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Isolation and Computer Analysis of the 5′-Regulatory Region of the Seed Storage Protein Gene from Buckwheat (*Fagopyrum esculentum* Moench)

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Using the modified rapid amplification of cDNA ends (5′-RACE) approach, a fragment containing the 955 bp long 5′-regulatory region of the buckwheat storage globulin gene (*FeLEG1*) has been amplified from the genomic DNA of buckwheat. The entire fragment was sequenced, and the sequence was analyzed by computer prediction of cis-regulatory elements possibly involved in tissue-specific and developmentally controlled seed storage protein gene expression. The promoter obtained might be interesting not only for fundamental research but also as a useful tool for biotechnological application.

KEYWORDS: Buckwheat; promoter; seed storage

INTRODUCTION

Legumins, or 11−13S globulins, are a major class of seed storage proteins that were first characterized in Fabaceae (*1*) and are widespread in both angiosperms and gymnosperms, including different conifers (*2*). The expression of these proteins is temporally regulated during embryogenesis and is restricted to seed tissues such as cotyledons or endosperm (*3*). Thus, globulin storage proteins provide an excellent model for the study of plant gene regulatory mechanisms (*4*−*6*). Their corresponding mRNAs accumulate to high levels during the maturation phase and are mainly under transcriptional regulation (*7*). The midmaturation stage of seed development in many monocots and dicots was characterized by a superabundant level of mRNAs for storage proteins (*4*). Therefore, storage proteins could be taken as marker genes/proteins for late embryogenesis.

The regulatory sequences of the storage protein genes are probably the most studied class of promoters. One reason is the broad spectrum of genes available, as the storage proteins are often present in large families. Another reason is the apparent practical utility of seed specific promoters in crop improvement, production of valuable proteins, or modification of seed storage compounds (*8*).

Storage proteins of buckwheat seed are divided into two classes: salt-soluble globulins, forming 70% of total seed proteins, and water-soluble 2S albumins (*9*, *10*). The main storage protein of buckwheat, 13S globulin, resembles the legumin-like seed storage proteins of other species, whereas a minor 8S globulin has a structure common for all vicilin-like storage proteins (*11*). In our previous paper (*12*), it was shown that storage polypeptides start to dominate the total protein spectrum of buckwheat seed from 14 days after flowering (DAF). From that stage to full maturation, the level of the total storage protein per seed increases dramatically to >80% of total proteins by the end of development. Progressive accumulation proceeds simultaneously for all storage protein fractions.

In our previous paper (*13*) we isolated and characterized a full-length cDNA (*FeLEG643*, database accession AY256960) for the legumin-like storage polypeptide from buckwheat seed and compared its deduced amino acid sequence with those from different representatives of dicots, monocots, and gymnosperms. *FeLEG643* was also used as a probe to define the specific expression profile throughout buckwheat seed development. In addition, the exon/intron structure of the corresponding gene, which was the first genomic clone for storage proteins isolated from buckwheat (*FeLEG1*, version AY359286.1), was analyzed. The analysis of both contributed to molecular evolution studies.

In this paper we concentrated on the 5′-regulatory region of the same gene and showed the procedure used for its isolation and cloning, as well as computer analysis of the sequence to define the cis-regulatory elements potentially involved in the regulation of developmental and tissue-specific gene expression.

MATERIALS AND METHODS

Plant Material. Buckwheat (*Fagopyrum esculentum* Moench cv. Darja) was field-grown in the garden of the Institute of Molecular Genetics and Genetic Engineering. Mature seeds were collected and used for DNA isolation.

DNA Isolation. Genomic DNA was isolated as described by Dellaporta (*14*). Plasmid DNA was isolated using a QIAprep Spin Miniprep kit (Qiagen).

Isolation of the 5′-Regulatory Region of *FeLEG1* and Cloning Procedure. The modified 5′-RACE method (*15*) using the Marathon cDNA system (Clontech) was employed for the isolation of the 5′-regulatory region of *FeLEG1*. The genomic DNA was first digested

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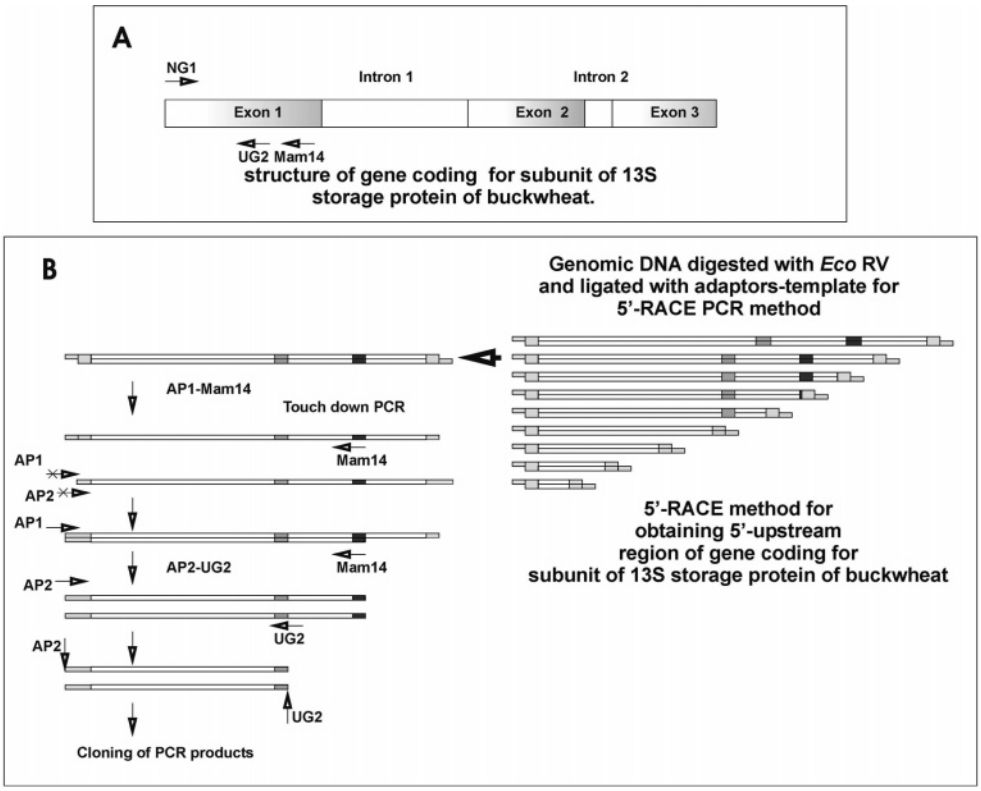
Scheme 1. 5′-RACE Method Applied for Isolation of the 5′-Regulatory Region of *FeLEG1*: (**A**) *FeLEG1* with Primer Positions UG2, Mam14, and NG1; (B) Isolation Procedure with Marathon Adaptor Primers AP1/AP2 and Gene-Specific Primers



with *Eco*RV. Then 1 μg of digested DNA restricted to 2−12 kb in length was ligated to a 200-fold molar excess of Marathon cDNA adaptor. The adaptor-ligated genomic DNA was used as the template for touchdown Polymerase Chain Reaction (PCR) amplification (Biometra T1 Thermocycler) in 50 μL reaction mixtures containing Advantage 2 polymerase mix, adaptor primer 1 (AP1), and gene-specific primer Mam14 (5′-GGCGACACCAGCACACTGGAACTGAT-3′), derived from the *FeLEG1* gene sequence and positioned 204 bp downstream from the ATG start codon. Reaction conditions were 94 °C for 3 min, (94 °C for 30 s; 72 °C for 4 min) × 5 cycles, (94 °C for 30 s; 70 °C for 4 min) × 5 cycles, (94 °C for 30 s; 68 °C for 4 min) × 20 cycles. Nested PCR amplification was performed with Nested Adaptor Primer 2 (AP2) and gene-specific primer UG2 (5′-ATTGGGCTGAGACGAGGTAAGTTGG-3′), positioned 126 bp downstream from ATG. Reaction conditions were 94 °C for 3 min, (94 °C for 15 s; 68 °C for 4 min) × 25 cycles, 72 °C for 10 min. The specific PCR products obtained were identified by Southern blot analysis using a gene-specific probe and then were eluted and cloned into the pGEM-T vector using the pGEM-T Vector System I (Promega). The specific clone representing the 5′-directed extension of the *FeLEG1* gene was finally identified by sequencing among several isolated clones. The complete nucleotide sequence of the 1081 bp long genomic fragment is available from the GeneBank database under accession no. AY359286 as an adaptation/elongation of the first notified sequence representing the coding region of the *FeLEG1* gene.

Southern Blot Analysis. After electrophoresis on agarose gel, DNA was depurinated in 0.25 M HCl for 15 min and denatured in 1.5 M NaCl/0.5 M NaOH for 30 min. Neutralization was performed twice in neutralizing buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2; 0.0001 M EDTA, pH 8) for 15 min. DNA was then blotted on a Pall Biodyne A membrane (Pall Corp.) in 10× SSC buffer (1.5 M NaCl; 0.15 M sodium citrate) overnight. DNA was fixed on the membrane by baking at 80 °C for 2 h.

Hybridization was performed overnight in hybridization buffer (5× SSC buffer: 0.1% *N*-laurylsarkosine, 0.02% SDs, and 1% casein) at 65 °C, using as the probe 204 bp from the 5′-region of clone *FeLEG1*, obtained by PCR with two gene-specific primers, Mam14 and NG1 (5′-G**ATG**CTTCATGGGGTGCTTCTATG-3′). The probe was labeled by the BioPrime DNA labeling system (Invitrogen). After the membrane had been washed in SSC buffers of decreasing ionic strengths, followed by incubation in 3% BSA solution at 65 °C for 1 h and incubation with streptavidine−alkaline phosphatase at room temperature for 15 min, the membrane was incubated with the BCIP-dye NBT (BRL) at room temperature for 15 min. After visualization of signals, the reaction was stopped by placing the membrane in Tris−EDTA buffer (20 mM Tris, pH 7.5; 0.5 mM EDTA).

DNA Sequencing. Both strands of DNA were sequenced according to the Sanger method using an automated laser fluorescence DNA sequencer,

Computer-Assisted Analysis. The obtained nucleotide sequence was subjected to the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) for sequence alignment; the Promoter Prediction program (http://www.fruitfly.org) for eukaryotic promoter prediction; Softberry (www.softberry.com); as well as PLACE (http://www.dna.affrc.go.jp)

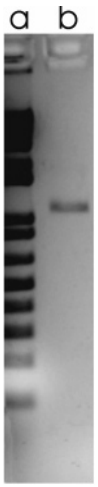


Figure 1. Electrophoretogram of the 5′-RACE PCR product: (**a**) DNA standard, 100 bp leader; (**b**) product of amplification.

Figure 2. Southern blot analysis of the 5′-RACE PCR product: (**a**) product of amplification; (**b**) PCR fragment NG1/Mam14 used as a probe-positive control.

and PlantCARE (http://sphinx.rug.ac.be:8080) databases for prediction of the promoter regulatory elements.

RESULTS AND DISCUSSION

Because no buckwheat genomic library was available, we applied the modified 5′-RACE method to isolate the 5′-regulatory region of the previously cloned and sequenced buckwheat legumin gene. The gene-specific primers, Mam14 and UG2, were designed according to the sequence of the *FeLEG1* representing a coding sequence of the buckwheat 13S storage globulin subunit gene. The positions of the primers and cloning strategy are presented in **Scheme 1**.

After the nested PCR protocol, the products of PCR amplification were analyzed by agarose gel electrophoresis (**Figure 1**). The high specificity of nested PCR with two gene-specific primers resulted in only one defined band ∼1 kb in length. The products of amplification were then analyzed by Southern blot with the 204 bp probe, representing the 5′-end of the *FeLEG1* (**Figure 2**).

The band that positively hybridized to the specific probe was eluted from the gel and introduced into the pGEM-T vector. After transformation of the XL1-blue *Escherichia coli* strain, several clones containing gene-specific inserts were isolated and further characterized. The identities of the inserts were finally confirmed by DNA sequencing. It was noted that a 126 bp 3′ part of the sequence was in complete concordance with the 5′ sequence of the *FeLEG1*, which was expected considering the position of the primers chosen for PCR amplification. That confirmed the identity of the cloned buckwheat genomic fragment as one containing the 5′-regulatory region of the defined buckwheat gene, which could be further analyzed. The sequence of a potential promoter region, 955 bp long, was analyzed by several computer programs mentioned under Material and Methods. Potentially important promoter elements are marked on the DNA sequence shown in **Figure 3**.

Although the transcriptional start site (TSS) has not been determined experimentally by primer extension, a potential TSS was predicted by the Promoter Prediction program 58 bp upstream from the ATG codon. The defined consensus sequence was also found in the promoter region of several storage protein genes of dicot plants (*Coffea arabica*, *Cicer arietinum* L., *Glycine max*, *Pisum sativum*, and *Vicia faba*) (**Figure 4**). Consequently, a TATAAA motif, located 26 bp upstream from the predicted transcriptional start site, is considered to be a likely candidate for a typical TATA box (*16*).

According to the PlantCare and PLACE databases, several cis-regulatory elements that had been shown to confer high levels of seed-specific expression in other species were predicted in the investigated region of the buckwheat *FeLEG1* gene. First,

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-955      CGCCCGGGCAGGTATCTCGGCTAGAGTCGTGTGAATTAAGGCTTAGGGTTTTCTC

-900 TAAGAAATTAAAGGCCGTCTTTATAATGATCTTGGATAAAACTATCTCTTAAAAATCTTA

-840 TCTTTTAACATTAATCTTTATTAAAAAATAAATTTTATTATCTAATAAATACGCACTCCC

-780 ATGCTATATCAAATTTGTTATTATAGCATATATATATATATTATATTTTCTTATTAAATG
                                                          ABRE
-720 ATCCATCTATCAACCCAATATGTGTGACTTTGTACAACCCGATAATAATAAACGTGAACT

-660 AATTTAGACCGTTGAATAAATTCAATCAACTACTAATAATGCTCCATAATTGATTACCAC

-600 AAGATCACTCCTTACTTAAACTACTTTCTTTATTAATTCTCTATAATAGTTTTTTCTTTT

-540 TATTTGTTTTAACTTCATACAAATTTCTTTTTTGGTGTGGTTGACTTCAATATGTTGATT

-480 TAATACCAAAATAGTGCGATTTGATCACAATTACATTTTTAATATCATAAAGTTATTAAT

-420 TGATTAAAAACTCTCAGTTTTAGATGCATTTATTTTATTTTTTAACATAATGACATCAAT
                                  RY
-360 TAAAATTTGAAAGTGCAAATCACATGCAACTCTTAGTCCAAAAACCCTCCATCATTTTCA
                    RY
-300 AAGACTTAAATGCATGCAAATTTGTCCCTTTTATGATCAGAAGCCTCTCGGCAAATGTAA
                                                          ABRE
-240 CCCTAAAACACAAACCTTTACCTCACAAATCAACAACACACACTCTTCAGTCCTCCCCAC
     ABRE                                 ABRE ABRE
-180 GTAGTCTCCTCTTTCCAACACATAACTTTCCTCTCCACGTACGTACTCGGTACTCATATG
                                    TATA
-120 TGGCATCTAAAAACTGCACTCAAAACCCATTATAAATACACCCGCAACACTACCTATCAA

 -60 ACCGCATCCAACAAACCTAAACCTCCAAAATACAATAGCACAACAATGAAGGGTTCTAAG
     ATG
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Figure 3. Nucleotide sequence of the 5′-regulatory region of *FeLEG1*. Positions of predicted cis-regulatory elements: RY and ABRE are in boxes; G-box is underlined; potential transcriptional start site is marked with an arrow; TATA box and ATG-translational start site are in bold.

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A. CAT TATAAAT ACACCCGCAACACTACCTATCAAA CCGCATCC AACAAAC
B. TGT TATAAA AGGAGCCATTTCCAAGCTCTAATCG CCGCATCC CCTCACC
C. CCC TATAAAT AACCACT--TCTCATTAAGGTTCT CCGCATC ACAAACCA
D. CCC TATAAAT AACCACG------CCTCAGGTTCT CCGCTTC ACAACACA
E. CTC TATAAAT TACCACT--TCTCATTAAGGTTCT CCGCATC ACAACCAA
F. TCC TATAAAT CACCACA------ACACAGCTTCT CCACTTC ACCACTTC
G. TCC TATAAAT CACCACA------CCACAACTTCT CCGGTTC AGCACTTC
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Figure 4. Alignment of computer-predicted transcriptional start site and TATA box sequences of *FeLEG1* with the corresponding sequences of different plant species: (**A**) *F. esculentum* (AY359286); (**B**) *Coffea arabica* (AF055300); (**C**) *Cicer arietinum* (Y13166); (**D**) *Glycine max* (X15122); (**E**) *Pisum sativum* (AJ276878); (**F**) *Pisum sativum (*X07014); (**G**) *Vicia faba* (X14238).

RY elements with a CATGCA core sequence were noted at two positions—283/288 and 333/338 upstream of ATG (**Figure 3**). The RY element may be assumed to be the "sine qua non" of seed-specific gene expression regulation, as it has been found in all sequenced genes specifically expressed in seeds up to now (*17*−*25*). In the legumin gene promoters, the RY motif represents the central core of the 28 bp legumin box (*21*) and its deletion abolishes most of the seed-specific promoter activity and results in low-level expression in leaves (*19*). Also in the napin promoter, destruction of two RY motifs drastically reduces promoter activity (*24*, *25*). Together, these data and the analysis of several other seed-specific promoters clearly demonstrated the importance of the RY motif for high-level expression of several seed-specific genes as well as the potential of this motif to function as a negative element repressing expression in nonseed tissues. The analysis of *Arabidopsis* promoter mutants (*26*−*28*) revealed the structural requirements for the function of the RY cis-element. It was shown that both the nucleotide sequence and the alternation of purine and pyrimidine nucleotides (RY character) are essential for the activity of the motif. It was also shown that FUS3 and ABI3 transcriptional factors can act independently of each other in controlling promoter activity and that the RY cis-motif is a target for both. The functional and biochemical data demonstrated that the regulators FUS3 and ABI3 are essential components of a regulatory network acting in concert through the RY element to control gene expression during late embryogenesis and seed maturation (*28*).

Besides RY, several other potentially active cis-elements were found in the cloned buckwheat gene fragment. Those that could be especially interesting are ABRE (with the core sequence ACGT located at four positions—137/140, 141/144, 178/182, and 666/669 upstream of the ATG—as well as overlapping the G-box motif). It has been established that ABA is a key regulator of gene expression during seed maturation (*29*, *30*). Promoter elements mediating ABA-responsive gene expression have been identified in seed-specific genes by transient analysis (*31*−*33*). Detailed analysis has revealed the composite nature of ABA-responsive complexes consisting of an ABRE and a coupling element, RY/G (*34*).

The G-box sequence was also identified in the upstream regions of plant storage protein genes, such as 7S phaseolin from common bean (*35*) and 2S storage proteins from *Brassica napus* and *Arabidopsis thaliana*, where the G-box is surrounded by two RY elements (*27*). The G-box was also found to be required for the differential expression of genes by stress and stimuli such as light (*36*), ABA (*37*), and ethylene (*38*). The diverse expression properties mediated by promoters containing identical G-box sequences clearly demonstrate that the function of this element varies according to the promoter context in which it resides. Thus, DNA context and additional elements are critical for the appropriate response. In the analyzed buckwheat promoter, the RY element together with the ABRE and G-box probably represent key "cis-players of the regulatory game". This assumption will be studied in our further experiments in which the interaction of promoter regions with buckwheat nuclear proteins as well as with purified transcriptional factors will be analyzed to confirm their involvement in specific gene regulation. In addition, the functionality of the potential promoter will be investigated in different transgenic constructs, which may be interesting for biotechnological application. In bioreactors, strong and constitutive promoters (such as CaMV 35S) are not ideal for the production of high accumulation levels of the heterologous protein, due to the "silencing" phenomenon characteristic for highly expressed genes. Nonconstitutive, highly specific SSP promoters able to regulate gene expression in a highly controlled manner ensuring spatial and temporal constraints have become a very attractive choice

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