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(54) Title: FEMALE GAMETOPHYTE SPECIFIC PROMOTER (ZMEA1)

(57) Abstract: The invention relates to sequences isolated from corn, which are expressed selectively in the female gametophyte, more particularly in the egg apparatus, and to a promoter capable of directing transcription of an operably linked foreign DNA sequence selectively in the female gametophyte of plants.

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Female gametophyte specific promoter (ZmEA1)

Field of the invention

The invention relates to sequences expressed selectively in the female gametophyte isolated from corn and to a promoter capable of directing transcription of an operably linked foreign DNA sequence selectively, preferably exclusively in the female gametophyte of plants. The invention also relates to the use of chimeric genes comprising the promoter of the invention for the selective expression of foreign DNA sequences in the female gametophyte of plants. Plants comprising the chimeric genes of the invention, in which foreign DNA is selectively expressed in the female gametophyte are also provided.

Background

Identification of genes involved in plant reproduction and/or flower development together with the regulatory elements which control their expression in reproductive tissue is of interest for the modulation of the reproductive capacity or embryo development of plants either by influencing endogenous gene expression or through the expression in reproductive tissue of heterologous genes. A number of genes which can be expressed specifically in the stamen have been described and have been proven useful in the development of e.g. male-sterility systems (Mariani et al. 1990, WO 00/26389; WO 00/68403; WO 00/77187; WO 01/12798). Similarly, female sterility systems have been developed making use of promoters of style- and stigma specific genes (WO 91/02068; WO 94/25613). Though female gametophyte development has been examined morphologically for different species, its complex structure and its localization deeply embedded in maternal tissue has made it more difficult to isolate specific genes.

In most plants, the female gametophyte or embryo sac develops from a haploid megaspore which undergoes three subsequent mitotic divisions, resulting in eight nuclei. These are arranged in two groups of four at each end of the gametophyte. One nucleus from each group migrates to the center to form the central cell. The three

remaining nuclei at the micropylar end become cellularized and organized as the egg apparatus, consisting of an egg-cell and two cellular synergids. The three nuclei at the chalazal end form separate cells called antipodals. This seven-celled structure (with eight nuclei) is called the embryo sac (polygonum type).

Upon reaching the embryo sac through the micropyle, the pollen tube penetrates one of the synergids, which subsequently starts to disintegrate allowing release of the two sperm cells. One sperm cell releases its nucleus into the egg-cell producing a zygote which will then develop into an embryo. The other sperm cell releases its nucleus into the central cell, which will then develop into the endosperm.

Different functions have been suggested for the cells of the gametophyte: guiding of the pollen tube to the ovule, targetting of the sperm cells to the egg cell and central cell respectively, prevention of polyspermy, accumulating mRNA for the development of the embryo and endosperm, and preventing autonomous embryo and endosperm development (Cordts et al. 2001). The involvement of female gametophyte cells in the control of embryogenesis is of particular interest for its application in the engineering of apomictic plants. Several mutants of genes affecting female gametophyte development have been described. The FIE/FIS3 (fertilization independent endosperm) gene mutant allows endosperm development without fertilization, suggesting the wild-type protein functions to suppress transcription of genes in the female gametophyte prior to fertilization (Ohad et al. 1999). Similarly, the MEA/FIS1 gene is believed to act by reducing cell proliferation in the embryo (Goodrich, 1998). The FIS1, FIS2 and FIS3 genes are believed to have a sequential regulatory role in the suppression of seed development genes (Luo et al. 1999).

A family of genes specifically expressed in the female gametophyte has been isolated from maize egg cells (ZmES1-4, Cordts et al. 2001). Expression of these genes was found to be high in the synergids, lower in the egg cell and central cell while the antipodal cells showed only weak expression.

The present invention relates to isolated nucleotide sequences specifically expressed in the female gametophyte of plants, preferably highly expressed in the egg cell and

expressed at a lower level or not expressed in the synergids, which sequences can be used for the modulation of endogenous expression of female gametophytic genes and the identification of further genes expressed in the female gametophyte. Furthermore, the promoter identified in these sequences or parts thereof have an application in the direction of expression of heterologous genes in the female gametophyte for the production of plants with modified endosperm and embryo development. A particular embodiment of the invention relates to the production of female sterile plants and of plants capable of apomictic propagation, which are of significant agricultural interest.

Summary of the invention

The present invention relates to isolated DNA sequences that are expressed in the female gametophyte of plants, preferably selectively in the egg apparatus.

A first aspect of the invention relates to promoters, capable of directing expression in the female gametophyte of plants, which can be used for expression of a heterologous DNA sequence in the female gametophyte, in particular in the female gametophyte of monocotyledonous plants, such as maize or rice.

A particular embodiment of the invention relates to the regulatory region of the ZmEA1 gene of SEQ ID No.1, more specifically to the promoter, which directs expression specifically in the female gametophyte of plants. According to another aspect of the invention the promoter of the ZmEA1 gene is reduced to a shorter promoter sequence which is still capable of directing expression in the egg apparatus of the female gametophyte of plants. The invention further relates to functional equivalents of the ZmEA1 promoter, which are capable of directing selective expression of a heterologous DNA in the female gametophyte of plants more specifically of monocots, such as corn and rice, preferably expressed selectively in the egg apparatus. Such functional equivalents can include but are not limited to:

a) sequences hybridizing to the nucleotide sequence of SEQ ID NO: 2 under stringent conditions. Such sequences can be isolated from different corn varieties,

or from other plant species. Such functional equivalents preferably have 90% sequence identity with SEQ ID NO: 2.

b) sequences which can be amplified using oligonucleotide primers comprising at least about 25, preferably at least about 50 or up to 100 consecutive nucleotides of SEQ ID NO:2 in a polymerase chain reaction.

c) Sequence isolated by using a cDNA of the transcript of the ZmEA1 gene of SEQ ID NO:1 or part thereof, as a probe to isolate the genomic DNA from a genomic library upstream of the nucleotide sequence corresponding to the nucleotide sequence of the cDNA.

d) sequences obtained in the method above, whereby the cDNA probe is obtained by screening a cDNA library with oligonucleotides that are deduced from the amino acid sequence of the protein encoded by the ZmEA1 gene of SEQ ID NO: 1, provided in SEQ ID NO: 3.

e) Sequences obtained by screening of a genomic library as described under (c) using a cDNA sequence obtained screening of a cDNA library with a probe which is a fragment amplified from oligonucleotides using a nested-PCR approach.

Functional equivalents of the ZmEA1 promoter can also be obtained by substitution, addition or deletion of nucleotides of the sequence of SEQ ID No. 2. They can be partly or completely synthesized.

According to a further embodiment of the present invention, the DNA sequences of the ZmEA1 gene described herein are used for the cloning and isolation of female gametophyte specific genes, regulatory regions or coding regions from plants. More specifically these sequences can be used for the isolation of functional equivalents of the ZmEA1 promoter from plants, preferably monocotyledonous plants, especially preferably cereal plants such as corn or rice.

According to a particular embodiment of the invention, the female gametophyte-specific promoter of the invention directs expression essentially in the egg apparatus, i.e. the egg cell and the synergids of the female gametophyte of plants.

The present invention further relates to chimeric genes comprising the ZmEA1 promoter or a functional equivalent thereof which directs expression of a heterologous DNA in the female gametophyte. Preferably, the heterologous DNA is a DNA encoding an RNA or protein capable of modifying reproductive development. Most preferably, the heterologous DNA is a DNA encoding an RNA or protein capable of modifying embryogenesis and/or endosperm development. Such constructs can be applied in engineering female sterility, seedless fruit, embryoless seed, haploid or doubled haploid plants or plants capable of apomictic propagation.
According to one embodiment, such a heterologous DNA can be a DNA encoding a cytotoxic molecule, whereby expression of the chimeric ZmEA1-heterologous DNA results in cell death of the female gametophytes. By linking such a gene to a transgene of interest, such constructs can also be applied to prevent transmission of that transgene through the female germline.

The present invention also relates to plant cells or plants and seeds or tissues of plants comprising a chimeric gene according to the invention.

It is yet another objective to provide a method for expressing a biologically active RNA, protein or polypeptide preferentially in the female gametophyte, more specifically the egg apparatus of the plant, comprising the steps of providing a plant cell with the chimeric gene of the present invention and regenerating a plant therefrom.

According to another aspect of the present invention, a method is provided for modifying reproduction of a plant, wherein the method comprises introducing into a plant cell a chimeric gene comprising the ZmEA1 promoter or a functional equivalent thereof operably linked to a heterologous DNA sequence which, upon expression, modifies the development of the egg apparatus, and growing the plant cell into a mature plant.

According to yet another embodiment of the invention a method is provided for modifying the development of the female gametophyte, wherein the method

comprises modulating the endogenous expression of the ZmEA1 gene or overexpression of a gene comprising he ZmEA1 coding region.

Detailed description

The present invention relates to sequences expressed specifically in the female gametophyte of plants, more particularly to the ZmEA1 gene and regulatory and coding sequences derived therefrom. More particularly the invention relates to the use of the female gametophyte-specific promoters, such as the ZmEA1 promoter sequences derived therefrom to direct selective expression of a heterologous DNA in the female gametophyte of plants.

The term "gene" as used herein refers to any DNA sequence comprising several operably linked DNA fragments such as a promoter, a 5' untranslated region (the 5'UTR), a coding region (which may or may not code for a protein), and an untranslated 3' region (3'UTR) comprising a polyadenylation site. Typically in plant cells, the 5'UTR, the coding region and the 3'UTR are transcribed into an RNA of which, in the case of a protein encoding gene, the coding region is translated into a protein. A gene may include additional DNA fragments such as, for example, introns.

The term 'regulatory region' as used herein refers to a DNA region which is involved in regulating the transcription, such as the specificity, timing or level, of a DNA sequence, such as, but not limited to, a DNA sequence encoding a protein. The 5'regulatory region is a region located upstream from a coding sequence which comprises the promoter and the 5' untranslated UTR. The 3' regulatory region is a sequence downstream of the coding sequence comprising suitable termination signals (e.g. one or more polyadenylation signals).

As used herein the term 'promoter' refers to a DNA region, a sequence of which is recognized (directly or indirectly) by a DNA-dependent RNA polymerase during initiation of transcription and which includes the transcription initiation site, binding sites for transcription initiation factors and RNA polymerase. The promoter may also comprise binding sites for other regulatory proteins, such as enhancers or inhibitors.

The term 'chimeric' when referring to a gene or DNA sequence is used to refer to the fact that the gene or DNA sequence comprises at least two functionally relevant DNA fragments (such as promoter, 5'UTR, coding region, 3'UTR, intron) that are not naturally associated with each other and/or originate, for example, from different sources. "Heterologous" referring to a gene or DNA sequence with respect to a plant species is used to indicate that the gene or DNA sequence is not naturally found in that plant species, or is not naturally found in that genetic locus in that plant species. An endogenous gene is a gene which is naturally found in a plant species. "Heterologous" when referring to a parts of a gene (such as coding region, promoter, 3' end) is used to indicate that the heterologous part of the gene is not naturally found associated with at least one other part of that gene.

'Expression' when referring to a gene (in a broad sense) or DNA sequence (in a specific sense) refers to transcription of a DNA region thereof into an RNA which itself is biologically active (eg antisense RNA, ribozyme or other kind of interaction with a DNA, RNA or protein sequence) or which is translated into a biologically active protein or polypeptide.

The term 'female gametophyte specific expression' as used herein refers to expression of a DNA sequence predominantly, preferably exclusively in the female gametophyte, including in the egg apparatus of plants. Expression selectively in the egg apparatus is referred to as 'egg apparatus specific expression'. An 'egg apparatus specific promoter' is a promoter capable of directing egg apparatus specific expression, i.e. expression predominantly, preferably exclusively in the egg apparatus (the egg-cell and the synergids) of the female gametophyte. According to a preferred embodiment of the invention, the female gametophyte specific promoter is characterized in that it is an egg apparatus specific promoter.

A particular embodiment of the present invention relates to a female gametophyte specific promoter, more specifically the promoter of the ZmEA1 gene isolated from corn, which confers female gametophyte specific expression and egg apparatus specific expression in monocots, more specifically in corn. The term 'functional

equivalent of the ZmEA1 promoter' as used herein refers the fact that the promoter is capable of directing female gametophyte specific expression of a gene. Preferably, a functional equivalent of the ZmEA1 promoter is capable of directing egg apparatus specific expression. A specific embodiment of the invention relates to promoters capable of directing female gametophyte specific expression in corn or rice. Such functional equivalents include 'shortened' ZmEA1 promoters, i.e. promoters which comprise only part of the sequence of SEQ ID NO:2, more particularly the fragments of about 1.5kbp, about 1.0kbp and about 0.5kbp upstream from the coding region, for the ZmEA1 protein as described herein. Most preferably, the fragments are of 1570 bp, 1014 bp, and 470 bp upstream from the coding region. However, other fragments which are functional equivalents of the ZmEA1 promoter are envisaged according to the present invention. Alternatively, promoter sequences hybridizing to the nucleotide sequence of SEQ ID NO:2 under stringent conditions and which are capable of directing female gametophyte specific expression are herein considered as functional equivalents of the ZmEA1 promoter. Such functional equivalents can be isolated from different corn varieties, or from other plant species. They can also be obtained by substitution, addition or deletion of nucleotides of SEQ ID NO: 2. They can be partly or completely synthesized.

Other functional equivalents of the ZmEA1 promoter comprise sequences which can be amplified using oligonucleotide primers comprising at least about 25, preferably at least about 50 or up to 100 consecutive nucleotides of SEQ ID NO:2 in a polymerase chain reaction.

Alternatively, a functional equivalent of the ZmEA1 promoter can be isolated by using a cDNA of the transcript of the ZmEA1 gene of SEQ ID NO:1 or part thereof, as a probe to isolate the genomic DNA upstream of the nucleotide sequence corresponding to the nucleotide sequence of the cDNA. Functional equivalents of the ZmEA1 promoter can also be obtained by screening a cDNA library with oligonucleotides that are deduced from the amino acid sequence of the protein encoded by the ZmEA1 gene. Finally, a nested-PCR approach can also be used, whereby the oligonucleotides are used to amplify a fragment, which can serve as a probe to screen a cDNA library. Other methods for obtaining functional equivalents of

the ZmEA1 promoter are based on hybridizations of DNA, cDNA, RNA or oligonucleotides deduced from the ZmEA1 gene of SEQ ID NO:1. Such methods have been described and are known to the person skilled in the art. Egg apparatus specific expression can be ascertained in different ways such as, but not limited to in situ hybridization, detection of GUS-expression after linkage of the promoter to the coding region of the *gus* gene, detection of cell-ablation after linkage of the promoter to a cytotoxic (or 'killer') gene and RNA detection.

'Stringent hybridization conditions' as used herein refers to the fact that hybridization will generally occur if there is at least 95% and preferably at least 97% sequence identity between the probe and the target sequence. Stringent hybridization conditions can for instance comprise the following steps: prehybridizing the filter for 1 to 2 hours at 42°C in 50% formamide, 5 X SSPE, 2 X Denhardt's reagent and 0.1% SDS, or for 1 to 2 hours at 68°C in 6 X SSC, 2 X Denhardt's reagent and 0.1% SDS, 3) adding the hybridization probe which has been labeled, 4) incubating for 16 to 24 hours, 5) washing the filter for 20 min. at room temperature in 1X SSC, 0.1 %SDS, and 6) washing the filter three times for 20 min. each at 68°C in 0.2 X SSC, 0.1 %SDS. Other hybridizing conditions as described by Sambrook et al. (1989).

The heterologous DNA of interest, to which the female gametophyte specific promoter is linked in the chimeric gene of the present invention, can encode a protein or polypeptide or a biologically active RNA, such as an antisense RNA, a sense RNA, a ds-RNA (comprising both sense and antisense sequences so as to form double stranded RNA, as in WO99/53050) which can be used for posttranscriptional gene silencing of a target sequence.

According to a preferred embodiment of the invention the heterologous DNA of interest is a DNA sequence which encodes a biologically active RNA, or a protein or polypeptide which, when expressed in the female gametophyte, is capable of significantly disturbing the metabolism and/or functioning of the cells of the female gametophyte, more particularly the cells of the egg apparatus, so as to modulate embryogenesis and/or endosperm development. Examples of DNA sequences encoding proteins modulating embryogenic and/or endosperm development include

but are not limited to the coding sequence of the FIS-genes (Luo et al, 1999, above), LEC-genes (WO 99/67405; WO 01/70777), BBM gene (WO 00/75330), ZmES genes (Cordts et al, 2001, above; WO/0164924), the WUS gene (Zuo et al., 2002) and the coding sequence of genes directly or indirectly resulting in increased levels of hormones, such as cytokinin, auxin, ethylene and/or brassinosteroids. Examples of DNA suitable for causing death of the cells in which they are expressed include the DNA sequences encoding cytotoxic molecules, such as but not limited to the ribonucleases barnase or Rnase T1, diptheria toxin A (as described, for instance in PCT patent publications WO 89/10396 and WO 91/02068).

The invention relates *inter alia* to methods for expressing a heterologous DNA of interest in the female gametophyte of plants, whereby the method comprises the following steps: introducing of the chimeric genes of the invention into plant cells so as to obtain stable integration in the genome of the plant cells and regeneration of the plant cells into plants.

Modulation of endogenous expression of the ZmEA1 gene can be suppression of expression by using an antisense RNA of the ZmEA1 gene of SEQ ID NO:1, or a ds-RNA (comprising both sense and antisense sequences so as to form double stranded RNA, as in WO99/53050) which can be used for posttranscriptional gene silencing of the ZmEA1 gene or by cosuppression using a sense RNA. Other methods of suppressing or eliminating gene expression (functional knock-outs) known in the art can also be envisaged. Alternatively the ZmEA1 gene product can be over-expressed by introducing one or more additional copies of the ZmEA1 gene in the genome of the plant.

The coding sequence of the ZmEA1 gene of the present invention can be used in the development of plants with modified embryogenesis and/or endosperm development. Thus the coding sequence of the ZmEA1 gene can be placed under control of a heterologous promoter in order to direct expression in cells not naturally expressing the ZmEA1 protein and/or to overexpress the ZmEA1 coding region in the female gametophyte. Preferably, the ZmEA1 coding region is the region encoding the protein

of SEQ ID NO:3, most preferably it corresponds to the coding region of SEQ ID NO:1.

Introduction of a foreign DNA into a plant cell can be obtained by conventional transformation methods described in the art. Such methods include but are not limited to *Agrobacterium* mediated transformation (US 6,074,877, Hiei et al., 1997), microprojectile bombardment (as described, for example by Chen et al., 1994; Casas et al., 1995; Christou, 1997, Finer et al., 1999, Vasil et al. 1999), direct DNA uptake into protoplasts (as described, for example by De Block et al. 1989; Poulsen, 1996, Datta et al., 1999), electroporation (D'Halluin et al., 1992, US 5,641,665, Bates 1999) or silicon whisker mediated DNA introduction (Dunwell, 1999) or other methods as generally reviewed by Potrykus (1990), Sawahel et al. (1995), Komari et al. (1998), Bogorad (2000) and Newell (2000).

Operably linking the DNA of interest to a female gametophyte specific promoter according to the invention can also be achieved by replacing the DNA naturally associated with the female gametophyte specific promoter by homologous recombination with the gene of interest, provided that the DNA of interest comprises a homology region with the DNA normally associated with the female gametophyte specific promoter. Such methods have been described in the art (eg US 5,744,336).

It will be appreciated that the means and methods of the invention are particularly useful for corn and rice, but may also be used in other plants.

The following non-limiting examples describe the isolation of a female gametophyte specific promoter and the construction of chimeric genes for expression in the female gametophyte, preferably the egg apparatus of plants. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook and Russell (2001) Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, NY, in Volumes 1 and 2 of Ausubel et al. (1994) Current Protocols in Molecular Biology, Current Protocols, USA and in Volumes I and II of Brown (1998) Molecular Biology LabFax, Second Edition, Academic Press (UK). Standard materials and methods for

plant molecular work are described in Plant Molecular Biology Labfax (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK. Standard materials and methods for polymerase chain reactions can be found in Dieffenbach and Dveksler (1995) PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, and in McPherson at al. (2000) PCR - Basics: From Background to Bench, First Edition, Springer Verlag, Germany.

Throughout the description and Examples, reference is made to the following sequences represented in the sequence listing:

SEQ ID NO:1 :	nucleotide sequence of the ZmEA1 gene isolated from corn
SEQ ID NO:2 :	nucleotide sequence of a ZmEA1 promoter
SEQ ID NO:3 :	deduced amino acid sequence of the ZmEA1 protein encoded by SEQ ID NO: 1
SEQ ID NO:4 :	primer CML5
SEQ ID NO:5 :	long RACE primer
SEQ ID NO:6 :	short RACE primer
SEQ ID NO:7 :	cDNA of ZmEA1
SEQ ID NO:8 :	adaptor 2R
SEQ ID NO:9 :	adaptor AP1
SEQ ID NO:10 :	primer P7
SEQ ID NO:11 :	adaptor AP2
SEQ ID NO:12 :	primer CML7
SEQ ID NO:13 :	primer P6
SEQ ID NO:14 :	primer RT1
SEQ ID NO:15 :	primer Qbr
SEQ ID NO:16 :	primer Gap1
SEQ ID NO:17 :	primer Gap2
SEQ ID NO:18 :	primer ZE-Bbs
SEQ ID NO:19 :	primer ZE1-Xsma
SEQ ID NO:20 :	primer ZE2-Xsma
SEQ ID NO:21 :	primer ZE3-Xsma

SEQ ID NO:22 : primer ZE-Asc

EXAMPLES

Example 1: Isolation of the ZmEA1 gene

a) Plant material, isolation of cells from the unfertilized and fertilized embryo sac and in vitro fertilisation

Maize (Zea mays L.) inbred lines A188 (Green and Phillips, 1975), CO159, TX303, CM37 and T232A (all described by Burr and Burr, 1991) were grown under standard greenhouse conditions. Cells of the embryo sac were mechanically isolated with glass needles from ovule tissues treated with a cell wall degrading enzyme mixture and transferred using a hydraulic microcapillary system according to Kranz et al. (1991). In vitro zygotes were generated after fusing isolated gametes by a short electric pulse and cultivated as described (Kranz and Lörz, 1993). In vivo zygotes were isolated as described by Cordts et al. (2001). The cells were collected and stored in 200 nl each at -80°C until usage.

b) Isolation of the ZmEA1 cDNA

With the aim to identify genes specifically upregulated in the unfertilized egg cell of maize, a differential plaque screening was conducted with a cDNA library of maize egg cells (Dresselhaus et al. 1994). A total of 29,000 plaque forming units was hybridized with cDNA populations of egg cells, in vitro zygotes (Dresselhaus et al. 1996) and seedlings (Cordts 2000). 250 clones gave strong signals with the egg cell cDNA population and were selected for a second screening round (reverse Northern blot analysis) according to the procedure described by Dresselhaus et al. (1996). 70 clones were selected which produced strong signals after hybridization with the egg cell cDNA population, but no or weak signals with cDNA populations of in vitro zygotes and seedlings, respectively. All 70 clones were fully sequenced. Partial cDNAs of the ZmEA1 gene were represented 17 times among these clones varying in length from 356-438 bp. All clones contained a 3' UTR (untranslated region) which varied in length due to different polyA sites used to terminate transcription.

In order to isolate the full length cDNA of ZmEA1, cDNA was generated from in vivo zygotes, 16 to 18 hours after fertilization. Poly(A)$^+$ mRNA isolation out of 9 zygotes was performed using the Dynabeads® mRNA DIRECT™ Micro Kit (Dynal). Immediately after isolation, poly(A)$^+$ mRNA was used for first strand cDNA synthesis. Reverse transcription and cDNA amplification by long distance PCR (LD PCR) was performed using the SMART™ cDNA synthesis Kit (Clontech, Palo Alto) according to the user manual. Quality of obtained cDNA was checked by separating 5µl of PCR reactions on a 0,8% agarose gel. After gel electrophoresis, cDNA was blotted onto Hybond NX™ nylon membrane (Amersham) and hybridized with a GAPDH-specific probe. The missing 5'-end was amplified from 10 ng/ul of this cDNA using a gene specific primer and a universal primer mix (UPM- a mixture of two specific race primers detailed below) in a standard PCR reaction with the following profile: 2 min 94°C followed by 5 cycles: 30 sec 94°C, 3 min 72°C; 5 cycles: 30 sec 94°C, 30 sec 70°C, 3 min 72°C; 20 cycles: 30 sec 94°C, 30 sec 68°C, 3 min 72°C and a final extension for 10 min at 72°C. PCR products were cloned and sequenced.

gene specific-primer:

CML5:

5'-ACGATCACTTGCTCACAGTCACAGCTAG-3' SEQ ID No. 4

RACE primers:

Long primer:

5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3'

SEQ ID NO: 5

Short primer:

5'-GTAATACGACTCACTATAGGGC-3' SEQ ID NO: 6

c) DNA sequencing and sequence analysis

All clones were sequenced from both directions using Taq DNA polymerase FS Cycle Sequencing Kit (PE Applied Biosystems) and the 373A and 377 automated DNA

sequencer (Applied Biosystems). DNA and amino acid sequence data were further processed using the DNASTAR program software packages (DNASTAR Inc.). Sequence data were compiled and compared with EMBL, GenBank, DDBJ, SwissProt, PIR and PRF databases with FASTA and BLAST algorithms (Pearson, 1990). Prediction of protein localization sites, identification of transmembrane domains and secondary structure prediction were performed using appropriate computer programs.

The cDNA of ZmEA1 is 534 bp in length with the largest open reading frame encoding 94 amino acids (position: 1571 – 1855 in SEQ ID NO:1). The 5' UTR is 107 bp (5' UTR: position 1464 - 1570 SEQ ID NO:1) in length, calculated from the putative start point of transcription at position 1464 as analyzed by Single Cell RT-PCR and excluding the ATG at position 1571-1573. The 3' UTR is 142 bp (3' UTR: position 1856 - 1997). The sequence of the full-length cDNA is given in SEQ ID NO: 7. The amino acid sequence of ZmEA1 is given in SEQ ID NO: 3. The ZmEA1 gene encodes a very hydrophobic peptide and contains no introns. Homology searches revealed that ZmEA1 does not match to any other ESTs in public data bases (February 2002).

d) Isolation of the ZmEA1 promoter

Genomic DNA from leaf material of Zea mays inbred line A188 was used to construct GenomeWalker - "libraries", based on the Universal Genome Walker™ Kit protocol (Clontech, Palo Alto). Separate aliquots of DNA were completely digested with 6 different restriction enzymes that leave blunt ends (EcoRV, DraI, HincII, PvuII, ScaI and SmaI). Each batch of digested genomic DNA was then ligated separately to the GenomeWalker Adaptor (Adaptor 2R: 5'-CTAATACGACTCACTATAGGGCAGCGTG GTCGCGGCCGAGGT-3', SEQ ID NO:8). These libraries served as templates for the synthesis of 1463 bp of promoter sequence. The amplifications were carried out with the outer adapter primer (AP1: 5'-CTAATACGACTCACTATAGGGC-3', SEQ ID NO:9) and outer, gene-specific primers (CML5, SEQ ID NO:4; P7: 5'-TGACCACGCTAACGAAGAGCCCTAGTCGC-3', SEQ ID NO:10) for the first or

primary PCR, and with the nested adaptor primer (AP2: 5'-AGCGTGGTCGCGGCCGAGGT-3', SEQ ID NO:11) and nested gene-specific primers (CML7: 5'-GCTGCTTAGTTGGAGGAGAGCGATCGGCT-3', SEQ ID NO:12; P6: 5'-GACCAGGGTCGGACGTCGAAACCTGATAG-3', SEQ ID NO: 13) for the secondary or "nested" PCR, according to the manufacturer's instructions, with the exception that 5% DMSO was added. At the end, the 1463 bp promoter fragment was cloned with the TOPO-TA PCR Cloning®Kit (Invitrogen, Leek) and sequenced as described above.

Example 2: Expression analysis of ZmEA1

Tissue in situ hybridization was performed according to Cordts et al. (2001) to investigate the expression of ZmEA1 in ovules at maturity. The in situ hybridization experiments were made with ovule tissues embedded in BMM (butyl-methyl methacrylate). A partial cDNAs of the ZmEA1 gene was used as a hybridization probe. Strong signals were detected in the cytoplasm of synergids and egg cell close to the micropyle region. Signals in nucellus cells, integuments or ovary tissues were never observed (Cordts 2000). In order to study ZmEA1 expression pattern in other tissues of maize, total RNA and poly(A)$^+$ RNA Northern blot analyses were performed. The investigated tissues included immature male and female inflorescences of different developmental stages, immature and mature ovaries, kernels at different developmental stages, immature and mature embryos, embryogenic and non-embryogenic suspension cultures, different seedling stages, light and dark green leaves, internodes, meristematic leaf base, root tips and roots without tips. A signal was not obtained in any sample tested.

Multiplex RT-PCR was performed according to Cordts et al. (2001) and applied to prove absense of ZmEA1 messenger RNA in most of the tissues investigated by Northern blot analysis and in addition with nodes, scutellum tissue, immature and mature pollen, microspores at the 2 nucleus stage, immature cob, immature and mature ovules. RT-PCR with single cells of the embryosac before and after fertilization was performed as described by Cordts et al. (2001) using the primers

(RT1: 5'-AGCGCCCGCTGTCCATTCAT-3', SEQ ID NO:14) and (Qbr: 5'-ACGACGATCACTTGCTCACAG-3', SEQ ID NO:15). A maize GAPDH gene was used as a control for the success of the RT-PCR using the forward primer Gap1: 5'-AGGGTGGTGCCAAGAAGGTTG-3' (SEQ ID NO:16) and the reverse primer Gap2: 5'-GTAGCCCCACTCGTTGTCGTA-3' (SEQ ID NO:17), as described by Richert et al. (1996). The data obtained from the Northern blot analysis were confirmed. Strong signals were observed exclusively in egg cells and synergids before fertilization and weaker signals in in vitro zygotes up to 68h after in vitro fertilization (Cordts 2000). Some 15 antipodal cells instead of single cells were used under the same RT-PCR conditions for a single reaction and a much lower or no signal was detected. ZmEA1 trancripts could not be detected in any other tissue tested.

Example 3: Genomic analysis of ZmEA1

Extraction of genomic DNA from maize inbred lines was performed according to Dellaporta et al. (1983). 10 µg genomic DNA was digested with restriction enzymes and resolved on 0.8 % agarose gels. DNA was transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech) with 0.4 M NaOH. Blots were hybridized overnight with radioactive probes derived from a partial ZmEA1 cDNA and prepared by Prime-It Random Primer Labelling Kit (Stratagene) in QuickHyb buffer (Stratagene) containing 100 µg/ml salmon sperm DNA. Filters were washed with decreasing concentrations of SSC with a final wash at 65 °C in 0.2 x SSC / 0.1% SDS. Filters were exposed at -70 °C to Kodak X-Omat AR films using intensifier screens. A partial cDNA of ZmEA1 was used as a probe in DNA gel blots to identify RFLPs between the parents of the inbred mapping populations CO159 x TX303 and CM37 x T232A (Burr and Burr, 1991). The resulting polymorphisms were scored within the corresponding loci placed on the Brookhaven National Laboratory map using the INBRED program (Burr et al. 1994). A single copy band was observed in A188 with HindIII, EcoRI, EcoRV and DraI, as well as in CO159 and TX303 with PstI and in CM37 and T232A with BglII. ZmEA1 maps on chromosome 7 L between the molecular markers isc(b32B) (119.3) and bnl8.39 (124.8). ZmEA1 is not co-localized with known phenotypic markers.

Example 4: Generation of constructs for rice transformation

Three GUS-promoter deletion contructs and one BARNASE-construct were generated for rice transformation.

For the GUS-contructs, different lengths of the ZmEA1 promoter corresponding to 1.57 kbp, 1.013 kbp and 0.47 kbp of the genomic sequences upstream of the AUG codon (positions: 1-1570, 558-1570, 1101-1570) were PCR amplified, using the following primers:

ZE-Bbs: CTCACTCACGAAGACGACATGCAGAATTCAGCGTC
(SEQ ID NO: 18)

ZE1-Xsma: CTCACTCCCCCCGGGGGGATCCACACGATTCTGCCTGCAT
(SEQ ID NO:19)

ZE2-Xsma: CTCACTCCCCCCGGGGGGAGTGTTGTGCGCACTCAGGTC
(SEQ ID NO: 20)

ZE3-Xsma: CTCACTCCCCCCGGGGGGACGGATTGCTGGTAGTGGACG.
(SEQ ID NO: 21)

The resulting different promoter fragments were cloned in front of the GUS reporter gene to obtain three rice T-DNA contructs: pCM-G1, pCM-G2 and pCM-G3.

The ZmEA1::GUS T-DNAs are designed as follows:

LB- 3'nos <BAR<cab22L<p35S2 <>ZmEA1promoter>GUS>3'nos-RB

The constructs are transformed into rice cells, followed by regeneration and analysis of GUS-expression. Based hereon it can be demonstrated that the 1.57 kbp upstream of the transcription start codon is sufficient to drive cell specific expression in the female gametophyte of rice.

Example 5: Use of the ZmEA1 promoter to engineer embryo sac lethality

Constructs are made to engineer embryo sac lethality in rice by placing a DNA encoding a cytotoxic molecule under the control of the ZmEA1 promoter.

A construct comprising the coding region of the barnase gene (Hartley et al., 1988) and the prophylactic barstar (WO 96/26283) was made for introduction into rice.

The "full-length" ZmEA1 promoter (1.57 kbp) was amplified using the following primers:

ZE-Bbs: CTCACTCACGAAGACGACATGCAGAATTCAGCGTC (SEQ ID NO: 18)

ZE-Asc: CTCACTTGGCGCGCCAATCCACACGATTCTGCCTGCAT (SEQ ID NO: 22)

The ZmEA1 promoter was cloned in front of the BARNASE coding sequence. The prophylactic BARSTAR under the control of the 35S promoter was included in the T-DNA. The final T-DNA construct was designed as follows:

LB-3'nos<bar<cab22L<P35S2<>pZmEA1>BARNASE>3''nos><3'NOS<BARSTAR<p35S3-RB

Transformation of the ZmEA1-Barnase construct into plant cells can result in plants in which female gametophyte development is disturbed, due to the expression of the barnase gene in the egg apparatus. Use of the prophylactic barstar construct is expected to increase transformation efficiency and can be of interest to counter-act possible non-specific expression of the ZmEA1 promoter.

Example 6: Use of the ZmEA1 promoter to prevent transmission through the female germline

Female transmission of a specific transgenic trait is eliminated or reduced by physically linking this trait to a construct comprising the ZmEA1 promoter driving expression of a cytotoxic gene (for ex. Barnase).

In hemizygous plants, all female gametophytes which carry the specific transgenic trait in their genome are killed by expression of the linked ZmEA1::cytotoxic gene construct. Thus, female transmission of the specific transgenic trait is reduced or eliminated.

5

The specific transgenic trait and the ZmEA1::cytotoxic gene construct can be transferred together into the genome of the plants as a single piece of DNA or as two separate events. In the latter, the two events should have complete genetic and or physical linkage.

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Claims:

1. An isolated DNA sequence that is expressed in the female gametophyte of plants, which hybridizes to the nucleotide sequence of SEQ ID NO: 1 under stringent conditions, or a part or complementary strand thereof.

2. The sequence of claim 1, which contains a regulatory region capable of directing expression of an operably linked DNA sequence in the female gametophyte of plants.

3. A promoter capable of directing expression in the egg apparatus of the female gametophyte of plants, comprising a nucleotide sequence selected from the following group of nucleotide sequences:

a) sequences hybridizing to the nucleotide sequence of SEQ ID NO: 2 under stringent conditions and having at least 90% sequence identity with SEQ ID NO: 2.

b) sequences which can be amplified using oligonucleotide primers comprising at least about 25 consecutive nucleotides of SEQ ID NO:2 in a polymerase chain reaction.

c) sequences isolated by using a cDNA of the transcript of the ZmEA1 gene of SEQ ID NO:1 or part thereof, as a probe to isolate the genomic DNA from a genomic library upstream of the nucleotide sequence corresponding to the nucleotide sequence of the cDNA.

d) sequences obtained in the method above, whereby the cDNA probe is obtained by screening a cDNA library with oligonucleotides that are deduced from the amino acid sequence of the protein encoded by the ZmEA1 gene of SEQ ID NO: 1, provided in SEQ ID NO: 3.

e) sequences obtained by screening of a genomic library as described under (c) using a cDNA sequence obtained screening of a cDNA library with a probe which is a fragment amplified from oligonucleotides using a nested-PCR approach.

4. A promoter sequence capable of directing expression in the egg apparatus of the female gametophyte of plants, comprising a nucleotide sequence having at least

90% sequence similarity with the sequence of SEQ ID NO: 2 and obtained by substitution, addition or deletion of nucleotides of the sequence of SEQ ID NO: 2.

5. The promoter of claim 3 or 4 comprising the sequence of SEQ ID NO: 2.

6. The promoter of any one of claims 3 to 5, which is isolated from corn.

7. The use of the sequence of SEQ ID NO: 1 for the cloning, isolation or expression of female gametophyte specific nucleotide sequences.

8. A chimeric gene comprising a heterologous DNA sequence under the control of the promoter of claim 3.

9. The chimeric gene of claim 8, wherein the heterologous DNA is a DNA encoding an RNA or protein capable of modifying embryogenesis and/or endosperm development.

10. The chimeric gene of claim 9, wherein said heterologous DNA is a DNA encoding a cytotoxic molecule.

11. A method for engineering female sterility, seedless fruit, embryoless seed, haploid plants or plants capable of apomictic propagation using the chimeric gene of claim 8.

12. A method for preventing transmission of a trait through the female germline, said method comprising linking the gene or genes encoding said trait to a chimeric gene comprising a DNA encoding a cytotoxic molecule under control of the promoter capable of directing expression in the female gametophyte of the plant.

13. The method of claim 12, wherein said promoter is the promoter according to claim 3.

14. A plant or cells, seeds or tissues thereof, comprising, stably integrated into its genome, the chimeric gene of claim 8.

15. A method for modifying reproduction of a plant, wherein the method comprises introducing into a plant cell a chimeric gene comprising the ZmEA1 promoter or a functional equivalent thereof operably linked to a heterologous DNA sequence which, upon expression, modifies the development of the egg apparatus, and growing the plant cell into a mature plant.

16. A method for modifying the development of the female gametophyte, wherein the method comprises modulating the endogenous expression of the ZmEA1 gene.

17. A method for modifying the development of the female gametophyte, wherein the method comprises overexpressing a heterologous gene comprising the ZmEA1 coding region.

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/06037

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C07K14/415 C12N15/29 A01H1/00 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 64924 A (CORDTS SIMONE ;LOERZ HORST (DE); AMIEN SUSENO (DE); DRESSELHAUS TH) 7 September 2001 (2001-09-07) page 7, line 1 - line 19 page 9, paragraph 2 -page 10, paragraph 1 examples 1,4 ---	1-17
X	WO 01 21785 A (JEFFERSON RICHARD A ;CAMBIA (AU); YANG WEI (AU); GROSSNICKLAUS UEL) 29 March 2001 (2001-03-29) page 2, line 5 - line 35 page 3, line 10 - line 15 example 4 --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
12 September 2003	30/09/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mundel, C

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	DROUAUD JAN ET AL: "A Brassica napus skp1-like gene promoter drives GUS expression in Arabidopsis thaliana male and female gametophytes." SEXUAL PLANT REPRODUCTION, vol. 13, no. 1, July 2000 (2000-07), pages 29-35, XP002253404 ISSN: 0934-0882 the whole document	1-17
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