime, it exhibited similar activity to the 35S promoter in seeds, which could be associated with seed-related *cis*-elements found in the 1-248 bp, 451-932 bp, and 1627-1803 bp regions of the promoter.

Key words: *FAD2-1B* gene, *Glycine max*, promoter, transgenic tobacco.

Introduction

The soybean is an important grain and oil crop, which is a type of nutrition-balanced food rich in proteins, lipids, vitamins and minerals. It is also a renewable source of biofuel and chemical production (1), and its components, such as saponin (2), isoflavones and lecithin (3), have medicinal value. However, the utilization of soybean seeds is often limited in soymilk because of the beany flavor (4) and in animal feed because of the anti-nutritional factors (5). The primary consideration for breeding new cultivars to increase or decrease the levels of certain substances in a genetically modified soybean is to select suitable promoter. A seed-specific promoter can regulate the expression of exogenous genes exclusively in the seed. For instance, a significant increase in oil content was observed in transgenic tobacco seeds under the control of a seed-specific Napin promoter (6), and phytic acid biosynthesis in transgenic rice was effectively suppressed under the control of the rice Ole18 promoter (7). Thus, use of a seed-specific promoter is an indispensable means to obtain a desired soybean cultivar.

As previously reported, there are some problems in the specificity and activity of seed-specific promoters. For instance, the activity of the USP promoter from *Vicia faba* in a transgenic *Arabidopsis thaliana* is detectable only in the embryo, but USP promoter activity in a transgenic pea is detected in pollen and cotyledons (8). The activity of α-globulin B gene promoter is highly seed-specific in cotton, but the activity was only 16.7% in Arabidopsis compared to in cotton seeds, and it was even less than 1% of cotton seeds in tobacco (9). Thus, it is necessary to select a native soybean seed-specific promoter in soybean transgenic engineering.

Microsomal omega-6 fatty acid desaturase (FAD2) is a enzyme that can alter the desaturation degree required for converting monounsaturated oleic acid to polyunsaturated linoleic acid (10). FAD2 has been characterized in several plants, and one or more FAD2 isoforms exist depending on the particular plant (11). There are two different FAD2 genes in the soybean, *FAD2-1* and *FAD2-2*. The *FAD2-1B* gene has been shown to be specifically expressed in the developing soybean seeds by semi-quantitative RT-PCR (12). Therefore, it is possible that the promoter regulated *FAD2-1B* gene can enhance gene expression in soybean seeds.

In this study, we found that the activity of *FAD2-1B* was extremely high only in soybean seeds, which was consistent with previously published data. To investigate the activity of the *FAD2-1B* promoter, we isolated the 5'-flanking sequence of *FAD2-1B* and characterized it using stable *gus* reporter gene expression in tobacco.

Materials and methods

Plant Materials

Glycine max (Jidou2), a species cultivated widely in northeastern China, was grown outdoors under natural conditions from early May to late September. The tobacco (*Nicotiana tabacum*) plants used for transformation were cultured in Murashige and Skoog (MS) solid medium at 25°C under a 14-h light /10-h dark. The sterilized seedlings of the transformed tobacco were transplanted in soil under greenhouse cultivation after roots developed.

QRT-PCR

Total RNA was extracted from the roots, stems, leaves, flowers, and immatured seeds of soybean collec-

ted on the 30th day after flowering (DAF) and from the matured seeds collected on the 90th DAF with RNAiso Reagent (Takara, China). The cDNA was synthesized with Reverse Transcriptase M-MLV and the oligo $(T)_{18}$ primer (Takara) by use of total RNA samples as the templates. qRT-PCR reactions were performed on an Agilent M×3000P instrument. The soybean *β*-tubulin (GenBank acc. no. GMU12286) was selected as a reference gene (forward: 5'-GGAAGGCTTTCTTGCATTGGTA-3'; reverse: 5'-AGTGGCATCCTGGTACTGC-3'). To detect the relative quantities of *FAD2-1B* gene expression among different organs, and a set of primers (forward: 5'-GTGGCTCACCATCTTTTCTCTA-3'; reverse: 5'-ACCAATACACGCCCTTCTCG-3') was designed according to the soybean *FAD2-1B* gene sequence (GenBank acc. No. DQ532370).

Promoter Cloning and bioinformatic prediction

Soybean genomic DNA was isolated from the leaves using a Genomic DNA Mini Preparation Kit (Anygen, China). According to the soybean genome (http://www.phytozome.net/soybean) and the soybean *FAD2-1B* gene sequence (GenBank acc. No. DQ532370), a pair of primers containing the *Pst*I and *Nco*I restriction enzyme sites (forward, F_1: 5'-GGGCTGCAGTGGTGTGCTTACTCACAAAGC-3'; reverse, F_2: 5'-GGGCCATGGGCCTAGTGGCTTGTAGTATCATTTC-3') was designed to amplify the 5' upstream sequence, approximately 1929 bp, which contains the putative promoter region of the *FAD2-1B* gene, named PF. Then, the PCR product was cloned into a pMD-18T vector, which was the recombinant plasmid pMD18-T-PF and was sequenced.

Combined with the promoter characteristics of eukaryotic cells, the BDGP (http//:www.fruitfly.org/) and SoftBerry-TSSP (http://www.softberry.com) websites were used to analyze the putative transcription start site. The *cis*-acting elements were predicted by the PLACE (http//:www. dna.affrc.go.jp/PLACE) website.

Construction of the expression vector and transformation of tobacco

The putative promoter fragment PF was inserted into the pCAMBIA1301 vector through digestion with *Pst*I and *Nco*I, replacing the 35S promoter upstream of *GUS* gene, which completed construction of the expression vector, named pCAM-PF.

The tobacco plant seeds were surface sterilized and grown on MS medium for approximately 30 days. The tobacco was then transformed with the introduction of the expression vectors pCAM-PF and pCAMBIA1301 into the *Agrobacterium tumefaciens* strain EHA105 via the leaf discs method. The seeds of regenerated plants were screened on MS medium with hygromycin. Regenerated plants were tested for the presence of the inserted putative promoters using PCR amplification of genomic DNA, and RT-PCR detection was performed according to the *gus* gene sequence.

Expression pattern analysis of the PF promoter

The expression pattern and activity among different organs and developmental stages of the PF promoter fragment were identified with qRT-PCR and GUS histochemical assay. The transcription level of the *GUS* was measured by qRT-PCR with the positive T_1 transformants various organs of the pCAM-PF vector. The tobacco *EF-1α* gene-specific forward (5'-TGAGATGCACCACGAAGCTC-3') and reverse (5'-CCAACATTGTCACCAGGAAGTG-3') primers were used to normalize the amount of total mRNA in all samples. The *gus* gene -specific forward (5'-GTAGAAACCCCAACCCGTGAA-3') and reverse (5'-CGTAATGAGTGACCGCATCGA-3') primers were designed according to the *gus* gene sequence (GenBank acc. No. AF354046). The experimental methods for total RNA extracted, cDNA synthesis and qRT-PCR were identical to the ones on the quantification of *FAD2-1B* gene in soybean organs.

GUS histochemical assay was performed according the Jefferson's method (13). The roots, stems, leaves, flowers and seeds of T_1 positive transgenic tobacco plants were incubated in GUS straining buffer containing 2 mM 5-bromo-4chloro-3indolyl-β-D-glucuronide, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 10 mM EDTA, 0.1% Triton-X 100 and 50 mM sodium phosphate buffer pH 7.0 for 10-12 h at 37°C. After staining, the materials were rinsed in ethanol to clear the chlorophyll, and images were then taken using a stereomicroscope (Nikon, Japan).

Statistical analysis

The statistical analysis of the relative amounts of *FAD2-1B* gene expression in soybean organs and the relative amounts of *gus* gene expression in tobacco organs were performed using the SPSS 20.0 (SPSS, Chicago, IL, USA) software. The data represented the mean of three independent experiments. Significant differences were shown by $P < 0.05$ and $P < 0.01$.

Results

Organ specific expression of the FAD2-1B gene

Because qRT-PCR is sensitive, quantitative and direct, it has become a preferred method for detecting gene expression patterns and activity. The relative quantities of the *FAD2-1B* gene expression in different organs and at different developmental stages were determined using qRT-PCR analysis and were shown in Figure 1. The results revealed that the relative *FAD2-1B* gene expression levels in the stems, leaves, flowers, immatured seeds and matured seeds were 0.90-, 31.30-, 0.89-, 4598.00- and 2389.00-fold compared to the expression level of roots, respectively. The relative amounts of *FAD2-1B* gene expression in the matured seeds or in the immatured seeds were extremely high compared to the other organs ($P<0.01$), which demonstrated that the *FAD2-1B* gene had a strictly seed-specific expression and extremely high activity in seeds.

Promoter Isolation

Some *cis*-acting elements, especially the enhancer, are sometimes located in the distant upstream sequence of the ATG, so a 5'-upstream 1929 bp fragment (PF: GenBank acc. No. JN982131) from the ATG of the *FAD2-1B* gene was cloned in this experiment. Comparison of PF with the corresponding fragment of the soybean genome showed 100% homology, which indicated that there was no difference between varieties.

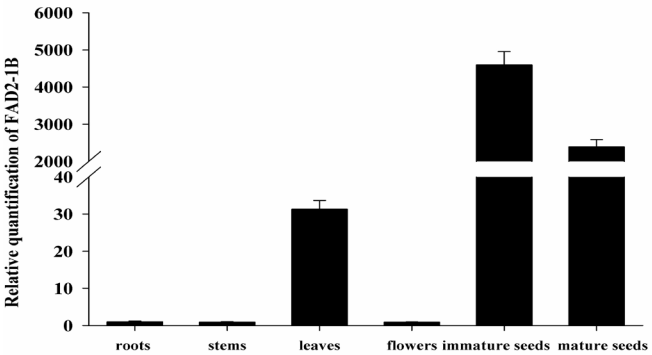

Figure 1. The relative quantification of *FAD2-1B* gene by qRT-PCR with three independent soybean lines. Each value represents the average of three measurements, with error bars representing standard deviation.

Bioinformatics Analysis of the PF Promoter

The presumed transcription start site (TSS) designated as +1 was in the 1883 bp (Figure 2). The PF promoter sequence contained a typical conserved TATA-box and CAAT-box (14-15), and more *cis*-elements related to seed-specific expression were also included, such as an element involved in triacylglycerol synthesis and presented in seed-specific promoters (E-box) (16), an embryo-related element (SEF4) (17), seed-specific elements (CCAA, ACGT, AACA, AACACA and Skn-1 motif) (18-22), a motif found in a seed germination-related gene promoter (AATAAA) (23) and the prolamin box, which is involved in the quantitative regulation of prolamin expression (24-25).

Expression pattern characterization of the PF promoter in transgenic tobacco

In transgenic tobacco plants, the qRT-PCR analysis of the stems, leaves, flowers and seeds showed that the

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1    TGGTGTGCTTACTCACAAAGCAGTCTTACACTAATCTCGAAATAAACTTATCAGATGGTCGAAAATCCTT
                                          AATAAA      E-box
71   TGGCACGTTAAAACACGTCGTACAAAAGATGCAGCTGATCTGATTCTCCACTTGTCTCAAGCAGACATCA
       ACGT    AACA/ACGT              E-box          E-box
141  CACTGATCACAGGTGGAAACCAAATTTGCCTAAGTTCCAAGGCCTCGGTGTGACTCAGCCCCAAGTGACG
                E-box                                                E-box
211  CCAACCAAACGCGTCCTAACTAAGGTGTAGAAGAAACAGATAGTATATAAGTATACCATATAAGAGGAGA
     CCAA/CCAA                          AACA                          AATAAA
281  GTGAGTGGAGAAGCACTTCTCCTTTTTTTTTTCTCTGTTGAAATTGAAAGTGTTTTCCGGGAAATAAATA
                                                                   AATAAA
351  AAATAAATTAAAATCTTACACACTCTAGGTAGGTACTTCTAATTTAATCCACACTTTGACTCTATATATG
       AATAAA
421  TTTTAAAAATAATTATAATGCGTACTTACTTTCTCATTATACTAAATTTAACATCGATGATTTTATTTTC
                                                  AACA
491  TGTTTCTCTTCTTTCCACCTACATACATCCCCAAAATTTAGGGTGCAATTTTAAGTTTATTAACACATGTT
                                   CCAA                          AACACA SEF4
561  TTTAGCTGCATGCTGCCTTTGTGTGTGCTCACCAAATTGCATTCTTCTCTTTATATGTTGTATTTGAATT
                                   CCAA
631  TTCACACCATATGTAAACAAGATTACGTACGTGTCCATGATCAAATACAAATGCTGTCTTATACTGGCAA
           E-box/prolamin box    ACGT/ACGT                E-box
701  TTTGATAAACAGCCGTCCATTTTTTCTTTTTCTCTTTAACTATATATGCTCTAGAATCTCTGAAGATTCC
         AACA
771  TCTGCCATCGAATTTCTTTCTTGGTAACAACGTCGTCGTTATGTTATTATTTTATTCTATTTTATTTTA
                                  AACA/ACGT                          SEF4
841  TCATATATATTTCTTATTTTGTTCGAAGTATGTCATATTTTGATCGTGACAATTAGATTGTCATGTAGGA
     Skn-1 motif                  Skn-1 motif                  Skn-1 motif
911  GTAGGAATATCACTTTAAAACATGATTAGTCTGTAGGCAATATTGTCTTCTTTTTCCTCCTTTATTAAT
                          AACA
981  ATATTTTGTCGAAGTTTTACCACAAGGTTGATTCGCTTTTTTTGTCCCTTTCTCTTGTTCTTTTTACCTC

1051 AGGTATTTTAGTCTTTCATGGATTATAAGATCACTGAGAAGTGTATGCATGTAATACTAAGCACCATAGC

1121 TGTTCTGCTTGAATTTATTTGTGTGTAAATTGTAATGTTTCAGCGTTGGCTTTCCCTGTAGCTGCTACAA

1191 TGGTACTGTATATCTATTTTTTGCATTGTTTTCATTTTTTCTTTTACTTAATCTTCATTGCTTTGAAATT

1261 AATAAAACAATATAATATAGTTTGAACTTTGAACTATTGCCTATTCATGTAATTAACTTATTCACTGACT
       AATAAA/AACA
1331 CTTATTGTTTTTCTGGTAGAATTCATTTTAAATTGAAGGATAAATTAAGAGGCAATACTTGTAAATTGAC

1401 CTGTCATAATTACACAGGACCCTGTTTTGTGCCTTTTTGTCTCTGTCTTTGGTTTTGCATGTTAGCCTCA
       Skn-1 motif
1471 CACAGATATTTAGTAGTTGTTCTGCATACAAGCCTCACACGTATACTAAACCAGTGGACCTCAAAGTCAT
                                                ACGT                Skn-1 motif
1541 GGCCTTACACCTATTGCATGCGAGTCTGTGACACAACCCCTGGTTTCCATATTGCAATGTGCTAOGCCGT

1611 CGTCCTTGTTTGTTTCCATATGTATATTGATACCATCAAATTATTATATCATTTATATGGTCTGGACCAT
                          E-box                          Skn-1 motif
1681 TACGTGTACTCTTTATGACATGTAATTGAGTTTTTTAATTAAAAAAATCAATGAAATTTAACTACGTAGC
       ACGT                                          CAAT-box          ACGT
1751 ATCATATAGAGATAATTGACTAGAAATTTGATGACTTATTCTTTCCTAATCATATTTTCTTGTATTGATA
       Skn-1 motif                                   Skn-1 motif
1821 GCCCCGCTGTCCCTTTTAAACTCCCGAGAGAGTATAAAAACTGCATCGAATATTACAAGATGCACTCTTGT
                                  TATA-box                          +1
1891 CAAATGAAGGGGGGGAAATGATACTACAAGCCACTAGGCATG
       E-box
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Figure 2. Regulatory elements analysis of the PF promoter. Putative TSS is indicated with +1. All putative *cis*-elements are underlined.

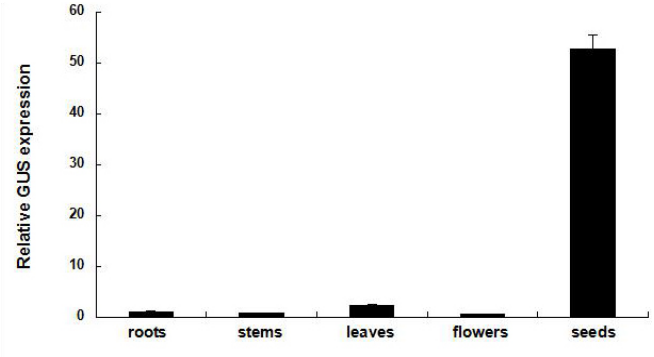

Figure 3. Relative GUS expression by qRT-PCR with three independent lines of the positive T_1 transformants with the pCAM-PF vector. Each value represents the average of three measurements, with error bars representing standard deviation.

expression levels of *gus* were 0.72-, 2.31-, 0.46- and 52.59-fold compared to the roots expression levels (Figure 3), respectively. The relative amount of *gus* gene expression in seeds was higher than the other organs (P<0.01), demonstrated that PF promoter had the characteristics of higher expression in the seed.

Several positive T_1 transformants with the pCAM-PF and pCAMBIA1301 vectors were characterized, and were used to perform histochemical assays to check the GUS activity in different organs. The results showed that GUS activity was well detected in the roots, stems , leaves, flowers and seeds of transgenic tobacco plants transformed by the pCaMV35S vector, confirming the constitutive expression of the CaMV 35S promoter in higher plants (Figure 4c), while no staining was observed in all organs from the non-transgenic control plants (Figure 4a). As shown in Figure 4b, GUS was slightly expressed in the roots, stems, leaves, and flowers, but strongly expressed in seeds, where the stain intensity was similar to that of the expression in the seeds of the pCaMV35S vector transformants, indicating that the PF promoter conferred a little activity in the stems, roots, leaves, and flowers, but the activity of the PF promoter in seeds was similar to the 35S promoter activity.

Discussion

The soybean *FAD2-1B* gene, which plays a predominant role in determining the polyunsaturated fatty acid content of the seed-storage oil, is one of the important

Figure 4. Histochemical analysis of GUS expression in transgenic tobacco. a. Wild type; b. GUS expression was driven by PF promoter; c. GUS expression was driven by 35S constitutive promoter.

seed quality genes. This may be related to its high expression in the soybean seed (Figure 1), and the expression activity of the *FAD2-1B* gene is inseparable from its upstream promoter sequence.

In this study, the PF promoter activity was determined though detection of *gus* gene and GUS activity in different organs of positive transgenic tobacco after stable expression with an expression vector. The PF promoter had tissue-specific expression characteristics, with higher expression in the seed and the expression activity of the PF promoter in the seed was similar to the 35S promoter. The PF promoter is derived from soybean, which may have much higher activity in soybean seeds. Therefore, the important theoretical basis was provided by this work for studying the PF promoter activity in soybean organs. The PF promoter could be a useful tool in cultivating new soybean varieties.

PF promoter activity is related to the *cis*-elements in its sequence. Combining prediction software, our knowledge of the characteristics of eukaryotic cells and the study by Li et al (12), the presumed TSS was located at 1883 bp and designated as +1 (Figure 2). Therefore, the true promoter region of PF promoter fragment was from 1 bp to 1882 bp. In addition to the typical TATA-box and CAAT-box motifs, all other *cis*-elements were related to seed-specific promoters, which were mainly located at 1-248 bp, 451-932 bp, and 1627-1803 bp. E-box was present five times at 1-248 bp, which help in the activation of seed-specific promoters for heterologous expression (19); Skn-1 motif was present three times at 451-932 bp and 1627-1803bp respectively, which are required for endosperm expression (26); SEF4 was present two times at 451-932 bp, which are recgnized by embryo-specific proteins such as soybean α′-conglycinin(23). The amounts and the locations of the seed-specific related elements may account for the high expression activity of the PF promoter in seeds. These three short sequences would provide a theoretical basis for studying core *cis*-elements with functional deletion analysis and the related transcription factors with a yeast hybrid library construction.

Acknowledgments

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