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(54) Titre : REGION REGULATRICE DE GENES PROMOUVANT UNE TRANSCRIPTION PRECOCE SPECIFIQUE DE GRAINES

(54) Title: GENE REGULATORY REGION THAT PROMOTES EARLY SEED-SPECIFIC TRANSCRIPTION

(57) Abrégé/Abstract:
Nucleic acid sequence capable of regulating transcription during embryogenesis in plants is provided. This sequence may be used in transgenic plants to promote high levels of expression of foreign and endogenous genes in developing seeds to affect seed lipid metabolism, protein or carbohydrate composition and accumulation, or seed development. In addition, these sequences may be useful for the production of modified seed containing novel recombinant proteins which have pharmaceutical, industrial or nutritional value, or novel products like plastics.

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(54) Title: GENE REGULATORY REGION THAT PROMOTES EARLY SEED-SPECIFIC TRANSCRIPTION

(57) Abstract: Nucleic acid sequence capable of regulating transcription during embryogenesis in plants is provided. This sequence may be used in transgenic plants to promote high levels of expression of foreign and endogenous genes in developing seeds to affect seed lipid metabolism, protein or carbohydrate composition and accumulation, or seed development. In addition, these sequences may be useful for the production of modified seed containing novel recombinant proteins which have pharmaceutical, industrial or nutritional value, or novel products like plastics.

GENE REGULATORY REGION THAT PROMOTES EARLY SEED-SPECIFIC TRANSCRIPTION

Field of the Invention

This invention relates to a nucleic acid sequence, which regulates transcription during embryogenesis in plants. More specifically, the nucleic acid sequence of the present invention can be used in transgenic plants to promote high levels of expression of foreign and endogenous genes in developing seeds to affect seed lipid metabolism, protein or carbohydrate composition and accumulation, or seed development. In addition, the nucleic acid sequcnce of the present invention can be useful for the production of modified seed containing novel recombinant proteins which have pharmaceutical, industrial or nutritional value, or novel products like plastics.

BACKGROUND

Most of the information about seed-specific gene expression comes from studies of genes encoding seed storage proteins like napin, a major protein in the seeds of *Brassica napus*, or conglycinin of soybean. Furthermore, upstream DNA sequences directing strong embryo-specific expression of these storage proteins have been used successfully in transgenic plants to manipulate seed lipid composition and accumulation (Voelker et al., 1996). However, expression of storage protein genes begins fairly late in embryogenesis.

Thus, promoters of seed storage protein genes may not be ideal for all seed-specific applications. For example, storage oil accumulation commences significantly before the highest level of expression of either napin (Stalberg et al., 1996) or conglycinin (Chen et al., 1988) is achieved. It is, therefore of interest to identify other promoters which control expression of genes in developing embryos with temporal specificity different from that of seed storage proteins.

SUMMARY OF THE INVENTION

The nucleic acid sequence of the present invention can be used to regulate transcription during embryogenesis in plants. By the present invention it is possible to promote high levels of expression of foreign and endogenous genes in developing seeds to affect seed lipid metabolism, protein or carbohydrate composition and accumulation, or seed development. The present invention can also be useful for the production of modified seed, which contains novel recombinant proteins.

BRIEF DESCRIPTION OF THE DRAWING

The Figure shows nucleic acid sequence of the insert in the plasmid pLfKCS3-GUS.

DETAILED DESCRIPTION

The inventors have determined that a more suitable gene regulatory region for directing gene expression aimed at seed oil modification would originate from a seed lipid

metabolic gene expressed in a seed-specific manner. One such gene is *LfKCS3*, which encodes a condensing enzyme of very long chain fatty acid biosynthesis in *Lesquerella fendleri*. *LfKCS3* condensing enzyme is thought to be localized in the endoplasmic reticulum where it catalyzes the sequential elongation of C18 fatty acyl chains to C20 in length. RNA blot analyses showed that the *LfKCS3* gene transcript was present only in developing embryos. The inventors isolated the 5' regulatory region of the *LfKCS3* gene and in the present application demonstrate that it is useful in promoting early seed-specific transcription of heterologous genes in *Arabidopsis*. Regulatory 5' DNA sequences promoting early seed-specific transcription found upstream of other plant *KCS* genes have also been isolated and disclosed previously.

Isolated transcription regulatory region from the *LfKCS3* gene is capable of directing expression of desired genes at an early stage of development in a seed-specific manner. Because this regulatory sequence can also promote transcription in developing seeds of a different plant species, it can be used in a variety of dicotyledonous plants for modification of the seed phenotype.

Examples of applications wherein the nucleic acid sequence of the present invention can be useful include, for example:

(1) altered seed fatty acid composition or seed oil composition and accumulation,

(2) altered seed protein or carbohydrate composition or accumulation,

(3) enhanced production of desirable seed products,

(4) suppression of production of undesirable seed products using antisense, co suppression or ribozyme technologies,

(5) production of novel recombinant proteins for pharmaceutical, industrial or nutritional purposes,

(6) production of novel compounds/products in the seed, ie. secondary metabolites, plastics, etc.

The methods employed in the isolation of the nucleic acid of the present invention and the uses thereof are discussed in the following non-limiting examples:

Examples:

Isolation of a seed-specific promoter region form *Lesquerella fendleri*

A *Lesquerella fendleri* genomic DNA library was obtained from Dr. Chris Somerville, Carnegie Institution of Washington, Stanford, CA. The genomic library was plated on *E. coli* LE392 (Promega) and about 150,000 clones were screened using *Arabidopsis FAE1* gene (James et al., 1995) as a probe. The probe was prepared by PCR using pGEM-7Zf(+)-FAE1 (Millar and Kunst, 1997) as a template with *FAE1* upstream primer, 5'-CCGAGCTCAAAGAGGATACATAC-3' and *FAE1* downstream primer, 5'-GATACTCGAGAACGTTGGCACTCAGATAC-3'. PCR was performed in a 10µl reaction containing 10 ng of the template, 2mM $MgCl_2$, 1.1 µM of each primer, 100 µM of (dCTP + dGTP + dTTP) mix, 50 µCi of [α-32P]dATP, 1X PCR buffer and 2.5 units of *Taq* DNA polymerase (Life Technologies). Amplification conditions were: 2 min of initial denaturation at 94°C, 30 cycles of 94°C for 15 sec, 55°C for 30 sec, 72°C for 1 min and 40 sec, followed by a final extension at 72°C for 7 min. The amplified radiolabeled probe was purified by QIAquick PCR Purification Kit (Qiagen) and denatured by boiling before adding to the hybridization solution. Hybridization took place overnight at 65°C in a solution containing 6X SSC, 20 mM NaH_2PO_4. 0.4% SDS, 5X Denhardt's solution, and 50 µg/ml sonicated,

denatured salmon sperm DNA (Sigma) and washing was performed three times for 20 min each in 2X SSC, 0.5% (w/v) SDS at 65°C.

Nine clones with sequences corresponding to the *Arabidopsis FAE1* gene were isolated from the *Lesquerella fendleri* genomic library. The phage DNA from those nine clones was extracted and purified using QIAGEN Lambda Mini Kit (Qiagen) according to the manufacturer's protocol. One of them was digested with *Eco*RI and a 4.3 kb fragment was subcloned into the pGEM-7Zf(+) vector (Promega) cut with *Eco*RI, resulting in the vector pMHS15. The whole insert was sequenced with ABI automatic 373 DNA sequencer using fluorescent dye terminators. Approximately 573 bp of the 5' upstream region of the 4.3 kb genomic DNA was amplified using the high fidelity *Pfu* polymerase (Stratagene) with a forward primer 5'-CGCAAGCTTGAATTCGGAAATGGGCCAAG-3' and a reverse primer 5'-CGCGTCGACTGTTTTGAGTTTGTGTCGGG-3'. The amplified fragment was inserted upstream of the GUS gene in pBI101 (Clontech) cut with *Hind*III and *Sal*I, resulting in the vector pLfKCS3-GUS. The sequence of the insert in the plasmid pLfKCS3-GUS is shown in Figure 1.

Functional analysis of the *LfKCS3* 5' upstream region

To evaluate the ability of the 5' upstream fragment of the *LfKCS3* gene to confer seed-specific and temporal regulation of gene expression in plants, the pLfKCS3-GUS construct was introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Koncz and Schell, 1986) by heat-shock and selected for resistance to kanamycin (50 μg/mL). *A. thaliana* ecotype Columbia was transformed with *A. tumefaciens* harbouring the pLfKCS3-GUS construct using floral dip method (Clough and Bent, 1998). Screening for transformed seed

was done on 50μg/mL kanamycin as described previously (Katavic et al., 1994). Approximately 100 transgenic lines were generated for each construct.

Histochemical localization of GUS activity in transgenic plants was done on tissue sections as follows. Sections were incubated in 50 mM sodium phosphate, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.05%(w/v)Triton™ X-100, and 0.35 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) for 4 to 7 hours at 37°C (Jefferson, 1987). Following staining the blue-stained samples were fixed in 70% ethanol.

Using this assay, over 30 independent transgenic *Arabidopsis* lines were examined for the embryo-specific expression of the GUS gene. In addition, leaves, stems, inflorescences, roots, and siliques at different stages of development were histochemically stained for β-glucuronidase activity. The GUS reporter gene fused to the *LfKCS3* promoter was not expressed in any of the vegetative tissues, whereas it was highly expressed in developing embryos. We also compared the *LfKCS3* promoter with the *LFAH12* promoter that was reported to be an early and seed-specific promoter active already at the torpedo stage of *Arabidopsis* (Broun *et al.*, 1998). Our results suggest that the *LfKCS3* promoter is active even earlier. Thus, the onset of the *LfKCS3* promoter activity coincides with or precedes that of storage oil accumulation. GUS activity in all the examined transgenic lines persisted throughout subsequent embryo development. Thus the *LfKCS3* promoter is useful for seed-specific expression of foreign genes in transgenic plants.

In conclusion, we have demonstrated that the elements which confer both tissue specific and developmental regulation of a reporter gene linked to the *LfKCS3* promoter reside within the 573 bp upstream of the AUG translation initiation codon. Our experiments also show that the *Lesquerella fendleri LfKCS3* promoter directs seed-specific expression at

least as early as the torpedo stage embryo and that the it is capable of promoting transcription in plants other than *Lesquerella fendleri.*

It should also be mentioned that the seed-specific expression conferred by the *LfKCS3* promoter is independent of the native terminator at the *LfKCS3* gene 3' end. In all our constructs, a terminator derived from the *Agrobacterium* nopaline synthase gene was used. Thus, the sequence in the 573 bp promoter construct is sufficient for the desired expression profile independent of ancillary sequences.

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SEQUENCE LISTING

GENERAL INFORMATION

APPLICANT: THE UNIVERSITY OF BRITISH COLUMBIA

TITLE OF INVENTION: GENE REGULATORY REGION THAT PROMOTES EARLY SEED-SPECIFIC TRANSCRIPTION

NUMBER OF SEQUENCES: 5

CORRESPONDENCE ADDRESS: Gowlings
P.O. Box 30, Suite 2300
550 Burrard Street
Vancouver, British Columbia
Canada V6C 2B5

COMPUTER-READABLE FORM
COMPUTER: Dell Dimension L400c
OPERATING SYSTEM: Windows Millennium
SOFTWARE: PatentIn 2.1

CURRENT APPLICATION DATA
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FILING DATE: 2000-05-24

PATENT AGENT INFORMATION
NAME: Gowlings
Attn: Dr. Alakananda Chatterjee

REFERENCE NUMBER: 08900442CA

INFORMATION FOR SEQ ID NO.: 1
SEQUENCE CHARACTERISTICS
LENGTH: 588
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY:
MOLECULE TYPE: DNA
HYPOTHETICAL:
ANTI-SENSE:
FRAGEMENT TYPE:
ORIGINAL SOURCE: Lesquerella fendleri
IMMEDIATE SOURCE:
POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

SEQUENCE DESCRIPTION: SEQ ID NO: 1

GAATTCGGAA ATGGGCCAAG TGAAATGGAA ATAGAGCTTC AATCCATTTA GTCCCACTCA 60
AAATGGTGCT CGAATTATAT TTAGTTACGT TCGAATCAGA CAACCAAGTA TTTGGTTAAT 120
AAAAACCACT CGCAACAAAG GAAAAACACC AAGCGCGTGC GTCCAACATC CGACGGAAGG 180
GGGGTAATGT GGTCCGAAAA CCTTACAAAA ATCTGACGTC ATCTACCCCC GAAAACGTTG 240
AATCGTCAAC GGGGGTAGTT TTCGAATTAT CTTTTTTTTA GGGGCAGTTT TATTAATTTG 300
CTCTAGAAAT TTTATGATTT TAATTAAAAA AAGAAAAAGA ATATTTGTAT ATTTATTTTT 360
TATACTCTTT TTTTGTCCAA CTATTTCTCT TATTTTGGCA ACTTTAACTA GACTAGTAAC 420
TTATGTCAAT GTGTATGGAT GCATGAGAGT GAGTATACAC ATGTCTAAAT GCATGCCTTA 480
TGAAAGCAAC GCACCACAAA ACGAAGACCC CTTTACAAAT ACATCTCATC CCTTAGTACC 540
CTCTTACTAC TGTCCCGACA CAAACTCAAA ACAATGACAT CTCTAAAC 588

INFORMATION FOR SEQ ID NO.: 2
SEQUENCE CHARACTERISTICS
LENGTH: 23
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY:
MOLECULE TYPE: DNA
HYPOTHETICAL:
ANTI-SENSE:
FRAGEMENT TYPE:
ORIGINAL SOURCE: Artificial Sequence
IMMEDIATE SOURCE:
POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:
OTHER INFORMATION: Description of Artificial Sequence: Primer

SEQUENCE DESCRIPTION: SEQ ID NO: 2

CCGAGCTCAA AGAGGATACA TAC 23

INFORMATION FOR SEQ ID NO.: 3
SEQUENCE CHARACTERISTICS
LENGTH: 29
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY:
MOLECULE TYPE: DNA
HYPOTHETICAL:
ANTI-SENSE:
FRAGEMENT TYPE:
ORIGINAL SOURCE: Artificial Sequence
IMMEDIATE SOURCE:
POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:
OTHER INFORMATION: Description of Artificial Sequence: Primer

SEQUENCE DESCRIPTION: SEQ ID NO: 3

GATACTCGAG AACGTTGGCA CTCAGATAC 29

INFORMATION FOR SEQ ID NO.: 4
SEQUENCE CHARACTERISTICS
LENGTH: 29
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY:
MOLECULE TYPE: DNA
HYPOTHETICAL:
ANTI-SENSE:
FRAGEMENT TYPE:
ORIGINAL SOURCE: Artificial Sequence
IMMEDIATE SOURCE:
POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:
OTHER INFORMATION: Description of Artificial Sequence: Primer

SEQUENCE DESCRIPTION: SEQ ID NO: 4

CGCAAGCTTG AATTCGGAAA TGGGCCAAG 29

INFORMATION FOR SEQ ID NO.: 5
SEQUENCE CHARACTERISTICS
LENGTH: 29
TYPE: nucleic acid
STRANDEDNESS: double
TOPOLOGY:
MOLECULE TYPE: DNA
HYPOTHETICAL:
ANTI-SENSE:
FRAGEMENT TYPE:
ORIGINAL SOURCE: Artificial Sequence
IMMEDIATE SOURCE:
POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:
OTHER INFORMATION: Description of Artificial Sequence: Primer

SEQUENCE DESCRIPTION: SEQ ID NO: 5

CGCGTCGACT GTTTTGAGTT TGTGTCGGG 29

What we claim is:

1. An isolated nucleic acid comprising a promoter sequence which directs seed-specific transcription of genes in plants, wherein the promoter sequence comprises SEQ ID NO: 1.

2. The isolated nucleic acid of claim 1 wherein the promoter sequence consists of SEQ ID NO: 1.

3. The isolated nucleic acid of claim 1 further comprising a gene under control of the promoter.

4. The isolated nucleic acid of claim 3 wherein the promoter sequence is directed to the seed specific transcription of genes in plants.

5. A vector comprising the nucleic acid of any one of claims 1 to 4.

6. The vector of claim 5 wherein the vector is a plasmid.

7. The plasmid of claim 6 wherein the plasmid is carried by *Agrobacterium tumefaciens*.

8. The plasmid of claim 7 wherein the plasmid is pLfKCS3-GUS.

9. A transformed plant cell comprising a nucleic acid of any one of claims 1 to 4.

10. The plant cell of claim 9 wherein the plant cell comprises a cell from *Arabidopsis thaliana*.

11. A method of transforming a plant cell comprising:

(i) providing a plant cell; and

(ii) stably transfecting the vector of claim 5 into the plant cell.

12. The method of claim 11 wherein the vector comprises a plasmid.

13. The method of claim 12 wherein the plasmid is pLfKCS3-GUS.

14. A method of transforming a plant cell comprising:

(i) providing a plant cell; and

(ii) contacting the plant cell with an Agrobacterium containing the plasmid of claim 6 for a time and under conditions sufficient to allow transformation.

15. The method of any one of claims 11 to 14 wherein the plant cell comprises a cell from *Arabidopsis thaliana*.

Figure 1. ***Lesquerella fendleri*** *LfKCS3* **promoter:**

(Length: 573 bp)

GAA TTCGGAAATG GGCCAAGTGA -573

AATGGAAATA GAGCTTCAAT CCATTTAGTC CCACTCAAAA TGGTGCTCGA -550

ATTATATTTA GTTACGTTCG AATCAGACAA CCAAGTATTT GGTTAATAAA -500

AACCACTCGC AACAAAGGAA AAACACCAAG CGCGTGCGTC CAACATCCGA -450

CGGAAGGGGG GTAATGTGGT CCGAAAACCT TACAAAAATC TGACGTCATC -400

TACCCCCGAA AACGTTGAAT CGTCAACGGG GGTAGTTTTC GAATTATCTT -350

TTTTTTAGGG GCAGTTTTAT TAATTTGCTC TAGAAATTTT ATGATTTTAA -300

TTAAAAAAAG AAAAAGAATA TTTGTATATT TATTTTTTAT ACTCTTTTTT -250

TGTCCAACTA TTTCTCTTAT TTTGGCAACT TTAACTAGAC TAGTAACTTA -200

TGTCAATGTG TATGGATGCA TGAGAGTGAG TATACACATG TCTAAATGCA -150

TGCCTTATGA AAGCAACGCA CCACAAAACG AAGACCCCTT TACAAATACA -100

TCTCATCCCT TAGTACCCTC TTACTACTGT CCCGACACAA ACTCAAAACA -50

ATGACATCTCTAAAC