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CLONING AND COMPUTER ANALYSIS OF THE PROMOTER REGION OF THE LEGUMIN–LIKE STORAGE PROTEIN GENE FROM BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH)

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Abstract — Using the modified 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 analysed 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.

Abbreviations: ABA — abscisic acid; ABRE — ABA responsive element; HSE — heat shock element; PCR — polymerase chain reaction; 5'–RACE — rapid amplification of cDNA ends, SSP — seed storage protein, TSS — transcription start site.

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INTRODUCTION

Legumins, or 11–13S globulins, are a major class of seed storage proteins that were first characterized in *Fabaceae* (Derbyshire et al., 1976) and are widespread in both angiosperms and gymnosperms, including different conifers (Shutov et al., 1999). The expression of these proteins is temporally regulated during embryogenesis and is restricted to seed tissues such as cotyledons or endosperm (Shewry, 1995). Thus, globulin storage proteins provide an excellent model for the study of plant gene regulatory mechanisms (Goldberg et al., 1989; Pang et al., 1988; Shirsat et al., 1989). Their corresponding mRNAs accumulate to high levels during the maturation phase and are mainly under transcriptional regulation (Tomas, 1993) The mid–maturation stage of seed development in many monocots and dicots is characterized by a super–abundant level of mRNAs for storage proteins (Goldberg et al., 1989). 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 (Truska et al., 2003).

Storage proteins of buckwheat seed are divided into two classes: salt soluble globulins, forming 70% of total seed proteins and water–soluble 2S albumins (Radović et al., 1996, 1999). The main storage protein of buckwheat, 13S globulin, resembles the legumin–like seed storage proteins of other species, while a minor 8S globulin has a structure common to all vicilin–like storage proteins (Milisavljević et al., 2004). In our previous paper (Maksimović et al., 1996), 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 up to full maturation, the level of the total storage protein per seed increases dramatically to more than 80% of total proteins by the end of development. Progressive accumulation proceeds simultaneously for all storage protein fractions.

In our previous paper (Samardžić et al., 2004) 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 analysed. The analysis of both contributed to molecular evolution studies.

In this paper we concentrate on the 5'–regulatory region of the same gene and describe the procedure used for its isolation and cloning, as well as computer analysis of the sequence in order 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 et al., (1983). Plasmid DNA was isolated using QIAprep® Spin Miniprep Kit (Qiagen).

Isolation of the 5'–regulatory region of FeLEG 1 and cloning procedure

The modified 5'–RACE method (Chenchik et al., 1996) using the Marathon cDNA system (Clontech) was employed for the isolation of the 5'–regulatory region of *FeLEG1*. The genomic DNA was firstly digested 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 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) x 5 cycles, (94°C for 30 s; 70°C for 4 min) x 5 cycles, (94°C for 30 s; 68°C for 4 min) x 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) x 25 cycles, 72°C for 10 min. The specific PCR products obtained were identified by Southern blot analysis using a gene specific probe, then were eluted and cloned into the pGEM–T vector using the pGEM–T Vector System I (Pomega). 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 number 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 denaturated in 1.5 M NaCl; 0.5 M NaOH for 30 min. Neutralisation 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 Pall Biodyne® A Membrane (Pall Corporation) in 10 x SSC buffer (1.5 M NaCl; 0.15 M Na–citrate) overnight. DNA was fixed on the membrane by baking at 80 °C for 2 h.

Hybridization was performed overnight in hybridization buffer (5 x SSC buffer; 0.1% N–laurylsarcosine; 0.02% SDS and 1% casein) at 65 °C, using as the probe 204 bp long fragment from the 5' region of clone *FeLEG1*, obtained by PCR with two gene specific primers Mam14 andNG1(5'–G**ATG**CTTCATGGGGTGCTTCTATG–3'). The probe was labeled by the BioPrime® DNA Labeling System (Invitrogen). After washing the membrane in SSC buffers of decreasing ionic strength, followed by incubation in 3% BSA solution at 65 °C for 1 h and incubation with streptavidin–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 by the Sanger method using an Automated Laser Fluorescence DNA sequencer

Computer–assisted analysis

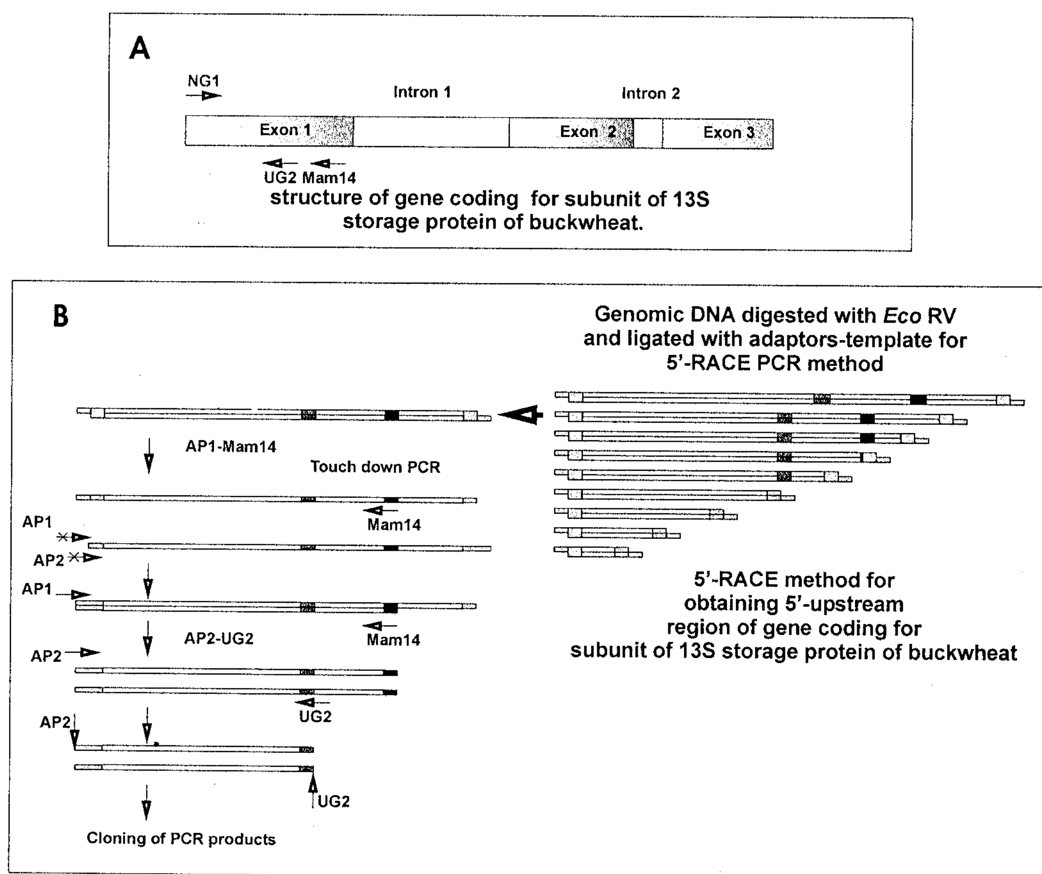
The obtained nucleotide sequence was subjected to the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST/) for sequence alignment; the Promoter Prediction program (http://www.fruitfly.org) for eukaryotic promoter prediction; Softberry (www.softberry.com), as well as PLACE (http://www.dna.affrc.go.jp), and PlantCARE (http://sphinx.rug.ac.be:8080) databases for prediction of the promoter regulatory elements.

RESULTS AND DISCUSSION

Since 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 NG2, 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 analysed by agarose gel electrophoresis (Figure 1). The high specificity of nested PCR with two gene specific primers, resulted in only one defined band about 1 Kb in length. The products of amplification were then analysed by Southern blot with the 204 bp probe, representing the 5'–end of FeLEG1 (Figure 2). The band that positively hybridised to the specific probe was eluted from the gel and introduced into the vector pGEM–T. After transformation of the XL1–blue *E. 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 noticed that a 126 bp 3' part of the sequence was in complete concordance with the 5' sequence of *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 analysed. The sequence of a potential promoter region, 955 bp long, was analysed by several computer programs mentioned in the Material and Methods section. 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, 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 (Shahmuradov et al., 2003).



Scheme 1. The 5'–RACE method applied for isolation of the 5'–regulatory region of FeLEG1: A) Scheme of the FeLEG1 with primer position — UG2, Mam14, NG1; B) Isolation procedure with Marathon adaptor primers AP1/AP2 and gene specific primers.

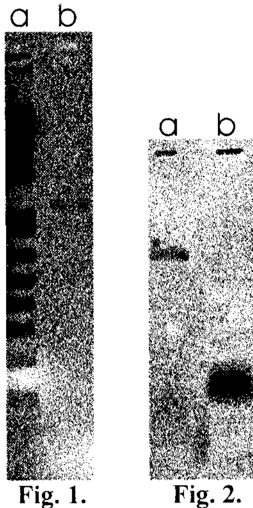


Fig. 1. Electrophoretogram of the 5'–RACE PCR product: A) DNA standard — 100 bp leader; B) product of amplification.

Fig. 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.

According to the PlantCare and PLACE data bases, several cis–regulatory elements which 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. Firstly, RY elements with a CATGCA core sequence were noticed 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

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A. CATTATAAATACACCCGCAACACTACCTATCAAACCGCATCCAACAAAC
B. TGTTATAAAAGGAGCCATTTCCAAGCTCTAATCGCCGCATCCCCTCACC
C. CCCTATAAATAACCACT--TCTCATTAAGGTTCTCCGCATCACAAACCA
D. CCCTATAAATAACCACG------CCTCAGGTTCTCCGCTTCACAACACA
E. CTCTATAAATTACCACT--TCTCATTAAGGTTCTCCGCATCACAACCAA
F. TCCTATAAATCACCACA------ACACAGCTTCTCCACTTCACCACTTC
G. TCCTATAAATCACCACA------CCACAACTTCTCCGGTTCAGCACTTC
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Fig. 4. Alignment of the computer predicted transcriptional start site and TATA box sequences of the FeLEG1 with the corresponding sequences of different plant species: A. Fagopyrum 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).

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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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Fig. 3. The nucleotide sequence of the 5'–regulatory region of the FeLEG1: Position of the predicted cis–regulatory elements: RY, ABRE, HSE are in boxes, the G–box is underlined; the position of the potential transcriptional start site is marked with an arrow; the TATA box and ATG–translational start site are in bold.

to now (Dickinson et al., 1988; Chamberland et al., 1992; Baumlein et al., 1992; Fujiwara and Beachy, 1994; Baumlein et al., 1986, 1991; Fiedler et al., 1993; Ellerström et al., 1996; Stalberg et al., 1993). In the legumin gene promoters, the RY motif represents the central core of the 28 bp legumin box (Baumlein et al., 1986) and its deletion abolishes most of the seed specific promoter activity and results in low–level expression in leaves (Baumlein et al., 1992). Also in the napin promoter, destruction of two RY motifs drastically reduces promoter activity (Ellerström et al., 1996; Stalberg et al., 1993). 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 non seed tissues. The analysis of Arabidopsis promoter mutants (Reidt et al., 2000; Ezcurra et al., 1999; Mönke et al., 2004) 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 (Mönke et al., 2004).

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 (Marion–Poll, 1997; Phillips et al., 1997) Promoter elements mediating ABA–responsive gene expression have been identified in seed–specific genes by transient analysis (Hattori et al., 1995; Shen and Ho, 1995; Ono et al., 1996). Detailed analysis has revealed the composite nature of ABA–responsive complexes consisting of an ABRE and a coupling element – RY/G (Roger and Roger, 1992).

The G–box sequence, was also identified in the upstream regions of plant storage protein genes, such as 7S phaseolin from common bean (Kawagoe and Murai, 1996) and 2S storage proteins from *B. napus* and *A. thalian,a* where the G–box is surrounded by two RY elements (Ezcura et al., 1999). The G–box was also found to be required for the differential expression of genes by stress and stimuli such as: light (Schindler and Cashmore, 1990), ABA (Mundy et al., 1990) and ethylene (Sessa et al., 1995), 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 analysed 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 analysed 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 applications. 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. Non–constitutive, 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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КЛОНИРАЊЕ И КОМПЈУТЕРСКА АНАЛИЗА ПРОМОТОРСКОГ РЕГИОНА ГЕНА ЗА РЕЗЕРВНИ ПРОТЕИН ХЕЉДЕ ЛЕГУМИНСКОГ ТИПА

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5'–регулаторни регион гена за резервни глобулин хељде (*FeLEG1*), дужине 955 бп, изолован је по умножавању фрагмента геномске ДНК хељде применом методе 5'–RACE . Комплетан фрагмент је секвенциран и секвенца је комјутерски анализирана у смислу дефинисања потенцијалних цус–регулаторних елемената укључених у ткивно специфичну и у развићу детерминисану генску експресију. Изоловани промотор је интересантан не само за фундаментална истраживања, већ и као потенцијално употребљив за биотехнолошку примену.