

IDENTIFICATION OF TWO EMBRYOGENESIS-RELATED
GENES IN SWEET PEPPER (*CAPSICUM ANNUUM* L.)
GENOME

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Abstract

Plant tissue culture techniques are essential part of modern plant biotechnology. Among them especially valuable are methods for production of haploid plants via anther cultures because they possess single copy of each gene and its functions can be easily manipulated and observed in the phenotype. *Capsicum annuum* (L.) produces sporadically successful anther cultures. Some cultivars produce embryos readily, while others do not respond at all. Many authors attributed variations in ability of plants to produce anther culture to differences in their genotype. It was also reported that the switch of microspores developmental programme from gametophyte- to sporophyte-type is regulated by transcription factors like BABY BOOM (BBM) and LEAFY COTYLEDON (LEC). Genes encoding such transcriptional factors have not been reported in sweet pepper yet. We used annotated in NCBI sequences of BBM and LEC to design degenerative primers for highly conservative regions in these genes and used them to search by PCR reactions for BBM- and LEC-like sequences in the genome of *Capsicum annuum* (L.), cultivar Stryama. The isolated PCR fragments were cloned and sequenced. The nblast in NCBI database revealed high similarity between our sequences and annotated BBM and LEC sequences. Next the obtained sequences were subjected to bioinformatics analyses to determine the exon-intron structure. Exons were translated in-silico in protein products. The protein blast of our BBM in NCBI revealed the presence of AP2-type functional domain, typical of BBM gene family. The LEC sequence carries CBFDFNFYB-HMF motive, typical of LEAFY COTYLEDON gene family. This is the

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first known report of existence of BBM-like and LEC-like genes in *Capsicum annuum* (L.) genome.

Key words: anther cultures, BABY BOOM gene, *Capsicum annuum* (L.), haploid plants, LEAFY COTYLEDON gene

Introduction. The plant tissue culture techniques are essential part of modern plant biotechnology. Among them especially valuable are the methods for establishing and regeneration of haploid and doubled haploid plants from anther cultures [1-4]. The haploid in vitro plant systems have many advantages in comparison with diploid plants. For instance the function of each gene can easily be manipulated and observed in the phenotype because it is presented as single copy in the haploid genome. The anther cultures are also more efficient tools for establishment of homozygous breeding lines and for production of hybrids in comparison with conversional breeding methods [5-7].

The ability of plants to switch their developmental programme from gametophyte- to sporophyte-type and to produce androgenic embryos with microspore origin depends on their genotype. Several genes have been reported to be related to zygotic and somatic embryogenesis [8-10]. BABY BOOM (BBM) gene family encodes AP2 type transcription factors that upon expression can induce embryogenesis in differentiated cells and possibly are key regulators of plant's embryonal development. So far two BBM genes were identified in *Brassica* and one – in *Arabidopsis*. BBM-transcripts were found in early embryogenic microspore cultures of Brassica, but they were not detected in non-embryogenic cultures [11,12]. The regenerative and embryogenic potential of tissue and microspore cultures also depend on expression of genes like LEAFY COTYLEDON (LEC). LEAFY COTYLEDON1 (LEC1) and LEAFY COTYLEDON2 (LEC2) participate in zygotic embryogenesis as well as in somatic embryogenesis. Their ectopic expression facilitates the embryogenesis from vegetative plant tissues. The expression of LEC1 and LEC2 genes was detected very early in microspore culture initiation [13-15].

Capsicum annuum (L.) cultivars differ in their ability to produce successful anther cultures. Some cultivars produce embryos readily, while others do not respond at all [16]. The frequency of direct embryogenesis also vary among cultivars from 0.5 to 75 embryos per 100 cultivated anther [17-20]. We assume that variations in ability of pepper cultivars to produce effective anther cultures and to regenerate from initiated microspores new plantlets depend on expression of genes like BBM and LEC. Since neither of these genes have been found yet in pepper, the aim of the research described in this article was to search for BBM- and LEC- like sequences in sweet pepper genome.

Materials and methods. Primers design. All BBM and LEC nucleotide sequences used to design degenerative primers were derived from the sequence databases of NCBI. The multiple alignments of homologous sequences were per-

formed using Vector NTI 10.1 software. The primers were ordered from Metabion International AG, Martinsried, Germany, and upon arrival were dissolved in DNase-free water to 100 mmol final concentration.

BBM NUCLEOTIDE SEQUENCES COMPARED. *Medicago truncatula* AP2/EREBP – (AY899909.1); *Brassica napus* AP2/EREBP transcription factor BABY BOOM (BBM1 – AF317904.1; BBM2 – AF317905.1); *Arabidopsis thaliana* AP2/EREBP clones (At5g17430; At3g20840); *Arabidopsis thaliana* BBM gene (AF317907).

LEC NUCLEOTIDE SEQUENCES COMPARED. *Helianthus annuus* leafy cotyledon 1-like gene (AJ879074.1); *Kalanchoe daigremontiana* leafy cotyledon 1-like (LEC1) gene (DQ674267.1); *Bixa orellana* partial mRNA for leafy cotyledon gene (AJ489457.1); *Oryza sativa* HAP3 transcriptional-activator (LEC1A – AY062183.1, LEC1B – AY062184.1, AY062185.1); *Oryza sativa* indica cultivar-group LEC1 gene (AY264284.1); *Arabidopsis thaliana* (At5g47670, LEC1 – AT1G21970; LEC2 – AT1G28300, AY568668.1); *Zea mays* LEC1 (AF410176.1); *Phaseolus coccineus* LEC1-like (AF533650.1). Exon-intron organization of the genes was deduced from *Arabidopsis thaliana* genes organization published in the TAIR database.

Plant material. Pepper plants, cultivar “Stryama” were grown till stage second true leaf in greenhouse conditions and the young leaves were harvested, frozen in liquid nitrogen and grind to fine powder. Next the material was immediately frozen at -80°C and stored until use.

DNA isolation. 100 mg of the frozen material was transferred into pre-cooled with liquid nitrogen microcentrifuge tubes and the DNA was extracted by DNeasy plant mini kit (Qiagen cat. No 69104) following the original protocol.

PCR reaction conditions. PCR reaction mix – in 250 μl PCR tubes were mixed 2 μl DNA template; 1 μl of each forward and reverse degenerative primers (100 mmol.l^{-1} concentration); 12.5 μl PCR master mix (Fermentas, Cat No K0171) and 8.5 μl DNase-free water (supplied with the master mix kit). PCR tubes were places in Thermal Cycler 2720 (Applied Biosystems) PCR apparatus and the PCR amplification was carried out by using the following programme: initial DNA melting at 94°C – 3 min 30 s; next 35 cycles of 94°C – 45 s; $48/57^{\circ}\text{C}$ – 45 s; 72°C – 3 min and final extension at 72°C for 6 min. PCR products were mixed with 2.5 μl of loading day (Fermentas #R0611) loaded onto 1.5% agarose gel containing 0.5 $\mu\text{g/ml}$ ethidium bromide (final concentration) covered with 0.5X TBE buffer and separated by applying 3.5 volts per cm electrical currency. The size of the products was determined by comparison with DNA ladder (Fermentas GeneRuler#SM0311). The PCR products were visualized by UV light and the bands of interest were sliced from the gel with pure surgical blades.

PCR product isolation, cloning and sequencing. The PCR products were isolated from the agarose by QIAquick Gel Extraction Kit (Qiagen, cat No 28704) following the original protocol. The concentration of PCR products was determined spectrophotometrically and 2–4 μl of them were used for A/T

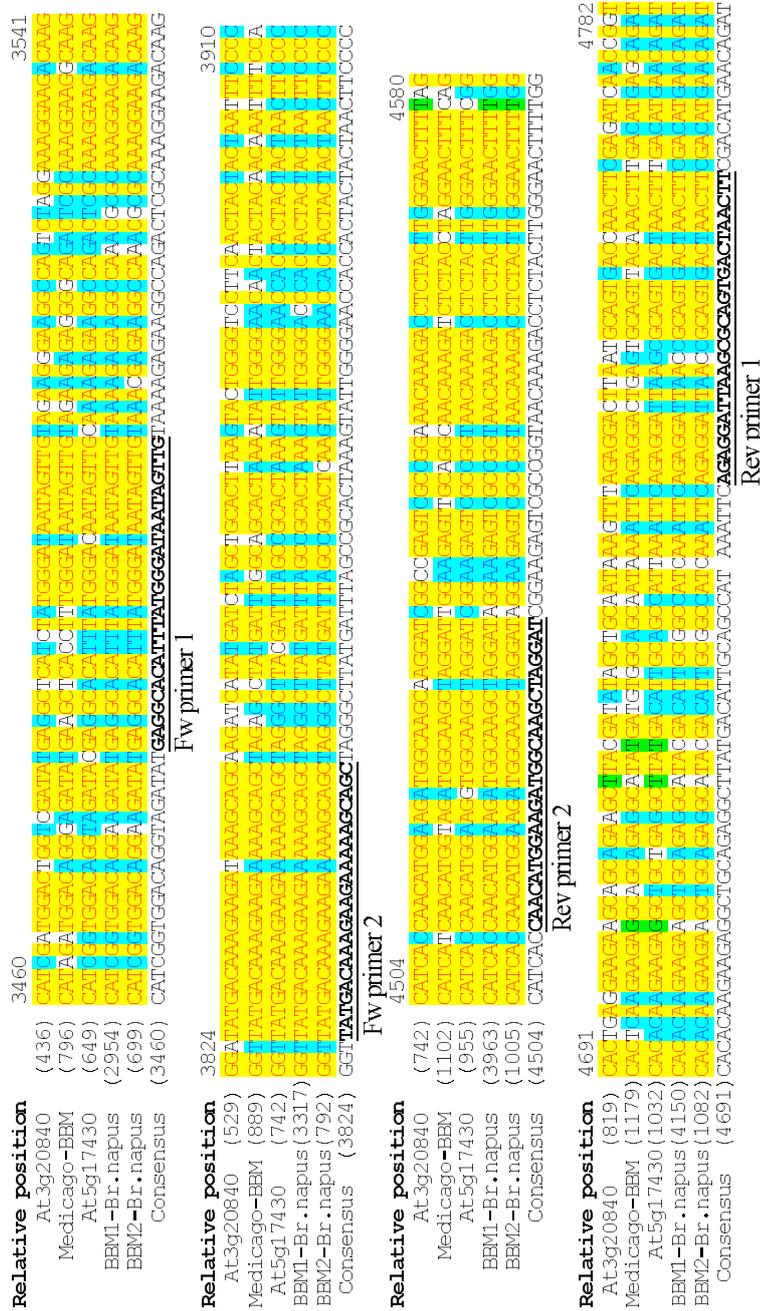


Fig. 1. A. Multiple sequence alignment containing regions with the highest degree of similarity amongst the annotated BABY BOOM (BBM) sequences. The consensus nucleotide sequence was used for degenerative primers design. Positions of the primers are indicated with bold & underlines symbols

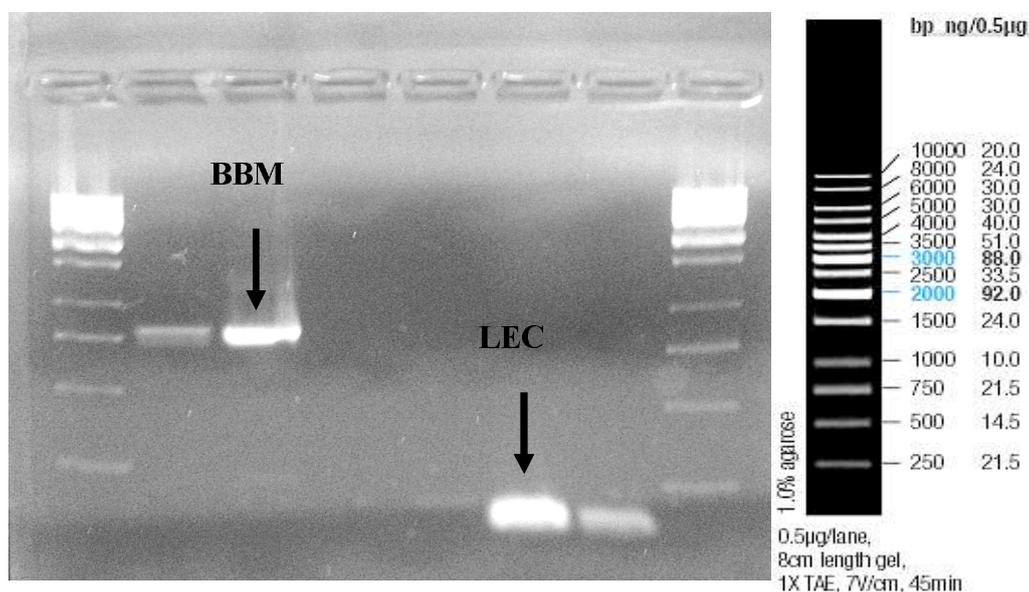


Fig. 2. The PCR products were mixed with 2.5 µl of loading day loaded onto 1.5% agarose gel containing 0.5 µg/ml ethidium bromide (final concentration) covered with 0.5X TBE buffer and separated by applying 3.5 volts per cm electrical currency. The size of the products was determined by comparison with DNA ladder (Fermentas GeneRuler#SM0311). The picture presents PCR products visualized by UV light

cloning. QIAGEN PCR Cloning Kit (cat No 231124) was used to clone the PCR products. Original protocol to the kit was followed. The ligation reactions were mixes with 250 µl freshly prepared competent bacterial cells (*E. coli*-TOP 10 – Invitrogen). The plasmids containing PCR products were isolated by QIAprep Spin Miniprep Kit (Cat No 27104) following the original protocol. The isolated plasmids were dissolved in 50 µl buffer (10 mM Tris · Cl, pH 8.5) and were sent to MWG – Biotech AG, Frankfurt, Germany, for sequencing.

Results. Bioinformatics analysis. There are no published sequences for BBM and LEC genes in *C. annuum* (L.). Therefore at the first stage of our investigations we used bioinformatics analysis to study the known sequences of such genes in other species and to search for conservative regions suitable for design of degenerative primers. We used Vector NTI 10.1 software to perform multiple alignments of homologous sequences. Since *C. annuum* (L.) belongs to the group of dicotyledons the sequences used in this study were mostly from dicotyledonous plants. For LEC genes, where the sequence data for dicotyledons were insufficient, sequences from monocots were included as well. The fragments of multiple sequence alignments containing regions with the highest degree of similarity amongst the species are presented in Fig. 1A for BBM and Fig. 1B for LEC genes. These consensus nucleotide sequences served as a start point for

degenerative primer design (Fig. 1A,B). During the next step of primer design we tried to keep primer degeneracy as low as possible in order to avoid amplification of nonspecific PCR products. That is why we selected amongst all “potential primers” those, whose consensus nucleotide sequences had the lowest degeneracy. In our effort to keep primer pools with the lowest possible degeneracy, we tried to avoid regions encoding amino acids like serine, lysine and arginine, which are encoded by up to six codons. Another important aim was to achieve an exact match between the primer and template near the 3'-end of the primer. For this purpose, we selected primers that ended on invariant nucleotides at their 3' ends. We have also tried to place at the 3'-ends consensus regions rich of cytosine and guanine pairs which confer greater stability of the primer-template DNA duplex during PCR reactions. Primers with annealing temperature higher than 70 °C were also eliminated. The list of selected sets of primers is presented in Table 1.

T a b l e 1

Degenerative primers designed for amplification of putative BBM and LEC genes. Wobble codes: **R** (A or G), **Y** (C or T), **M** (A or C), **K** (G or T), **S** (G or C), **W** (A or T), **H** (A or C or T), **B** (G or T or C), **V** (G or C or A), **D** (G or T or A), **N** (G or A or T or C)

1	BBM forward primer 1	5' GAGGC W CATY T TATGGG A YAATAGTTG 3'
2	BBM forward primer 2	5' TATGACAAAGAAG W AAAGCAGC 3'
3	BBM reverse primer 1	5' AAGTTCGT N ACTG C R B TGARTCCTCT 3'
4	BBM reverse primer 2	5' ATCCT W GCTTGCC A YCT W CCATGTTG 3'
5	LEC forward primer 1	5' GAGCAAGAC M G V YTKATGCCGAT 3'
6	LEC forward primer 2	5' GC V AA C GTGAT M M G VATCATGCG 3'
7	LEC reverse primer 1	5' TAGAA V CCGAG C Y K GCTCAT R GC 3'
8	LEC reverse primer 2	5' GTCTTG C KCTGCT C VCGBTGGCA 3'

Several different conditions were tested to achieve optimal PCR amplification. We did vary the amounts of DNA template from 50 to 300 ng and annealing temperatures from 48 to 57 °C. The optimal amplification of both genes was achieved with 150 ng (2 µl) DNA template and annealing temperature 55 °C. PCR products (Fig. 2) were cloned in pDrive vectors as described in Materials and methods and sent for sequencing.

We used online nblast in NCBI to compare our putative BBM and LEC sequences with those annotated in the databases. The returned results demonstrated high similarity of our BBM-like sequence to *Arabidopsis thaliana* BABY BOOM gene and *Brassica napus* AP2/EREBP transcription factor BABY BOOM 1 (Expect = $2e^{-29}$ and $3e^{-26}$). Our LEC-like sequence had high similarity with *Arabidopsis thaliana* LEC1 (LEAFY COTYLEDON 1) and *Helianthus annuus* putative leafy cotyledon 1-like protein (Expect = $5e^{-31}$ and $2e^{-36}$ respectively).

AATGTCGACA CGCGTTACGT ATCGGATCCA GAATTCGTGA TTGAGGCTCA TTTATGGGAT AATAGTTGCA AGAAGGAAGG GCAGACTAGG AAAGGGAGAC
 TACGACGTCT GCGCAATGCA TAGCCTAGGT CTTAAGCACT AACCTCGAGT AATAGCCCTA TTAACAAGTT CTT CCTT CC CGTCTGATCC TTTCCTCCTG
 AAGGTTAGCT AATAAGAAATCTTTATTCAA TCCACACAAA TCTTTGACIC TTTATTTTT AGTCCCAT CTGTTCATAG TCTGACAATT GTTATGAATT
 TTCCAAATCGA TTAATACCTAG AAAATAAGTIT AAGTGTGTTT AGAACTGAG AATAAAAAA TCCAGGGTAA GACAAGTATC AGACTGTATA CAATACITAA
 TTTTGTGAT GTAICTGCTT GTTGGTGGAT ATGCTATCTT AATCAACTCA GTTATCTTGT GTGATGGCT TTGTCAACAT TCAAATTTCA AGTAACAATGC
 AAAAACAGTA CATAGACGA CAACCAGCTA TACGATAAGA TTAGTTGAGT CAATAGAAC CACTCACCGA ACAGATHTGA AGHTTAAAGT TCATHTGACG
 TTTTATTTTC TTTACTTAAT TAATAGCAC TAAACTATAA AATGATGTGG AATTAAGGG GCTATGATAT GGAAGATAAA GCTGCAAGAG CATATGAATCA
 AAAATAAAG AAATGAATTA ATTAATGCTG ATTTGATAT TTACTAGACC TTAATATCCC CGATACTATA CCTTCTA TTT CGACGHTCTC GTATACATAGT
 AGCTGATTA AAGTACTGGG GTCTTCAAG TCACATTAAT TTCCAGTAA GTGTAATAC TTACCTCAAG TTATTTATAT TAAATGCCITT GGCTAACATA
 TCGACCTAAT TTCATGACCC CAGGAAGTTG AGTGTAAITA AAAGGTCAT CACATTAATG AATGGAGTTC AATAATATAA ATTACGGAAA CCGAATGTAT
 GGACAATAAT TTCCTTTGGT ACTTTTGGCA GTTGGAAAAT TATCAGAAAAG AGCTTGAAGA AATGAAGAAI AIGACCAGAC AAGAATATGT TGCACACTTG
 CCTGTAATA AAGGAACGA TGAATAACGT CAACCTTTA ATAGCTTTC TCGAACTTCT TTACTTCTTA TACTGGTCTG TTTCTTATACA ACGHTGAAAC
 AGAAGGTGAG TTT GTTACT ACICAAACTT TGTC TATAT TCT TGTG TTC ATTTT ATIAA TTAGCTGTG TTGAATAICA TT GTTANT AIT CAGGAAAAG
 TCT TT CCACTC AAAACAATGA TGAGTTTGA ACAGAAATA AGAACACAAG TAAATATAAT AATCGAACAC AACTATAGT AACAATAATAA GTTCTTTTTC
 TAGTGGATTT TCAAGAGGTG CTTCAATTT A CAGAGGAGTA ACAAGGTATA TTTGACATTT TCAATATGAAT AAATATGTT TTGAATAGCT ATGAACAGTIC
 ATCACCTAAA AGTTCCTCCAC GAAAGTAAAT GTCT CCT CAT TGTTCCTAAT AT AAACGTAAA AGTATACTTA TTTATAACA AACTTATCGA TACTTGTGAC
 CTTTCTAAAA TGAATAGTGT TAT TTTAAT TAT AGGCACC ATCAACATGG TAGATGGCAA GCTAGGAT AA TCTGAAITCG TCGACAAGCT TCTCGAGCCT
 GAAAGATTT ACTAATCACA ATAAAATTA ATA T CCGTGTAGTIT GTACC AT CTACCGIT CGATCCTA TT AGACTTAAGC AGCTGTTCGA AGACTCCGA
 TTGGGC TCGCTACGGT GCAACGGTC ATTGGCTTCG CCGGTAACGA AGCTGATGAA CTCGGACACG CATTCCTGAA TGGTCT CTTT CGAGTCTGICA
 AACGGACGAGTGGCA CCGTGTCCAG TAACCAGGAGG GGGCATTGTCT TCGACTACTT GAGCCGTGTC GTAAGGACTT ACCAGAGAAA GTCACAGCAGT
 GATACTTGG CATGGGAGG GTCC TAAAAA GAGTACTATGCTTAGTGCAA CCGATAGCCG ATCAACCCGHT CTGTCTAA
 CTATAGAACCC GTACGCCCTCC CAGGAATTTT CTCATGATAC GAATCACGTT GGCTATCGC TAGTTGGCCA GAACGAGT T

Fig. 3. A. The isolated from *C. annuum* (L.) BBM partial sequence. Exons are underlined

Fig. 3. B. The isolated from *C. annuum* (L.) LEC partial sequence. Exons are underlined

Annotated *A. thaliana* BBM1 and LEC1 sequences were used to determine exon-intron structure of our sequences. The comparison revealed that we managed to isolate 825 bp fragment of sweet pepper BBM-like gene containing two introns, one complete exon (position 692–749) and fragments two other exons at the left and right ends of our sequence (Fig. 3A). The comparison of our LEC-like sequence with the one of *A. thaliana* revealed one single 168 bp-long exon (Fig. 3B).

Vector NTI 10.1 software was used to perform in-silico translation of the coding sequences in proteins. Protein-protein blasts in NCBI revealed the presence of one AP2-type functional domain in our BBM-like sequence – a feature typical of transcription factors of BBM gene family. Our LEC-like sequence possess CBFD-NFYB-HMF motive, typical of LEAFY COTYLEDON gene family.

This is the first known report of the existence of BBM-like and LEC-like genes in *Capsicum annuum* (L.) genome. The obtained sequences were submitted to NCBI database.

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