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(54) Title: PLANT EGG CELL TRANSCRIPTIONAL CONTROL SEQUENCES

(57) Abrégé/Abstract:
The present invention relates generally to transcriptional control sequences. Generally, the present invention relates to transcriptional control sequences that specifically or preferentially direct expression of a nucleotide sequence of interest in a plant egg cell. The present invention is predicated, in part, on the identification of transcriptional control sequences derived from EC1 genes which, in preferred embodiments, direct preferential expression in an egg cell of at least one plant taxon.

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(54) Title: PLANT EGG CELL TRANSCRIPTIONAL CONTROL SEQUENCES

(57) Abstract: The present invention relates generally to transcriptional control sequences. Generally, the present invention relates to transcriptional control sequences that specifically or preferentially direct expression of a nucleotide sequence of interest in a plant egg cell. The present invention is predicated, in part, on the identification of transcriptional control sequences derived from EC1 genes which, in preferred embodiments, direct preferential expression in an egg cell of at least one plant taxon.

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PLANT EGG CELL TRANSCRIPTIONAL CONTROL SEQUENCES

FIELD OF THE INVENTION

The present invention relates generally to transcriptional control sequences. Generally, the present invention relates to transcriptional control sequences that specifically or preferentially direct expression of a nucleotide sequence of interest in a plant egg cell.

BACKGROUND OF THE INVENTION

The primary emphasis in genetic modification has been directed to prokaryotes and mammalian cells. For a variety of reasons, plants have proven more intransigent than other eukaryotic cells to genetically manipulate. However, in many instances, it is desirable to effect transcription of an introduced nucleotide sequence of interest either specifically or preferentially in a particular plant part or at a particular developmental stage of the plant. Accordingly, there is substantial interest in identifying transcriptional control sequences, such as promoters or enhancers, which specifically or preferentially direct transcription in particular plant organs, tissues or cell types or at particular developmental stages of the plant.

Expression of heterologous DNA sequences in a plant is dependent upon the presence of an operably linked transcriptional control sequence, such as a promoter or enhancer, which is functional within the plant. The choice of transcriptional control sequence will determine when and where within the organism the heterologous DNA sequence is expressed. For example, where continuous expression is desired throughout the cells of a plant, constitutive

promoters are utilized. In contrast, where gene expression in response to a stimulus is desired, an inducible promoter may be used. Where expression in specific tissues or organs is desired, a tissue-specific promoter may be used.

Frequently, it is desirable to effect expression of a DNA sequence in particular cells, tissues or organs of a plant. For example, male and/or female sterility in a plant might be accomplished by genetic manipulation of the plant's genome with a male or female gamete specific promoter operably linked to a toxic protein.

Alternatively, it might be desirable to inhibit expression of a native DNA sequence within particular plant tissues to achieve a desired phenotype. In this case, such inhibition might be accomplished by transformation of the plant with a tissue-specific promoter operably linked to an antisense or RNAi nucleotide sequence, such that expression of these sequences produces an RNA transcript that interferes with translation of the mRNA of the native DNA sequence.

Promoter sequences that can be used to drive egg cell specific expression of a nucleotide sequence of interest in higher plants are not presently available. This may be at least in part attributed to the difficulty in isolating female gametes from seed plants. As a consequence of this difficulty, the transcripts of plant egg cells are poorly represented in current databases of expressed sequence tags (ESTs), which have been mainly generated through sequencing from cDNA libraries produced from complex tissues, e.g. whole floral organs. Though more than 1.5 million *Poaceae* ESTs were present in the public EST database (by March 2004) the use of complex tissues resulted in under representation of genes expressed at low levels and in only one or a few cell types.

However, the isolation and characterization of egg cell-specific transcriptional control sequences would be desirable for use in the genetic manipulation of plants.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

SUMMARY OF THE INVENTION

The present invention is predicated, in part, on the identification of transcriptional control sequences derived from EC1 genes which, in preferred embodiments, direct preferential expression in an egg cell of at least one plant taxon.

Accordingly, in a first aspect, the present invention provides an isolated nucleic acid comprising:

(i) a nucleotide sequence defining a transcriptional control sequence, wherein said transcriptional control sequence is derived from a gene which encodes an EC1 polypeptide; or

(ii) a nucleotide sequence defining a functionally active fragment or variant of (i).

In accordance with the present invention, a consensus sequence for EC1 polypeptides has been determined. Accordingly, in one particularly preferred embodiment, the isolated nucleic acid of the first aspect of the invention comprises a transcriptional control sequence derived from a gene which encodes a polypeptide comprising the amino acid sequence:

$[X_N]$CWXXXXX[LI]X[SH]C[TS]X[DE] [IL] [ILV]XFF[LIV] $[X_N]$ [LI]
XXXCCX[AS] [ILV]XXXXXXCW $[X_N]$ [ILV]G[FL]TXXEXXXLXXXC $[X_N]$
(SEQ ID NO: 1)

wherein X is any amino acid residue; $[X_N]$ is one or more amino acid

residues of any type; [LI] or [IL] is a leucine or isoleucine residue; [SH] is a serine or histidine residue; [TS] is a threonine or serine residue; [DE] is an aspartic acid or glutamic acid residue; [ILV] or [LIV] is a leucine, isoleucine or valine residue; [AS] is an alanine or serine residue; and [FL] is a phenylalanine or leucine residue.

In further preferred embodiments the isolated nucleic acid comprises one or more sequence motifs comprising the nucleotide sequence set forth in SEQ ID NO: 33 and/or SEQ ID NO: 69.

In a preferred form, the transcriptional control sequences of the present invention, or functionally active fragments or variants thereof, are plant egg cell specific or plant egg cell preferential transcriptional control sequences.

In a second aspect, the present invention provides an isolated nucleic acid selected from the list consisting of:

(i) a nucleic acid comprising the nucleotide sequence set forth in any of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32;

(ii) a nucleic acid comprising a nucleotide sequence which is at least 50% identical to any of the nucleotide sequences mentioned in (i);

(iii) a nucleic acid which hybridizes to any of the nucleic acids mentioned in (i) under stringent conditions;

(iv) a nucleic acid comprising a nucleotide sequence which is the complement or reverse complement of any one of (i) to (iii); and

(v) a fragment of any of (i), (ii), (iii) or (iv).

In a third aspect, the present invention provides a nucleic acid construct comprising the isolated nucleic acid of the first and/or second aspects of the invention.

In a preferred embodiment, the isolated nucleic acid comprises a nucleotide sequence defining a transcriptional control sequence and further comprises a nucleotide sequence of interest operably connected to the transcriptional control sequence. More preferably, the nucleotide sequence of interest is heterologous with respect to said transcriptional control sequence.

In a fourth aspect, the present invention provides a cell comprising:

(i) the nucleic acid construct of the third aspect of the invention; and/or

(ii) a genomically integrated form of the construct mentioned at (i).

In a particularly preferred embodiment, the cell is a plant egg cell. Even more preferably, the level, rate and/or pattern of expression of at least one nucleotide sequence is altered in said plant egg cell relative to a wild type form of said plant egg cell.

In a fifth aspect, the present invention provides a multicellular structure comprising one or more cells of the fourth aspect of the invention. Preferably, the multicellular structure comprises a plant or a part, organ or tissue thereof.

In a sixth aspect, the present invention provides a method for specifically or preferentially expressing a nucleotide sequence of interest in a plant egg cell, the method comprising effecting transcription of the nucleotide sequence of interest in a plant under the transcriptional control of the nucleic acid of any of the first, second, or third aspects of the invention, wherein said nucleic acid comprises a plant egg cell specific or plant egg cell preferential transcriptional control sequence.

In a seventh aspect, the present invention provides a method for promoting female sterility in a plant, the method comprising expressing a nucleotide

sequence encoding a cytotoxic or cytostatic protein or a cytotoxic of cytostatic non-translated RNA, specifically or preferentially in an egg cell of the plant; wherein said nucleotide sequence is operably connected to a nucleic acid of any of the first, second or third aspects of the invention and wherein said nucleic acid comprises a plant egg cell specific or plant egg cell preferential transcriptional control sequence.

In an eighth aspect, the present invention provides a method for modulating embryo development and/or embryo size in a plant, the method comprising expressing a nucleotide sequence encoding a transcriptional regulator that acts during embryo development, specifically or preferentially in an egg cell of the plant; wherein said nucleotide sequence encoding a transcriptional regulator is operably connected to a nucleic acid of any of the first, second or third aspects of the invention and wherein said nucleic acid comprises a plant egg cell specific or plant egg cell preferential transcriptional control sequence.

In a ninth aspect, the present invention provides a method for promoting apomixis in a plant, the method comprising expressing an apomixis-promoting nucleotide sequence specifically or preferentially in an egg cell of the plant; wherein said apomixis-promoting nucleotide sequence is operably connected to a nucleic acid of any of the first, second or third aspects of the invention and wherein said nucleic acid comprises a plant egg cell specific or plant egg cell preferential transcriptional control sequence.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to herein by a sequence identifier number (SEQ ID NO:). A summary of the sequence identifiers is

provided in Table 1. A sequence listing is provided at the end of the specification.

TABLE 1 – Summary of Sequence Identifiers

Sequence Identifier	Sequence
SEQ ID NO: 1	EC1 polypeptide consensus amino acid sequence
SEQ ID NO: 2	AtEC1.1 polypeptide amino acid sequence
SEQ ID NO: 3	AtEC1.2a polypeptide amino acid sequence
SEQ ID NO: 4	AtEC1.2b polypeptide amino acid sequence
SEQ ID NO: 5	AtEC1.4 polypeptide amino acid sequence
SEQ ID NO: 6	AtEC1.5 polypeptide amino acid sequence
SEQ ID NO: 7	MtEC1.1 polypeptide amino acid sequence
SEQ ID NO: 8	OsEC1.1 polypeptide amino acid sequence
SEQ ID NO: 9	OsEC1.2 polypeptide amino acid sequence
SEQ ID NO: 10	OsEC1.3 polypeptide amino acid sequence
SEQ ID NO: 11	TaEC1 polypeptide amino acid sequence
SEQ ID NO: 12	HvECA1 polypeptide amino acid sequence
SEQ ID NO: 13	*AtEC1.1* open reading frame nucleotide sequence
SEQ ID NO: 14	*AtEC1.2a* open reading frame nucleotide sequence
SEQ ID NO: 15	*AtEC1.2b* open reading frame nucleotide sequence
SEQ ID NO: 16	*AtEC1.4* open reading frame nucleotide sequence
SEQ ID NO: 17	*AtEC1.5* open reading frame nucleotide sequence
SEQ ID NO: 18	*MtEC1.1* open reading frame nucleotide sequence
SEQ ID NO: 19	*OsEC1.1* open reading frame nucleotide sequence
SEQ ID NO: 20	*OsEC1.2* open reading frame nucleotide sequence
SEQ ID NO: 21	*OsEC1.3* open reading frame nucleotide sequence
SEQ ID NO: 22	*TaEC1* cDNA nucleotide sequence
SEQ ID NO: 23	*HvECA1* open reading frame nucleotide sequence
SEQ ID NO: 24	*pAtEC1.1* nucleotide sequence
SEQ ID NO: 25	*pAtEC1.2a* nucleotide sequence
SEQ ID NO: 26	*pAtEC1.2b* nucleotide sequence
SEQ ID NO: 27	*pAtEC1.4* nucleotide sequence
SEQ ID NO: 28	*pAtEC1.5* nucleotide sequence
SEQ ID NO: 29	*pMtEC1.1* nucleotide sequence
SEQ ID NO: 30	*pOsEC1.1* nucleotide sequence
SEQ ID NO: 31	*pOsEC1.2* nucleotide sequence
SEQ ID NO: 32	*pOsEC1.3* nucleotide sequence

SEQ ID NO: 33	EC1 promoter nucleotide sequence motif #1
SEQ ID NO: 34	TaGAP1 primer
SEQ ID NO: 35	TaGAP2 primer
SEQ ID NO: 36	TaEC1fw2 primer
SEQ ID NO: 37	TaEC1rev2 primer
SEQ ID NO: 38	Act3fw primer
SEQ ID NO: 39	Act3rev primer
SEQ ID NO: 40	AtEC1.1fw primer
SEQ ID NO: 41	AtEC1.1rev primer
SEQ ID NO: 42	AtEC1.2a/bfw primer
SEQ ID NO: 43	AtEC1.2a/brev primer
SEQ ID NO: 44	AtEC1.4fw primer
SEQ ID NO: 45	AtEC1.4rev primer
SEQ ID NO: 46	AtEC1.5fw primer
SEQ ID NO: 47	AtEC1.5rev primer
SEQ ID NO: 48	pAtEC1.1 primer
SEQ ID NO: 49	AtEC1.1rev1 primer
SEQ ID NO: 50	pAtEC1.2a primer
SEQ ID NO: 51	tAtEC1.2a primer
SEQ ID NO: 52	AtEC1.1-PstI primer
SEQ ID NO: 53	AtEC1.2a-BglII primer
SEQ ID NO: 54	GUS start rev primer
SEQ ID NO: 55	E1F primer
SEQ ID NO: 56	E1R primer
SEQ ID NO: 57	EC1-PF2 primer
SEQ ID NO: 58	EC1-R primer
SEQ ID NO: 59	GFP-seq primer
SEQ ID NO: 60	LH1 primer
SEQ ID NO: 61	GUS3 primer
SEQ ID NO: 62	GUS4 primer
SEQ ID NO: 63	bar-fw primer
SEQ ID NO: 64	bar-rev primer
SEQ ID NO: 65	1-1fwXbaI primer
SEQ ID NO: 66	1-1revXbaI primer
SEQ ID NO: 67	At2a-BglIIfw primer
SEQ ID NO: 68	At2a-SalIrev primer
SEQ ID NO: 69	EC1 promoter nucleotide sequence motif #2

DESCRIPTION OF PREFERRED EMBODIMENTS

It is to be understood that the following description is for the purpose of describing particular embodiments only and is not intended to be limiting with respect to the above description.

As set out above, the present invention is predicated, in part, on the identification of transcriptional control sequences which are derived from EC1 genes that are preferentially expressed in the egg cells of at least some plants.

As used herein, the term “transcriptional control sequence” should be understood as any nucleotide sequence that modulates at least the transcription of an operably connected nucleotide sequence. Furthermore, the transcriptional control sequence of the present invention may comprise any one or more of, for example, a leader, promoter, enhancer or upstream activating sequence.

As referred to herein, the term “transcriptional control sequence” preferably at least includes a promoter.

A “promoter” as referred to herein, encompasses any nucleic acid that confers, activates or enhances expression of an operably connected nucleotide sequence in a cell.

As used herein, the term “operably connected” refers to the connection of a transcriptional control sequence, such as a promoter, and a nucleotide sequence of interest in such as way as to bring the nucleotide sequence of interest under the transcriptional control of the transcriptional control sequence. For example, promoters are generally positioned 5' (upstream) of a nucleotide sequence to be operably connected to the promoter. In the construction of heterologous transcriptional control sequence/nucleotide sequence of interest combinations, it is generally preferred to position the promoter at a distance from the transcription start site that is approximately the same as the distance

between that promoter and the gene it controls in its natural setting, ie. the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function.

Accordingly, in a first aspect, the present invention provides an isolated nucleic acid comprising:

(i) a nucleotide sequence defining a transcriptional control sequence, wherein said transcriptional control sequence is derived from a gene which encodes an EC1 polypeptide; or

(ii) a nucleotide sequence defining a functionally active fragment or variant of (i).

In the present invention, “isolated” refers to material removed from its original environment (eg., the natural environment if it is naturally occurring), and thus is altered “by the hand of man” from its natural state. For example, an isolated polynucleotide could be part of a vector or a composition of matter, or could be contained within a cell, and still be isolated because that vector, composition of matter, or particular cell is not the original environment of the polynucleotide. An “isolated” nucleic acid molecule should also be understood to include a synthetic nucleic acid molecule, including those produced by chemical synthesis using known methods in the art or by *in-vitro* amplification (eg. polymerase chain reaction and the like).

The isolated nucleic acid of the present invention may comprise any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. For example, the isolated nucleic acids of the invention may comprise single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions.

In addition, the isolated nucleic acids may comprise triple-stranded regions comprising RNA or DNA or both RNA and DNA. The isolated nucleic acid molecules may also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically, or metabolically modified forms.

As set out above, the present invention contemplates, among other things, a nucleotide sequence defining a transcriptional control sequence, wherein said transcriptional control sequence is derived from a gene which encodes an EC1 polypeptide. As referred to herein, an “EC1 polypeptide” refers to a polypeptide which is substantially specifically expressed in an egg cell of a plant. Generally, EC1 polypeptides are small (generally less than about 200 amino acid residues) and are putatively secreted. In some embodiments, the EC1 polypeptides contemplated herein further comprise about six conserved cysteine residues and may further comprise a signal peptide for extracellular localization.

The nucleic acids of the present invention comprise nucleotide sequence defining which is “derived from a gene which encodes an EC1 polypeptide”. The term “derived from”, as it is used herein, refers to a source or origin for the transcriptional control sequence. As such, a transcriptional control sequence “derived from a gene which encodes an EC1 polypeptide” refers to a transcriptional control sequence which, in its native state, exerts at least some transcriptional control over a gene which encodes an EC1 polypeptide in an organism, preferably a plant.

In accordance with the present invention, a consensus EC1 polypeptide sequence has been determined. Accordingly, in one particularly preferred embodiment, the isolated nucleic acid of the first aspect of the invention comprises a transcriptional control sequence derived from a gene which encodes a polypeptide comprising the amino acid sequence:

[X_N]CWXXXXX[LI]X[SH]C[TS]X[DE] [IL] [ILV]XFF[LIV] [X_N] [LI]
XXXCCX[AS] [ILV]XXXXXXCW[X_N] [ILV]G[FL]TXXEXXXLXXXC[X_N]
(SEQ ID NO: 1)

wherein X is any amino acid residue; [X_N] is one or more amino acid residues of any type; [LI] or [IL] is a leucine or isoleucine residue; [SH] is a serine or histidine residue; [TS] is a threonine or serine residue; [DE] is an aspartic acid or glutamic acid residue; [ILV] or [LIV] is a leucine, isoleucine or valine residue; [AS] is an alanine or serine residue; and [FL] is a phenylalanine or leucine residue.

Accordingly, in one embodiment, the present invention provides an isolated nucleic acid comprising:

(i) a nucleotide sequence defining a transcriptional control sequence, wherein said transcriptional control sequence is derived from a gene which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 1; or

(ii) a functionally active fragment or variant of (i).

In further embodiments, the transcriptional control sequence is derived from a gene which encodes a polypeptide comprising the amino acid sequence set forth in any of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 and SEQ ID NO: 11.

In yet further embodiments, the transcriptional control sequence is derived from a gene which comprises an open reading frame comprising the nucleotide sequence set forth in any of SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22 and SEQ ID NO: 23.

In specific embodiments, the present invention provides an isolated nucleic comprising a nucleotide sequence defining a transcriptional control sequence, wherein the transcriptional control sequence comprises the nucleotide sequence set forth in any one of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, or a functionally active fragment or variant thereof.

In yet further embodiments the isolated nucleic acid comprises one or more sequence motifs comprising the nucleotide sequence set forth in SEQ ID NO: 33 and/or SEQ ID NO: 69.

As set out above, the present invention also provides a nucleic acid comprising a nucleotide sequence defining a functionally active fragment or variant of the subject transcriptional control sequences.

As referred to herein, a “functionally active fragment or variant” refers to a fragment or variant which retains the functional activity of a transcriptional control sequence.

“Functionally active fragments”, as contemplated herein, may be of any length wherein the transcriptional control sequence retains the capability to affect expression of an operably connected nucleotide sequence. The fragment may comprise, for example, at least 50 nucleotides (nt), at least 100 nt or at least 200 nt. For example, in specific embodiments, a fragment at least 50 nt in length comprises fragments which include 50 or more contiguous bases from, for example, the nucleotide sequence of any of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32.

“Functionally active variants” of the transcriptional control sequence of the invention include orthologs, mutants, synthetic variants, analogs and the like

which retain the capability to affect expression of an operably connected nucleotide sequence. For example, the term “variant” should be considered to specifically include, for example, orthologous transcriptional control sequences from other organisms; mutants of the transcriptional control sequence; variants of the transcriptional control sequence wherein one or more of the nucleotides within the sequence has been substituted, added or deleted; and analogs that contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. “Modified” bases include, for example, tritylated bases and unusual bases such as inosine.

In some embodiments, the functionally active fragment or variant comprises may comprise, for example, at least 50% sequence identity, at least 65% sequence identity, at least 80% sequence identity or at least 95% sequence identity to the nucleotide sequence set forth in any of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32.

When comparing nucleotide sequences to calculate a percentage identity, the compared nucleotide sequences should be compared over a comparison window of at least 50 nucleotide residues, at least 100 nucleotide residues, at least 200 nucleotide residues, at least 500 nucleotide residues or over the full length of any of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32. The comparison window may comprise additions or deletions (ie. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms such the BLAST family of programs as, for example, disclosed by Altschul *et al.* (*Nucl. Acids Res.* 25: 3389-3402, 1997). A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* ("Current Protocols in Molecular Biology" John Wiley & Sons Inc, 1994-1998, Chapter 15,1998).

In some specific embodiments, the transcriptional control sequences provided by the present invention, or functionally active fragments or variants thereof, comprise plant egg cell specific or plant egg cell preferential transcriptional control sequences.

As used herein, a “plant egg cell specific” transcriptional control sequence refers to a transcriptional control sequence which directs the expression of an operably connected nucleotide sequence of interest substantially only in an egg cell of a plant. A “plant egg cell preferential” transcriptional control sequence refers to a transcriptional control sequence which directs the expression of an operably connected nucleotide sequence at a higher level in a plant egg cell than in one or more other tissues of the plant, eg. leaf tissue or root tissue. Generally, preferential expression in an egg cell includes expression of a nucleotide sequence of interest in a plant egg cell at a level of at least twice, at least 5 times or at least 10 times the level of expression seen in at least one other tissue of a plant, eg. leaf or root tissue.

As referred to herein, the term “plant egg cell” should be understood to include a cell which is a component of the female gametophyte in a plant. For example, in angiosperm plants, the term “plant egg cell” may include a female gamete, a synergid, a central cell or an antipodal cell of the female gametophyte (embryo sac). In one specific embodiment, the term “plant egg cell” should be understood to refer to a female gamete of a plant.

In a second aspect, the present invention provides an isolated nucleic acid selected from the list consisting of:

(i) a nucleic acid comprising the nucleotide sequence set forth in any of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32;

(ii) a nucleic acid comprising a nucleotide sequence which is at least 50% identical to any of the nucleotide sequences mentioned in (i);

(iii) a nucleic acid which hybridizes to any of the nucleic acids mentioned in (i) under stringent conditions;

(iv) a nucleic acid comprising a nucleotide sequence which is the complement or reverse complement of any one of (i) to (iii); and

(v) a fragment of any of (i), (ii), (iii) or (iv).

In one embodiment, the isolated nucleic acid defined at (ii) comprises at least 50% sequence identity, at least 65% sequence identity, at least 80% sequence identity or at least 95% sequence identity to the nucleotide sequence set forth in any of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32.

In another embodiment, the isolated nucleic acid of the second aspect of the invention comprises a nucleotide sequence which defines a transcriptional control sequence or a complement, reverse complement or fragment thereof. In yet another embodiment, the isolated nucleic acid of the second aspect of the invention comprises a nucleotide sequence which defines an egg cell specific or egg cell preferential transcriptional control sequence or a complement, reverse complement or fragment thereof.

As set out at (iii) above, the second aspect of the invention provides isolated nucleic acids which hybridize to any of the nucleic acids mentioned at (i). As used herein, “stringent” hybridisation conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least 30℃. Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Stringent hybridisation conditions may be low stringency conditions, medium stringency conditions or high stringency conditions. Exemplary low stringency conditions include hybridisation with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl

sulphate) at 37°C., and a wash in 1x to 2xSSC (20xSSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridisation in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C., and a wash in 0.5x to 1xSSC at 55 to 60°C. Exemplary high stringency conditions include hybridisation in 50% formamide, 1 M NaCl, 1% SDS at 37°C., and a wash in 0.1xSSC at 60 to 65°C. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity of hybridisation is also affected by post-hybridization washes, with influencing parameters including the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (*Anal. Biochem.* 138: 267-284, 1984), ie. T_m =81.5°C +16.6 (log M)+0.41 (% GC)-0.61 (% form)-500/L; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization, and/or wash conditions can be adjusted to hybridize to sequences of different degrees of complementarity. For example, sequences with ≥90% identity can be hybridised by decreasing the T_m by about 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, high stringency conditions can utilize a hybridization and/or wash at, for example, 1, 2, 3, or 4°C lower than the thermal melting point (T_m); medium stringency conditions can utilize a hybridization and/or wash at, for example, 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at, for example, 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of

ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (*Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes*, Pt I, Chapter 2, Elsevier, New York, 1993), Ausubel *et al.*, eds. (*Current Protocols in Molecular Biology*, Chapter 2, Greene Publishing and Wiley-Interscience, New York, 1995) and Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Plainview, NY, 1989).

As set out above, the second aspect of the present invention also contemplates nucleic acid fragments.

"Fragments" of a nucleotide sequence may be, for example, at least 5 nucleotides (nt), at least 10 nt, at least 20 nt, at least 50, at least 100, at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450 or at least 500 nt in length. These fragments have numerous uses that would be evident to one of skill in the art and include, for example, diagnostic probes and primers. Of course, larger fragments, may also be useful, as are fragments corresponding to most, if not all, of the nucleotide sequences set forth in any of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32. For example, a fragment at least 5 nt in length may refer to a fragment which includes 5 or more contiguous bases from, for example, the nucleotide sequence of any of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32.

The isolated nucleic acids (or fragments or variants thereof) of the present invention may be derived from any source. For example, the nucleic acids may

be derived from an organism, such as a plant. Suitable plants include, for example, monocotyledonous angiosperms (monocots), dicotyledonous angiosperms (dicots), gymnosperms and the like.

Exemplary dicots include, for example, *Arabidopsis* spp., *Medicago* spp., *Nicotiana* spp., soybean, canola, oil seed rape, sugar beet, mustard, sunflower, potato, safflower, cassava, yams, sweet potato, other Brassicaceae such as *Thellungiella halophila*, among others.

In one embodiment, the isolated nucleic acids of the present invention may comprise a transcriptional control sequence derived from a gene which encodes an EC1 polypeptide in an *Arabidopsis* sp. plant. For example the transcriptional control sequences defined herein as *pAtEC1.1* (SEQ ID NO: 24), *pAtEC1.2a* (SEQ ID NO: 25), *pAtEC1.2b* (SEQ ID NO: 26), *pAtEC1.4* (SEQ ID NO: 27) and *pAtEC1.5* (SEQ ID NO: 28) are derivable from genes comprising the *AtEC1.1* (SEQ ID NO: 13), *AtEC1.2a* (SEQ ID NO: 14), *AtEC1.2b* (SEQ ID NO: 15), *AtEC1.4* (SEQ ID NO: 16) and *AtEC1.5* (SEQ ID NO: 17) open reading frame nucleotide sequences, respectively, from *Arabidopsis thaliana*.

In another embodiment, the isolated nucleic acid of the present invention may comprise a transcriptional control sequence derived from a gene which encodes an EC1 polypeptide in a *Medicago* sp. plant. For example the transcriptional control sequence defined herein as *pMtEC1* (SEQ ID NO: 29) is derivable from a gene comprising the *MtEC1.1* (SEQ ID NO: 18) open reading frame nucleotide sequence from *Medicago truncatula*.

In further preferred embodiments, the plant is a monocot, more preferably a cereal crop plant.

As used herein, the term “cereal crop plant” includes members of the order Poales, and more preferably the family Poaceae, which produce edible grain for human or animal food. Examples of cereal crop plants that in no way limit the

present invention include barley, wheat, rice, maize, millets, sorghum, rye, triticale, oats, teff, rice, spelt and the like. However, the term cereal crop plant should also be understood to include a number of non-Poales species that also produce edible grain, which are known as pseudocereals, and include, for example, amaranth, buckwheat and quinoa.

In one embodiment, the isolated nucleic acid of the present invention may comprise a transcriptional control sequence derived from a gene which encodes an EC1 polypeptide in an *Oryza* sp. plant. For example the transcriptional control sequences defined herein as *pOsEC1.1* (SEQ ID NO: 30), *pOsEC1.2* (SEQ ID NO: 31) and *pOsEC1.3* (SEQ ID NO: 32), are derivable from genes comprising the *OsEC1.1* (SEQ ID NO: 19), *OsEC1.2* (SEQ ID NO: 20) and *OsEC1.2b* (SEQ ID NO: 21) open reading frame nucleotide sequences, respectively, from *Oryza sativa*.

In further embodiments, the present invention also contemplates synthetic nucleic acids.

In a third aspect, the present invention provides a nucleic acid construct comprising the isolated nucleic acid of the first and/or second aspects of the invention.

The nucleic acid construct of the present invention may comprise any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. For example, the nucleic acid construct of the invention may comprise single- and/or double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, the nucleic acid construct may comprise triple-stranded regions comprising RNA or DNA or both RNA and DNA. The nucleic acid construct may

also comprise one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus the term "nucleic acid construct" embraces chemically, enzymatically, or metabolically modified forms.

In one embodiment, the nucleic acid construct comprises DNA. Accordingly, the nucleic acid construct of the present invention may comprise, for example, a linear DNA molecule, a plasmid, a transposon, a cosmid, an artificial chromosome and the like. Furthermore, the nucleic acid construct of the present invention may be a separate nucleic acid molecule or may be a part of a larger nucleic acid molecule.

In another embodiment, the isolated nucleic acid comprises a nucleotide sequence defining a transcriptional control sequence and further comprises a nucleotide sequence of interest operably connected to the transcriptional control sequence.

In some embodiments, the nucleotide sequence of interest is heterologous with respect to the transcriptional control sequence.

As used herein, the term “heterologous with respect to the transcriptional control sequence” refers to the nucleotide sequence of interest being a nucleotide sequence other than that which the transcriptional control sequence is operably connected to in its natural state.

For example, in its natural state, *pAtEC1.1* (SEQ ID NO: 24) is operably connected to a gene comprising the *AtEC1.1* open reading frame (SEQ ID NO: 13). Accordingly, in this example, any nucleotide sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 13 should be considered heterologous with respect to SEQ ID NO: 24. Similarly, any nucleotide

sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 14 should be considered heterologous with respect to SEQ ID NO: 25; any nucleotide sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 15 should be considered heterologous with respect to SEQ ID NO: 26; any nucleotide sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 16 should be considered heterologous with respect to SEQ ID NO: 27; any nucleotide sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 17 should be considered heterologous with respect to SEQ ID NO: 28; any nucleotide sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 18 should be considered heterologous with respect to SEQ ID NO: 29; any nucleotide sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 19 should be considered heterologous with respect to SEQ ID NO: 30; any nucleotide sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 20 should be considered heterologous with respect to SEQ ID NO: 31; and any nucleotide sequence other than a nucleotide seqence comprising an open reading frame consisting of the nucleotide sequence set forth in SEQ ID NO: 21 should be considered heterologous with respect to SEQ ID NO: 32.

Accordingly, a sequence that is heterologous with resepect to a particular transcriptional control sequence may therefore include, for example, orthologous EC1 sequences, reporter genes, selectable marker genes, heterologous protein-encoding nucleic acid sequences; heterologous non-translated RNA encoding nucleic acid sequences and the like.

In a further embodiment, the nucleic acid construct may further comprise a

nucleotide sequence defining a transcription terminator.

The term “transcription terminator” or “terminator” refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences generally containing a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3'-end of a primary transcript. As with promoter sequences, the terminator may be any terminator sequence which is operable in the cells, tissues or organs in which it is intended to be used. Examples of suitable terminator sequences which may be useful in plant cells include: the nopaline synthase (*nos*) terminator, the CaMV 35S terminator, the octopine synthase (*ocs*) terminator, potato proteinase inhibitor gene (*pin*) terminators, such as the *pinII* and *pinIII* terminators and the like.

In one specific embodiment, the nucleic acid construct of the third aspect of the invention comprises an expression cassette comprising the structure:

$$([N]_w – TCS – [N]_x – SoI – [N]_y – TT – [N]_z)$$

wherein:

$[N]_w$ comprises one or more nucleotide residues, or is absent;

TCS comprises the nucleic acid of the first and/or second aspects of the invention, wherein the nucleic acid defines a transcriptional control sequence;

$[N]_x$ comprises one or more nucleotide residues, or is absent;

SoI comprises a nucleotide sequence of interest which encodes an mRNA or non-translated RNA, wherein the nucleotide sequence, SoI, is operably connected to TCS;

$[N]_y$ comprises one or more nucleotide residues, or is absent;

TT comprises a nucleotide sequence defining a transcription terminator;

$[N]_z$ comprises one or more nucleotide residues, or is absent.

The nucleic acid constructs of the present invention may further comprise

nucleotide sequences such as, an origin of replication for one or more hosts; a selectable marker gene which is active in one or more hosts and the like.

As used herein, the term “selectable marker gene” includes any gene that confers a phenotype on a cell, in which it is expressed, to facilitate the identification and/or selection of cells which are transfected or transformed with a genetic construct of the invention.

“Selectable marker genes” include any nucleotide sequences which, when expressed by a cell, confer a phenotype on the cell that facilitates the identification and/or selection of transformed cells. A range of nucleotide sequences encoding suitable selectable markers are known in the art. Exemplary nucleotide sequences that encode selectable markers include: antibiotic resistance genes such as ampicillin-resistance genes, tetracycline-resistance genes, kanamycin-resistance genes, the AURI-C gene which confers resistance to the antibiotic aureobasidin A, neomycin phosphotransferase genes (eg. *nptI* and *nptII*) and hygromycin phosphotransferase genes (eg. *hpt*); herbicide resistance genes including glufosinate, phosphinothricin or bialaphos resistance genes such as phosphinothricin acetyl transferase encoding genes (eg. *bar*), glyphosate resistance genes including 3-enoyl pyruvyl shikimate 5-phosphate synthase encoding genes (eg. *aroA*), bromyxnil resistance genes including bromyxnil nitrilase encoding genes, sulfonamide resistance genes including dihydropterate synthase encoding genes (eg. *sul*) and sulfonylurea resistance genes including acetolactate synthase encoding genes; enzyme-encoding reporter genes such as GUS and chloramphenicol acetyltransferase (CAT) encoding genes; fluorescent reporter genes such as the green fluorescent protein-encoding gene; and luminescence-based reporter genes such as the luciferase gene, amongst others.

The genetic constructs of the present invention may include further nucleotide sequences intended for the maintenance and/or replication of the genetic

construct in prokaryotes or eukaryotes and/or the integration of the genetic construct or a part thereof into the genome of a eukaryotic or prokaryotic cell.

In one embodiment, the construct of the invention is adapted to be at least partially transferred into a plant cell *via Agrobacterium*-mediated transformation. Accordingly, in a further embodiment, the nucleic acid construct of the present invention comprises left and/or right T-DNA border sequences.

Suitable T-DNA border sequences would be readily ascertained by one of skill in the art. However, the term "T-DNA border sequences" should be understood to encompass any substantially homologous and substantially directly repeated nucleotide sequences that delimit a nucleic acid molecule that is transferred from an *Agrobacterium* sp. cell into a plant cell susceptible to *Agrobacterium*-mediated transformation. By way of example, reference is made to the paper of Peralta and Ream (*Proc. Natl. Acad. Sci. USA*, 82(15): 5112-5116, 1985) and the review of Gelvin (*Microbiology and Molecular Biology Reviews*, 67(1): 16-37, 2003).

The present invention also contemplates any suitable modifications to the genetic construct which facilitate bacterial mediated insertion into a plant cell *via* bacteria other than *Agrobacterium* sp., for example, as described in Broothaerts *et al.* (*Nature* 433: 629-633, 2005).

Those skilled in the art will be aware of how to produce the constructs described herein and of the requirements for obtaining the expression thereof, when so desired, in a specific cell or cell-type under the conditions desired. In particular, it will be known to those skilled in the art that the genetic manipulations required to perform the present invention may require the propagation of a genetic construct described herein or a derivative thereof in a prokaryotic cell such as an *E. coli* cell or a plant cell or an animal cell. Exemplary methods for cloning nucleic acid molecules are described in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual,* Cold Spring Harbor Laboratory Press, New York, 2000).

In a fourth aspect, the present invention provides a cell comprising:

(i) the nucleic acid construct of the third aspect of the invention; and/or

(ii) a genomically integrated form of the construct mentioned at (i).

The nucleic acid construct may be maintained in the cell as a nucleic acid molecule, as an autonomously replicating genetic element (eg. a plasmid, cosmid, artificial chromosome or the like) or it may be integrated into the genomic DNA of the cell.

As used herein, the term “genomic DNA” should be understood in its broadest context to include any and all DNA that makes up the genetic complement of a cell. As such, the genomic DNA of a cell should be understood to include chromosomes, mitochondrial DNA, plastid DNA, chloroplast DNA, endogenous plasmid DNA and the like. As such, the term “genomically integrated” contemplates chromosomal integration, mitochondrial DNA integration, plastid DNA integration, chloroplast DNA integration, endogenous plasmid integration, and the like.

The cells contemplated by the fourth aspect of the invention include any prokaryotic or eukaryotic cell.

In one embodiment, the cell is a plant cell. As used herein, the term “plant” includes any plant which comprises an egg cell, including, for example, dicotyledonous or monocotyledonous angiosperms and gymnosperms.

In another embodiment, the plant cell is a dicotyledonous plant cell, for example, an *Arabidopsis* sp. cell. In yet another embodiment the cell is a monocotyledonous plant cell and/or a cereal crop plant cell.

In one specific embodiment, the cell is a plant egg cell. In a further embodiment, the level, rate and/or pattern of expression of at least one nucleotide sequence is altered in the plant egg cell relative to a wild type form of said plant egg cell.

In yet another embodiment, the cell may also comprise a prokaryotic cell. For example the prokaryotic cell may include an *Agrobacterium* sp. cell which carries the nucleic acid construct and which may, for example, be used to transform a plant. In yet another embodiment, the prokaryotic cell may include an *E. coli* cell, which may, for example, be used in the construction or cloning of a nucleic acid construct.

In a fifth aspect, the present invention provides a multicellular structure comprising one or more cells of the fourth aspect of the invention.

In one embodiment, the multicellular structure comprises a plant or a part, organ or tissue thereof.

As referred to herein, “a plant or a part, organ or tissue thereof” should be understood to specifically include a whole plant; a plant tissue; a plant organ; a plant part; plant reproductive material (including, for example, cuttings, seed, flowers, pollen and the like); and cultured plant tissue such as a callus or suspension culture.

The multicellular structure may comprise one or more plant egg cells. For example, the multicellular structure may comprise a plant, a flower, a carpel or pistil, a plant ovary, an ovule or a female gametophyte (embryo sac). In one embodiment, the level, rate and/or pattern of expression of at least one nucleotide sequence is altered in said one or more plant egg cells of the multicellular structure relative to a wild type form of said egg cell.

As set out above, the present invention is predicated, in part, on effecting transcription of the nucleotide sequence of interest under the transcriptional

control of the nucleic acid of the first, second or third aspects of the invention, wherein said nucleic acid comprises a transcriptional control sequence.

Accordingly, in a sixth aspect, the present invention provides a method for specifically or preferentially expressing a nucleotide sequence of interest in a plant egg cell, the method comprising effecting transcription of the nucleotide sequence of interest in a plant under the transcriptional control of the nucleic acid of any of the first, second or third aspects of the invention, wherein said nucleic acid comprises a plant egg cell specific or plant egg cell preferential transcriptional control sequence.

Generally, specific or preferential expression of a nucleotide sequence of interest is effected by introducing said nucleic acid into a plant cell such that the nucleotide sequence of interest is operably connected to a transcriptional control sequence of the present invention.

The present invention contemplates any method to effect operable connection of a nucleotide sequence of interest to the transcriptional control sequence of the invention. For example, a nucleotide sequence of interest may be incorporated into the nucleic acid molecule that comprises the transcriptional control sequence, and be operably connected thereto. In this way, the nucleotide sequence of interest and transcriptional control sequence are both introduced into the plant. Alternatively, the nucleic acid sequence of the present invention may be inserted into the plant genome such that it is placed in operable connection with an endogenous EC1 transcriptional control sequence. As would be recognised by one of skill in the art, the insertion of the transcriptional control sequence into the plant genome may be either by non-site specific insertion or by site-specific insertion (for an example of site-specific insertion see Terada *et al*., *Nat Biotechnol* 20: 1030-1034, 2002).

The nucleic acid may be introduced into a plant *via* any suitable method. For example, an explant or cultured plant tissue may be transformed with a nucleic

acid molecule, and the transformed explant or cultured plant tissue subsequently regenerated into a mature plant which produces seed including the nucleic acid molecule; a nucleic acid may be directly transformed into a plant, either stably or transiently; a nucleic acid may be introduced into a plant *via* breeding using a parent plant that carries the nucleic acid molecule; and the like.

In one embodiment, the nucleic acid molecule is introduced into a plant cell *via* transformation. Plant cells may be transformed using any method known in the art that is appropriate for the particular plant species. Common methods include *Agrobacterium*-mediated transformation, microprojectile bombardment based transformation methods and direct DNA uptake based methods. Roa-Rodriguez *et al*. (Agrobacterium-*mediated transformation of plants, 3rd Ed.* CAMBIA Intellectual Property Resource, Canberra, Australia, 2003) review a wide array of suitable *Agrobacterium*-mediated plant transformation methods for a wide range of plant species. Microprojectile bombardment may also be used to transform plant tissue and methods for the transformation of plants, particularly cereal plants, and such methods are reviewed by Casas *et al*. (*Plant Breeding Rev.* 13: 235-264, 1995). Direct DNA uptake transformation protocols such as protoplast transformation and electroporation are described in detail in Galbraith *et al*. (eds.), *Methods in Cell Biology Vol. 50*, Academic Press, San Diego, 1995). In addition to the methods mentioned above, a range of other transformation protocols may also be used. These include infiltration, electroporation of cells and tissues, electroporation of embryos, microinjection, pollen-tube pathway, silicon carbide- and liposome mediated transformation. Methods such as these are reviewed by Rakoczy-Trojanowska (*Cell. Mol. Biol. Lett.* 7: 849-858, 2002). A range of other plant transformation methods may also be evident to those of skill in the art and, accordingly, the present invention should not be considered in any way limited to the particular plant transformation methods exemplified above.

The nucleotide sequence of interest, which is placed under the regulatory

control of the transcriptional control sequence of the present invention, may include any nucleotide sequence of interest. General categories of nucleotide sequences of interest may include, for example:

(i) cytotoxin genes such as barnase, RNase or diphtheria toxin which may be used to induce female sterility and/or embryoless fruits in a plant;

(ii) genes encoding transcriptional regulators acting during later stages of embryo development such as BBM or LEC1/LEC2, AP2 transcription factors which may be used to modify embryo development and/or increase embryo size;

(iii) genes encoding cell cycle regulators such as RB or E2F, transcriptional regulators acting during later stages of embryo development such as BBM, LEC1/LEC2 or chromatin remodelling factors such as DNA methyltransferases, histone modifying enzymes and the like, which may be used to effect apomictic embryo development (eg. parthenogenesis; autonomous embryogenesis);

(iv) reporter genes, such as those encoding GUS, GFP and the like;

(v) genes involved in cellular metabolism such as Zinc finger proteins, kinases, heat shock proteins and the like;

(vi) genes involved in agronomic traits such as disease or pest resistance or herbicide resistance;

(vii) genes involved in grain characteristics such as grain biomass, nutritional value, post-harvest characteristics and the like;

(viii) genes encoding heterologous proteins, such as proteins encoding heterologous enzymes or structural proteins or proteins involved in biosynthetic pathways for heterologous products;

(ix) nucleotide sequences encoding non-translated RNA, for example an siRNA, miRNA, antisense RNA and the like.

Generally, the nucleotide sequence of interest is heterologous with respect to

the transcriptional control sequence.

In a seventh aspect, the present invention provides a method for promoting female sterility in a plant, the method comprising expressing a nucleotide sequence encoding a cytotoxic or cytostatic protein or a cytotoxic of cytostatic non-translated RNA, specifically or preferentially in an egg cell of the plant; wherein said nucleotide sequence is operably connected to a nucleic acid of any of the first, second or third aspects of the invention and wherein said nucleic acid comprises a plant egg cell specific or plant egg cell preferential transcriptional control sequence.

As referred to herein, the “cytotoxic or cytostatic protein” or “cytotoxic or cytostatic non-translated RNA” refers to any protein or non-translated RNA that inhibits or prevents the growth, division, metabolic function or fertilisation of the egg cell. In some embodiments, the nucleotide sequence encodes a cytotoxic protein selected from the list consisting of a barnase, an RNAse or a diphtheria toxin.

In an eighth aspect, the present invention provides a method for modulating embryo development and/or embryo size in a plant, the method comprising expressing a nucleotide sequence encoding a transcriptional regulator that acts during embryo development, specifically or preferentially in an egg cell of the plant; wherein said nucleotide sequence encoding a transcriptional regulator is operably connected to a nucleic acid of any of the first, second or third aspects of the invention and wherein said nucleic acid comprises a plant egg cell specific or plant egg cell preferential transcriptional control sequence.

In one embodiment, the nucleotide sequence encoding a transcriptional regulator comprises a transcriptional regulator which acts during later stages of embryo development, more preferably the transcriptional regulator comprises a BBM, LEC1/LEC2, AP2 or other transcription factor.

In a ninth aspect, the present invention provides a method for promoting apomixis in a plant, the method comprising expressing an apomixis-promoting nucleotide sequence specifically or preferentially in an egg cell of the plant; wherein said apomixis-promoting nucleotide sequence is operably connected to a nucleic acid of the first second or third aspects of the invention and wherein said nucleic acid comprises a plant egg cell specific or plant egg cell preferential transcriptional control sequence.

As used herein, the term “apomixis-promoting nucleotide sequence” includes any nucleotide sequence which promotes apomixis when expressed in a plant, and, more particularly, when expressed in a plant egg cell. The apoxmis-promoting nucleotide sequence may include, for example, a nucleotide sequence which encodes any of:

(i) a cell cycle regulator, such as RB or E2F;

(ii) a transcriptional regulator that acts during later stages of embryo development, such as BBM, LEC1/LEC2;

(iii) a chromatin remodeling factor such as a DNA methyltransferase; or

(iv) a histone modifying enzyme.

The methods of the sixth, seventh, eighth and ninth aspects of the present invention may be performed using any suitable plant. However, in one preferred embodiment, the plant is a dicotyledonous plant and, in another embodiment, an *Arabidopsis* sp. plant. In further embodiments, the plant may be a monocotyledonous plant and/or a cereal crop plant.

Finally, reference is made to standard textbooks of molecular biology that contain methods for carrying out basic techniques encompassed by the present invention, including DNA restriction and ligation for the generation of the various genetic constructs described herein. See, for example, Maniatis *et al*., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press,

New York, 1982) and Sambrook *et al.* (2000, *supra*).

The present invention is further described by the following non-limiting examples:

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows an expression profile of C06_3 transcripts (*TaEC1*) originally derived from the wheat egg cell cDNA library. Expression of *TaEC1* was examined by RT-PCR using DNAse treated total RNA from different tissues of wheat and gene specific primers for C06_3. As controls, cDNA from egg cells, central cells and 2-celled pro-embryos have been used. Quality and quantity of generated cDNA was verified using primers for the ubiquitously expressed GAP-DH. Lane 1: egg cell, 2: pro-embryo, 3: central cell, 4: coleoptile, 5: primary leaf, 6: mature leaf, 7: stem, 8: root without tip, 9: root tip, 10: anther, 11: pistil, 12: kernel 12 dap (days after pollination), 13: negative control.

Figure 2 shows an expression profile of *AtEC1-like* genes in *Arabidopsis*. Expression was examined by RT-PCR using DNAse treated mRNA from different tissues of *Arabidopsis* and gene specific primers for *AtEC1.1*, *AtEC1.2a/b*, *AtEC1.4* and *AtEC1.5*. All five genes of *Arabidopsis* are exclusively expressed in tissues which contain the female gametophyte. Quality and quantity of generated cDNA was verified using primer for the ubiquitously expressed *Actin 3* gene. Lane 1: leaf, 2: stem, 3: root, 4: flower bud, 5: mature flower, 6: flower 1-3 dap, 7: pistil, 8: anther, 9: negative control.

Figure 3 shows transcript localisation of *AtEC1-like* genes in *Arabidopsis* ovules. *In situ* hybridization was performed using embedded sections of mature pistils hybridized with an *AtEC1.2a* antisense probe (A) and *AtEC1.1* antisense probe (B). As control, sense probes of *AtEC1.1* (C) and *AtEC1.2a* were used. Arrows point towards egg cell, showing egg cell specific signals in (A) and (B).

Transcripts of *AtEC1-like* genes could not be detected in other cells of the unfertilized embryo sac or in young embryos, early after fertilization (not shown).

Figure 4 shows promoter activity of *AtEC1-like* genes in *Arabidopsis*. Promoters *pAtEc1.1* and *pAtEc1.2* were cloned as translational fusions 5´ upstream of the ß-Glucuronidase gene. Cloned constructs were used for stable transformation of *Arabidopsis*. Arrows point at egg cells showing GUS staining in *pAtEc1.1*::*GUS* (A) and *pAtEc1.2a*::*GUS* ovules before fertilisation (B) and a zygote after fertilisation (C). GUS activity was only observed in egg cells of unfertilised ovules. After fertilisation, GUS activity was still visible in the zygote (C); however, this was due to the high stability of ß-Glucuronidase. A schematic of an unfertilized *Arabidopsis* ovule is shown in (D) (Drews and Yadegari, *Annu. Rev. Genet*. 36: 99-124, 2002). Abbreviations: (ac) antipodal cells; (cc) central cell; (ch) chalaza; (ec) egg cell; (emb) embryo; (f) funiculus; (mp) micropyle; (sc) synergid; (sn) secondary nucleus; (zyg) zygote.

Figure 5 shows the expression of EGFP (enhanced green fluorescent protein) as a C-terminal fusion to the open reading frame of *AtEC1.1*, under control of the promoter of *pAtEC1.1*. The construct was used for transformation of *Arabidopsis thaliana*. Ovules of transgenic plants were analyzed microscopically for green fluorescence using DIC (A, C) and UV-light (B, D) as well as UV-light for CLSM (confocal laser scanning microscopy) (E-G). Green fluorescence was visible at the chalazal pole of egg cells in unfertilized ovules (arrow in B) before fertilization. After fertilization, secreted fluorescent protein is visible between the zygote (zyg; arrow) and the neighbouring endosperm (C and D). CLSM shows the protein within vesicles of the unfertilized egg cell. (F) is an enlargement of (E) and (G) shows EGFP fluorescence of the egg cell. Red fluorescence was removed.

Figure 6 shows an alignment of *EC1* cDNA and predicted genomic sequences encoding open reading frames (ORFs) derived from wheat *(TaEC1*), barley (*HvECA1*), rice (*OsEC1*) and Arabidopsis (*AtEC1*).

Figure 7 shows an alignment of *EC1* ORFs derived from cereal cDNA and predicted genomic sequences.

Figure 8 shows an alignment of *EC1* predicted ORFs derived from *Arabidopsis thaliana* genomic sequences.

Figure 9 shows a protein-alignment of EC1 proteins from wheat (TaEC1), barley (HvECA1), rice (OsEC1), Arabidopsis (AtEC1) and *Medicago truncatula* (MtEC1.1).

Figure 10 is a graphical representation showing novel over-represented motifs in the upstream region of egg cell specific expressed genes identified by MotifSampler. The motif finding algorithm uses Gibbs sampling to find the position probability matrix that represents the motif.

EXAMPLE 1

Identification of genes specifically expressed in plant egg cells

To order to identify genes that are specifically expressed in the egg cell, female gametophytes of wheat were initially microdissected to isolate egg cells. Using the messenger RNA from 12 egg cells, a cDNA library was constructed and single-run partial sequencing of 960 randomly selected cDNA clones was performed. After DNA sequencer trace data passed an automated cleanup pipeline, a total of 735 ESTs were used for bioinformatical analysis. The 735 ESTs formed 404 independent clusters including 310 singletons.

The consensus sequences of the clusters were used for BLASTN and BLASTX searches at the NCBI nonredundant database (nr), dbEST and SwissProt database. Some cDNA sequences resulted in limited sequence information from non-coding regions. Therefore, searches were performed against the TIGR Wheat Gene Index Release 8.0, using the BLASTN algorithm. If a match with >95% sequence identity over the total length of the query sequence was found, the matching sequence was retrieved and used in subsequent BLASTX searches in place of the original EST. BLASTN searches against the NCBI database category of non-mouse and non-human ESTs resulted in 629 egg cell ESTs (333 clusters) matching significantly to annotated ESTs mainly generated from different vegetative tissues of wheat, barley or rice (NCBI dbEST *Poaceae*). 106 egg cell ESTs (71 clusters) did not match annotated ESTs and were thus considered as "novel" transcripts.

Transcripts were selected which did not match to any EST generated from vegetative plant tissues, and which matched to so-called "hypothetical" proteins (computer-predicted open reading frames from the *Arabidopsis* and/or rice genome sequences). It was assumed that the corresponding genes of some of these transcripts might be specifically expressed in egg cells of seed plants.

Significant similarities to "hypothetical" proteins of *Arabidopsis* and/or rice were identified for 98 egg cell clusters. Of these, 11 clusters were not similar to any published EST but only to annotated "hypothetical" genes detected in genomes of *Arabidopsis* and/or rice and it was concluded that these might be candidate genes that are specifically or preferentially expressed in the female gametes or gametophyte of *Arabidopsis* and/or rice.

Using this strategy a very large cluster of transcripts from the wheat egg cell (*TaEC1*) was identified, which did not match to ESTs from any vegetative tissues, but which displayed significant similarity to hypothetical proteins from *Arabidopsis, Medicago truncatula* and rice. In total, there are five *TaEC1*-like hypothetical genes in *Arabidopsis*, which are located on chromosomes 1, 2, 4 and 5. These were designated *AtEC1.1* (SEQ ID NO: 13), *AtEC1.2a* (SEQ ID

NO: 14), *AtEC1.2b* (SEQ ID NO: 15), *AtEC1.4* (SEQ ID NO: 16) and *AtEC1.5* (SEQ ID NO: 17). One hypothetical *TaEC1*-like gene in *Medicago truncatula* was designated *MtEC1.1* (SEQ ID NO: 18) and three hypothetical *TaEC1*-like genes in rice are located on chromosomes 3, 11, and 12 and were designated Os*EC1.1* (SEQ ID NO: 19), Os*EC1.2* (SEQ ID NO: 20) and *OsEC1.3* (SEQ ID NO: 21).

The specific expression of *TaEC1* was investigated by RT-PCR using DNAse treated total RNA from different tissues of wheat and gene specific primers for the egg cell cDNA cluster C06_3 (*TaEC1*: SEQ ID NO: 22). Expression of *TaEC1* was not detected in any vegetative tissue, in anthers or 12 day old developing caryopsis of wheat. Transcripts were only found in the tissue containing the unfertilized egg cell (pistil), and isolated egg cells. After fertilization, *TaEC-1* is down-regulated (see Figure 1). A similar expression profile was observed for the *AtEC1*-like genes in *Arabidopsis*. Expression was examined by RT-PCR using DNAse treated mRNA from different tissues of *Arabidopsis* and gene specific primers for *AtEC1.1*, *AtEC1.2a/b*, *AtEC1.4* and *AtEC1.5*. All five genes of *Arabidopsis* are exclusively expressed in tissues containing the female gametophyte (see Figure 2).

EXAMPLE 2

Confirmation of egg-cell specific expression for *AtEC1*-like transcripts

Egg cell-specificity of *AtEC1*-like transcripts was confirmed by *in situ* hybridization using embedded sections of mature *Arabidopsis* pistils hybridized with an *AtEC1.2a* and *AtEC1.1* antisense probe, respectively. As shown in Figure 3, no mRNA of *AtEC1-like* genes could be detected in other cells of the unfertilized embryo sac or in young embryos after fertilization.

EXAMPLE 3

Specificity of EC1-derived promoters

Promoter specificity of *AtEC1-like* genes was analysed by cloning a defined 5´ upstream region of *AtEC1.1* (*pAtEC1.1*: SEQ ID NO: 24) and a defined 5´ upstream region of *AtEC1.2* (*pAtEC1.2*: SEQ ID NO: 25) upstream of the ß-Glucuronidase (GUS) gene. Cloned constructs were used for stable transformation of *Arabidopsis*. GUS activity was detected in egg cells of unfertilized ovules. After fertilization, however, GUS activity was still visible in the zygote, due to the high stability of ß-Glucuronidase (see Figure 4).

In addition, EGFP (enhanced green fluorescence protein) was C-terminal fused to the open reading frame of *AtEC1.1* (SEQ ID NO: 13), under control of the *AtEC1.1* promoter (SEQ ID NO: 24). The construct was used for transformation of *Arabidopsis*. Ovules of transgenic plants were analysed microscopically for green fluorescence under DIC and UV-light. In addition, EGFP signals were observed by Confocal Laser Scanning Microscopy (CLSM). A shown in Figure 5, green fluorescence was visible exclusively in egg cells of unfertilized ovules. Early after fertilization, some fluorescent protein was still visible in the zygote as well as secreted between the zygote and the neighbouring endosperm. No EGFP could be detected in other cells of the embryo sac or in later stages of embryo development.

EXAMPLE 4

Materials and Methods - Isolation of wheat embryo sac cells before and after fertilization

Spikes of *Triticum aestivum* cv ´Florida´ were emasculated 2-4 days before anthesis and covered with bags to prevent fertilization. Egg cells were isolated mechanically from microdissected ovules in 0.55 M sterile mannitol using fine-tipped glass needles and an inverted microscope, as described by Kumlehn *et al*. (*Protoplasma* 208: 156-162, 1999). Single cells were transferred into 0.5 ml

reaction tubes by using a glass capillary interfaced with a hydraulic system to a micropump. Collected cells were immediately frozen in liquid nitrogen.

EXAMPLE 5

Materials and Methods - mRNA isolation and cDNA synthesis

mRNA was isolated from 12 egg cells using the Dynabeads® mRNA DIRECT™ Micro kit (Dynal) following the manufacturer's guidelines, but scaled down to 50 µl. Annealed mRNA was isolated using a magnetic particle transfer device (PickPen™, Bio-Nobile). Subsequently, the SMART™ PCR cDNA synthesis kit (BD Biosciences) was used for cDNA synthesis. First-strand cDNA, long distance-PCR, and determination of optimal cycle numbers for generating a population of representative cDNAs was performed according to the manufacturer's guidelines, but using a digoxigenin-11-dUTP (Roche Applied Science) labeled fragment of wheat *GAPDH* as a probe.

EXAMPLE 6

Materials and Methods - Library construction and sequencing

150 µl of cDNA was used for polishing, according to instructions of the SMART™ PCR cDNA synthesis kit (BD Biosciences). Subsequently, 3 µg of *EcoR*I (NotI) adapters (Invitrogen) were ligated to blunt-end cDNA, using T4 ligase (New England Biolabs). Remaining adapters and fragments below 0.3 kb were removed by electrophoresis in 0.8% low-melting point agarose (Seaplaque GTG). Afterwards, cDNA was extracted using ß-agarase I (New England Biolabs). After phosphorylation of EcoRI cohesive ends (10 U/µl T4 polynucleotide kinase, New England Biolabs), a second purification step using Chromaspin™ columns (BD Biosciences) was performed. The cDNA was then ligated into predigested lambda ZAP® II/EcoRI/CIAP vector (Stratagene). The titre of the unamplified library was 1.43×10^6 pfu/ml. After amplification and *in vivo* excision, clones were randomly picked and used to generate ESTs. Insert

sizes ranged from 300 to 3000 bp, with an average of 900 bp. The average readable sequence length of ESTs was about 500 bp. DNA sequencer trace data subsequently passed an automated cleanup pipeline including PHRED to call bases and assign quality values, followed by CROSS_MATCH to align sequences and to eliminate vector sequences.

EXAMPLE 7

Materials and Methods – Bioinformatics

The sequences were clustered using blastclust (NCBI) and assembled into contigs using Vector NTI 8 (Invitrogen software package). The contig's consensus sequence or the longest representative was used for BLASTN searches against NCBI's nonredundant (nr) database and the EST-database, and for BLASTX searches against NCBI's nr database and SWISSPROT (March 2004). A number of cDNAs resulted in limited sequence information (100 - 250 bp) from non-coding regions. Therefore, BLASTN searches against the TIGR Wheat Gene Index Release 8.0 (Quackenbush *et al.*, *Nucleic Acids Res* 29: 159-164, 2001) were performed, using the BLASTN algorithm. If a match with >95% sequence identity over the total length of the query sequence was found, the matching sequence was retrieved and used in subsequent BLASTX searches in place of the original EST. A sequence was considered novel if it did not show a significant match with a sequence of the NCBI databases (nr, EST) or to the TIGR assembled wheat consensus sequences using the BLASTN algorithm (Altschul *et al.*, 1997, *supra*). The significance threshold used for BLASTN searches were: Score > 115, Expect-value < e^{-25}.

For BLASTX searches, the cutoff for a significant match for all but the short sequences was an e-value of < e^{-15} , Score >= 80. Matches to short query sequences (below 260 bp) were inspected and categorized manually. Clusters encoding proteins of known function were manually categorized into broad functional groups using the MIPS (Munich Information Centre for Protein Sequences) classification as guidance.

EXAMPLE 8

Materials and Methods – Expression analysis by RT-PCR

Wheat RNA was isolated from vegetative and generative tissues using TRIzol® reagent (Invitrogen), essentially following the manufacturer's protocol. Starch containing tissues such as caryopsis were extracted twice, using 3 ml of TRIzol® reagent per 100 mg of tissue. The quality of the total RNA preparation was analysed by denaturing agarose gel electrophoresis. Before RT-PCR, 1µg of total RNA was digested with DNAse (RNAse free; Invitrogen) and subsequently used for first-strand cDNA synthesis using Oligo$(dT)_{23}$ (Sigma) and Superscript II reverse transcriptase (Invitrogen), following the manufacturer's protocol but adding RNAseOUT™ (Invitrogen). Quality and amount of generated cDNAs was analysed by PCR with the following intron-spanning primers directed against wheat *GAPDH:*

TaGAP1 5´-AGGGTGGTGCCAAGAAGGTCA-3´ (SEQ ID NO: 34)

TaGAP2 5´-TATCCCCACTCGTTGTCGTA-3´ (SEQ ID NO: 35)

Expression of *TaEC1* was analysed using the primer pair:

TaEC1fw2 5´-CCGAGCGGCTGCAGGGAGTGG-3´ (SEQ ID NO: 36)

TaEC1rev2 5´-GCGTCGGAGTAGCCCTTGAGCA-3´ (SEQ ID NO: 37)

PCR reactions were carried out for 30 cycles (*GAPDH*) and 38 cycles (*TaEC1*) respectively, using 2.5 µl of cDNA as template.

Arabidopsis mRNA was isolated from up to 5mg tissue using the Dynabeads® mRNA DIRECT™ Micro kit (Dynal) following the manufacturer's guidelines. Annealed mRNA was isolated using a magnetic particle transfer device (PickPen™, Bio-Nobile). Before RT-PCR, the annealed mRNA was treated with

DNAse (RNAse free; Invitrogen) in a volume of 10µl. First-strand cDNA synthesis was carried out using Oligo(dT)$_{20}$ (Invitrogen) and Superscript II reverse transcriptase (Invitrogen), following the manufacturer's protocol but adding RNAseOUT™ (Invitrogen). Quality and amount of generated cDNAs was analysed by PCR with the following intron-spanning primers directed against *Arabidopsis* Actin (At2g37620):

Act3fw 5´-GATTTGGCATCACACTTTCTACAATG-3´ (SEQ ID NO: 38)
Act3rev 5´-GTTCCACCACTGAGCACAATG-3´ (SEQ ID NO: 39)

AtEC1-like cDNAs were amplified using the following gene specific primer pairs for each of *AtEC1.1*, *AtEC1.2a/b*, *AtEC1.4* and *AtEC1.5*:

AtEC1.1fw 5´-ACAGTGACAGCTCGCCCTCTC-3´ (SEQ ID NO: 40)
AtEC1.1rev 5´- AGTCATTGCCATCATAGTAACCTT-3´ (SEQ ID NO: 41)
AtEC1.2a/bfw 5´- AGTTTCCTCTTTGCCACCATC-3´ (SEQ ID NO: 42)
AtEC1.2a/brev 5´- CACCGTTGAGGAAGAAGAGAA-3´ (SEQ ID NO: 43)
AtEC1.4fw 5´- CCAGCGGAGTCATCAACCAACATA -3´ (SEQ ID NO: 44)
AtEC1.4rev 5´- GGAGACGGAGCCGGAGAAGAGT -3´ (SEQ ID NO: 45)
AtEC1.5fw 5´- GCGCCGGAAACTTGATGGACT-3´ (SEQ ID NO: 46)
AtEC1.5rev 5´- GGCGCCGGTGAAGGAGATAAT-3´ (SEQ ID NO: 47)

2µl of cDNA was used as template for each PCR reaction.

EXAMPLE 9

Materials and Methods – Cloning of promoter-GUS fusion constructs

Genomic DNA of *A. thaliana* (ecotype Columbia-0) was isolated according to Li and Chory, in *Methods in Molecular Biology* (Vol. 82) Eds. Martínez-Zapater, and Salinas, pp55-60, 1997). Genomic fragments of AtEC1.1 and AtEC1.2a

were amplified from genomic DNA by PCR, using “proof-reading” Taq DNA Polymerase (MBI Fermentas). Promoter primer:

pAtEC1.1 5´-TGCCTTATGATTTCTTCGGTTTC-3´ (SEQ ID NO: 48)

and gene specific primer:

AtEC1.1rev1 5´-TCAGAGTCATTGCCATCACAGTAACCTT-3´ (SEQ ID NO: 49)

were used for amplification of the promoter region and part of AtEC1.1 gene (837bp). Promoter primer:

pAtEC1.2a 5´- AAGCATTTGCGTTTGGTTTATC-3´ (SEQ ID NO: 50)

and terminator primer:

tAtEC1.2a 5´- AATGCGGTTTTAGTCACACG-3´ (SEQ ID NO: 51)

were used for amplification of AtEC1.2a (1371bp). Genomic amplification products were cloned into pCR®2.1-TOPO® (Invitrogen), after adding 3´ adenines (TOPO-gAtEC1.1 and TOPO-gAtEC1.2a). 3´A-addition, ligation and transformation of competent TOP10F´ cells was performed according the manufacturer’s guidelines.

Gene specific primers containing restriction sites were designed to clone the promoters in front of the ß-glucuronidase gene (*uid*A; Jefferson *et al. EMBO J* 6: 3901-3907, 1987). 457bp 5´ upstream of the *AtEC1.1* start codon was amplified from TOPO-gAtEC1.1 using the primers M13rev (vector primer) and:

AtEC1.1-PstI 5´-CCATTTCTCTGCAGATTGATAA-3´ (SEQ ID NO: 52)

893bp 5´ upstream of the *AtEC1.2a* start codon was amplified from TOPO-gAtEC1.2a using the primers M13rev (vector primer) and:

AtEC1.2a-BglII 5´-CCATAGATCTTTCTTTTTGGGG-3´ (SEQ ID NO: 53)

After precipitation of PCR reactions (1/10 vol. 3M NaAc, pH 5.2 / 1 vol. Isopropanol), the purified fragments were restricted with *PstI* (*AtEC1.1* promoter) and *BamHI/BglII* (*AtEC1.2a* promoter), respectively. The same enzymes were used for restriction of the GUS containing vector pMG2002 (Manfred Gahrtz, unpublished), thereby removing the maize ubiquitin promoter in front of the GUS gene. Restricted fragments and vectors were purified using the "Easy Pure" DNA purification kit (Biozym). Before ligation, PstI and BamHI/BglII digested plasmids were dephosphorylated using CIAP (Calf Intestine Alkaline Phosphatase; MBI Fermentas), following the manufacturer's guidelines. Promoters were ligated into digested and dephosphorylated vectors using T4 ligase (1 U/µl; Invitrogen), following the manufacturer's protocol. Ligation reactions were used for transforming competent Top10F' cells (Invitrogen). Positive clones were selected by colony PCR, using gene specific primers:

GUS start rev 5´-ATCCAGACTGAATGCCCACA-3´ (SEQ ID NO: 54),

pAtEC1.1 (SEQ ID NO: 48) and pAtEC1.2a (SEQ ID No: 50).

All plasmid preparations were performed either using the E.Z.N.A. Plasmid Miniprep Kit II (Peqlab), or the QIAGEN plasmid Midi Kit 100 (Qiagen). All cloned fragments and constructs were verified by sequencing, using either flanking primers M13fw and M13rev (TOPO-gAtEC1.1 and TOPO-gAtEC1.2a), or the gene specific primers GUS Start rev (SEQ ID NO: 54), pAtEC1.1 (SEQ ID NO: 48) and pAtEC1.2a (SEQ ID NO: 50).

EXAMPLE 10

Materials and Methods – Cloning of GFP-fusion protein

EGFP (enhanced green fluorescence protein; Pang *et al. Plant Physiol* 112: 893-900, 1996) was C-terminal fused to the open reading frame of *AtEC1.1*, under control of the *AtEC1.1* promoter. The promoter and open reading frame of *AtEC1.1* was amplified from genomic DNA using "proof reading" *Taq* polymerase (MBI Fermentas) and the primers:

E1F 5´-GCCTTATGATTTCTTCGGTT-3´ (SEQ ID NO: 55)
E1R 5´-GCAGGAGTGTAAAGATGAAT-3´ (SEQ ID NO: 56)

A second amplification of *AtEC1.1* was performed using the modified primers:

EC1-PF2 5´-CCCCGAATTCCTTATGATTTCTTCGGT-3´ (SEQ ID NO: 57)
EC1-R 5´-CTCGGATCCGGGTTAGAAGGAGAA-3 (SEQ ID NO: 58).

After restriction with EcoRI and BamHI, the fragment was cloned into the EcoRI-BamHI sites of the vector p7U-GFP (DNA Cloning Service). The C-terminal fusion of EGFP and the sequence of cloned fragment was verified by sequencing using the primers:

GFP-seq 5´-CCAGTTCCACCAGGATTG-3´ (SEQ ID NO: 59)
LH1 5´-CCCAAGATCTGGCCCTT-3´ (SEQ ID NO: 60).

EXAMPLE 11

Materials and Methods – Stable transformation of *Arabidopsis*

For plant transformation, vectors were transferred into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, *Mol Gen Genet* 204: 383-396, 1986). Transformations were performed on ecotype Columbia-0 by a "floral dip" procedure according to Clough and Bent (*Plant J* 16: 735-743, 1998). The

seeds obtained from the T0 transformants were germinated on soil after a cold-treatment of 2 days at 2°C. Three days after germination, transgenics were selected by spraying 200 mg/l BASTA® (Bayer Crop Science) supplemented with 0.1 % Tween. BASTA® treatment was repeated two times after three days, each. Surviving seedlings were transferred to single pots. BASTA® resistant plants of the T1 and T2 generation were analysed for the presence of the T-DNA by PCR using GUS primers:

GUS3 5´-GCGTGGTGATGTGGAGTATTG-3´ (SEQ ID NO: 61)
GUS4 5´-TCACCGAAGTTCATGCCAGTC-3´ (SEQ ID NO: 62)

or primers:

bar-fw 5´-CCGTACCGAGCCGCAGGAAC-3´ (SEQ ID NO: 63)
bar-rev 5´-CAGATCTCGGTGACGGGCAGGAC-3´ (SEQ ID NO: 64)

EXAMPLE 12

Materials and Methods – *GUS staining*

Activity of β-glucuronidase (GUS; Jefferson *et al*., 1987, *supra*) was performed according to a protocol of Vielle-Calzada *et al*. (*Nature* 404: 91-94, 2000). Inflorescences, siliques, leaves, and stems from soil-grown plants were transferred to microtiter wells containing 500µl of GUS staining buffer. Pistils and siliques were cut open lengthwise with a hypodermic needle (0.4 x 20mm, Braun) before transferring into GUS staining buffer. Microtiter dishes were placed under vacuum for 5 min. After release of vacuum, plates were covered with a lid and incubated at 37°C in the dark for 6 hours, or up to 3 days. Afterwards, the solution was removed and the tissues were cleared in 70% ethanol. Ovules were isolated on a glass slide by dissecting the pistils with a syringe in a drop of sodium phosphate buffer, pH 7.0. Ovules were cleared using either Hoyers solution (Liu and Meinke, *Plant J* 16: 21-31, 1998) or

Chloral hydrate clearing buffer (80g Chloral hydrate, 20ml H_2O, 10ml glycerol) and analysed under a Zeiss Axioskop microscope under differential interference contrast (DIC) optics. Images were captured on an Axiocam camera (Zeiss) using the Axiovision program AC Release 4.1 (Zeiss).

EXAMPLE 13

Materials and Methods – *GUS staining*

Ovules of transgenic *Arabidopsis* plants were dissected on glass slides in phosphate buffered saline, pH 7.4 (8g NaCl, 0.2g KCl, 1.,44g Na_2HPO_4, 0.24g KH_2PO_4). Fluorescence was observed using a Confocal Laser Scanning Microscope (CLSM), as described by Knebel *et al*. (*Eur J Cell Biol* 52: 328-340, 1990).

EXAMPLE 14

Materials and Methods – In situ Hybridization

Non radioactive *in situ* hybridization with DIG labeled RNA probes of *AtEC1.1* and *AtEC1.2a* was performed as described by Vielle-Calzada (*Genes Dev* 13*:* 2971-2982*,* 1999). For generating RNA probes by *in vitro* transcription, the open reading frame and 3´-UTR of *AtEC1.1* and *AtEC1.2a* was amplified from genomic DNA by PCR and subsequently cloned into pCR®II-TOPO® (Invitrogen).

Primers used for amplification of *AtEC1.1* were:

1-1fwXbaI	5´-ATCTGTCTAGAAATGGCTTC-3´	(SEQ ID NO: 65)
1-1revXbaI	5´-TTTATTCTAGAAAGTAATAACAG-3´	(SEQ ID NO: 66)

Primers used for amplification of *AtEC1.2a* were:

At2a-BglIIfw 5´-AAAGAAAGATCTATGGCTTCTAAC-3´ (SEQ ID NO: 67)

At2a-SalIrev 5´-TCATTAGTCGACTTTGCATACATC-3´ (SEQ ID NO: 68)

Ligation of PCR products into pCR®II-TOPO® and transformation of competent cells was performed according the manufacturer's guidelines.

Positive colonies were selected by colony PCR, using the vector primers M13-20 and M13rev. Plasmids were prepared using the QIAGEN plasmid Midi Kit 100 (Qiagen). Cloned fragments were verified by sequencing, using the vector primers M13fw and M13rev. The plasmids were linearized using BamHI (sense) and XhoI (antisense) and purified by two times of phenol/chloroform extractions followed by precipitation. Run off transcripts of linearized plasmids (0,5µg/µl in DEPC-treated water) were generated by using T7 and SP6 polymerases for *in vitro* transcription. *In situ* hybridization of *Arabidopsis* ovules with sense and antisense probes was performed using 8µm sections of embedded mature unfertilized and fertilized pistils, essentially following a protocol of Jackson, D. (in: *Molecular Plant Pathology, A Practical Approach***.** (Bowles, D.J., Gurr, S.J. and McPhereson, M., eds.), Oxford University Press, U.K., 163-174, 1991).

DNA and protein sequences were aligned using ClustalW software (Higgins *et al*., *Nucl. Acids Res*. 22: 4673-4680, 1994) and alignments drawn by GeneDoc version 2.6.02 (Nicholas *et al*., *Embnew. News* 4: 14, 1997).

EXAMPLE 15

Detection of conserved motifs in EC1-derived transcriptional control sequences

Each of the EC1-derived transcriptional control sequences defined herein (ie. SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32) were inspected for known *cis*-regulatory elements by PLACEdb 26.0

(http://www.dna.affrc.go.jp/PLACE/), a database of motifs found in plant cis-acting regulatory DNA elements.

Identification of novel sequence motifs was performed computationally using the sequence analysis software Lasergene/MegAlign (DNASTAR, Inc., 1228 South Park Street, Madison, WI 53715, USA).

All of the transcriptional control sequences were aligned pair wise with each other using two algorithms, Martinez Needleman-Wunsch DNA Alignment (Minimum Match: 9; Gap Penalty: 1.10; Gap Length Penalty: 0.33) and Wilbur-Lipman DNA Alignment (Ktuple: 3; Gap Penalty: 3; Window: 20). In addition, over-represented motifs in all of the upstream regions of the transcriptional control sequences were analyzed by the motif finding algorithm MotifSampler (Thijs et al., *Journal of Computational Biology*, 9(2), 447-464, 2002) at http://homes.esat.kuleuven.be/~thijs/Work/MotifSampler.html (see Figure 10).

Two motifs were identified in the upstream regions of SEQ ID NO: 24, SEQ ID NO: 25, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 30 and SEQ ID NO: 31. The consensus sequences of two novel sequence motifs identified were as follows:

EC1 promoter nucleotide sequence motif #1: 5'-CCACTAAT-3' (SEQ ID NO: 33)
EC1 promoter nucleotide sequence motif #2: 5'-kTAATTAm-3' (SEQ ID NO: 69)

As the expression of the subject EC1 genes is regulated in a similar manner, the identified motifs represent putative *cis*-regulatory elements for egg cell specificity of the transcriptional control sequences.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features,

compositions and compounds referred to, or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features.

Also, it must be noted that, as used herein, the singular forms “a”, “an” and “the” include plural aspects unless the context already dictates otherwise. Thus, for example, reference to “a nucleotide sequence of interest” includes a single nucleotide sequence as well as two or more nucleotide sequences; “an egg cell” includes a single egg cell as well as two or more egg cells; and so forth.

Future patent applications may be filed in Australia or overseas on the basis of the present application, for example by claiming priority from the present application, by claiming a divisional status and/or by claiming a continuation status. It is to be understood that the following claims are provided by way of example only, and are not intended to limit the scope of what may be claimed in any such future application. Nor should the claims be considered to limit the understanding of (or exclude other understandings of) the invention or inventions inherent in the present disclosure. Features may be added to or omitted from the example claims at a later date, so as to further define the invention or inventions.

THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A nucleic acid construct comprising a plant female gamete-specific transcriptional control sequence, wherein said transcriptional control sequence comprises:

(i) the nucleotide sequence set forth in SEQ ID NO:24, or

(ii) a variant of (i), said variant comprising a nucleotide sequence which is at least 80% identical to SEQ ID NO:24 over the full length of SEQ ID NO:24 and having plant female gamete-specific transcription control activity

and wherein said transcriptional control sequence is operably connected to a nucleotide sequence of interest which is heterologous with respect to the transcriptional control sequence.

2. The nucleic acid construct of claim 1, wherein the nucleic acid construct further comprises a nucleotide sequence defining a transcription terminator.

3. The nucleic acid construct of claim 1 or claim 2, wherein the nucleic acid construct comprises an expression cassette comprising the structure:

$$([N]_W – TCS – [N]_X – Sol – [N]_Y – TT – [N]_Z)$$

wherein:

$[N]_W$ comprises one or more nucleotide residues, or is absent;

TCS comprises the transcriptional control sequence;

$[N]_X$ comprises one or more nucleotide residues, or is absent;

Sol comprises a nucleotide sequence of interest which encodes an mRNA or non-translated RNA, wherein the nucleotide sequence of interest is operably connected to the TCS;

$[N]_Y$ comprises one or more nucleotide residues, or is absent;

TT comprises a nucleotide sequence defining a transcription terminator;

$[N]_Z$ comprises one or more nucleotide residues, or is absent.

4. A cell comprising the nucleic acid construct of any one of claims 1 to 3; or a genomically integrated form of said construct.

5. The cell of claim 4, wherein the cell is a plant cell.

6. The cell of claim 5, wherein the cell is a dicotyledonous plant cell.

7. The cell of claim 6, wherein the cell is an *Arabidopsis* sp. cell.

8. The cell of claim 5, wherein the cell is a monocotyledonous plant cell.

9. The cell of claim 8, wherein the cell is a cereal crop plant cell.

10. The cell of any one of claims 4 to 9, wherein the cell is a plant egg cell.

11. The plant egg cell of claim 10, wherein the level, rate and/or pattern of expression of at least one nucleotide sequence is altered in said plant egg cell relative to a wild type form of said plant egg cell.

12. A method for specifically or preferentially expressing a nucleotide sequence of interest in a plant egg cell, the method comprising effecting transcription of the nucleotide sequence of interest, in a plant, under the transcriptional control of a transcriptional control sequence comprising:

(i) the nucleotide sequence set forth in SEQ ID NO:24, or

(ii) a variant of (i), said variant comprising a nucleotide sequence which is at least 80% identical to SEQ ID NO:24 over the full length of SEQ ID NO:24 and having plant female gamete-specific transcription control activity.

13. The method of claim 12, wherein the nucleotide sequence of interest is heterologous with respect to said transcriptional control sequence.

14. A method for promoting female sterility in a plant, the method comprising expressing a nucleotide sequence encoding a cytotoxic protein, specifically or

preferentially in an egg cell of the plant, wherein said nucleotide sequence is operably connected to a transcriptional control sequence comprising:

(i) the nucleotide sequence set forth in SEQ ID NO:24, or

(ii) a variant of (i), said variant comprising a nucleotide sequence which is at least 80% identical to SEQ ID NO:24 over the full length of SEQ ID NO:24 and having plant female gamete-specific transcription control activity, wherein the cytotoxic protein is selected from the group consisting of a barnase, an RNAse and a diphtheria toxin.

15. A method for modulating embryo development and/or increase embryo size in a plant, the method comprising expressing a nucleotide sequence encoding a transcriptional regulator that acts during embryo development, specifically or preferentially in an egg cell of the plant, wherein said nucleotide sequence encoding a transcriptional regulator is operably connected to a transcriptional control sequence comprising:

(i) the nucleotide sequence set forth in SEQ ID NO:24, or

(ii) a variant of (i), said variant comprising a nucleotide sequence which is at least 80% identical to SEQ ID NO:24 over the full length of SEQ ID NO:24 and having plant female gamete-specific transcription control activity,

wherein the nucleotide sequence encoding a transcriptional regulator comprises a BBM, LEC1/LEC2 or AP2.

16. A method for promoting apomixes in a plant, the method comprising expressing an apomixes-promoting nucleotide sequence specifically or preferentially in an egg cell of the plant, wherein said apomixes-promoting nucleotide sequence is operably connected to a transcriptional control sequence comprising:

(i) the nucleotide sequence set forth in SEQ ID NO:24, or

(ii) a variant of (i), said variant comprising a nucleotide sequence which is at least 80% identical to SEQ ID NO:24 over the full length of SEQ ID NO:24 and having plant female gamete-specific transcription control activity, and

wherein said apomixes-promoting nucleotide sequence comprises a nucleotide sequence which encodes RB, E2F, BBM, LEC1/LEC2, AP2 or a DNA methyltransferase.

17. The method of any one of claims 12 to 16, wherein the plant is a dicotyledonous plant.

18. The method of claim 17, wherein the plant is an *Arabidopsis* sp. plant.

19. The method of any one of claims 12 to 16, wherein the cell is from a monocotyledonous plant.

20. The method of claim 19, wherein the cell is a cell from a cereal crop plant.

FIGURE 1

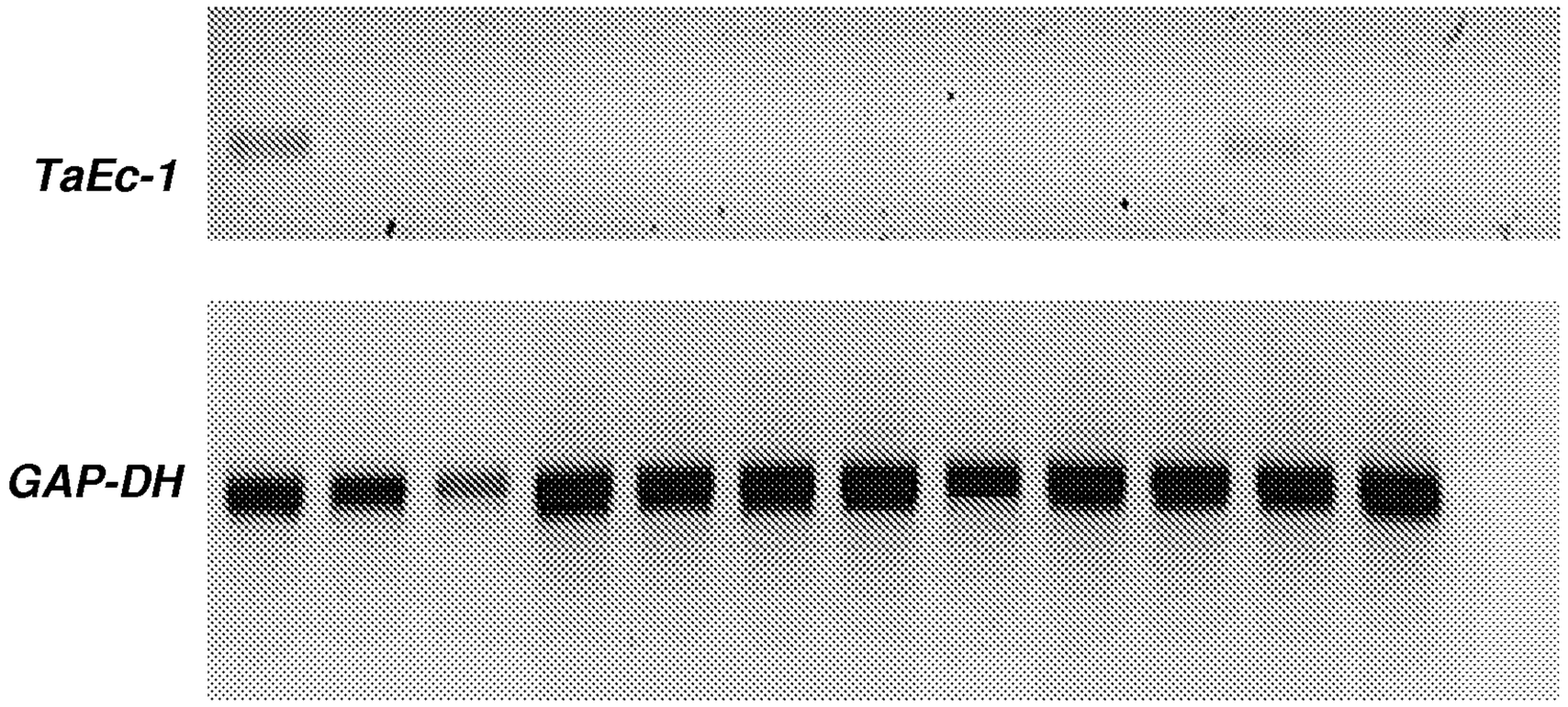
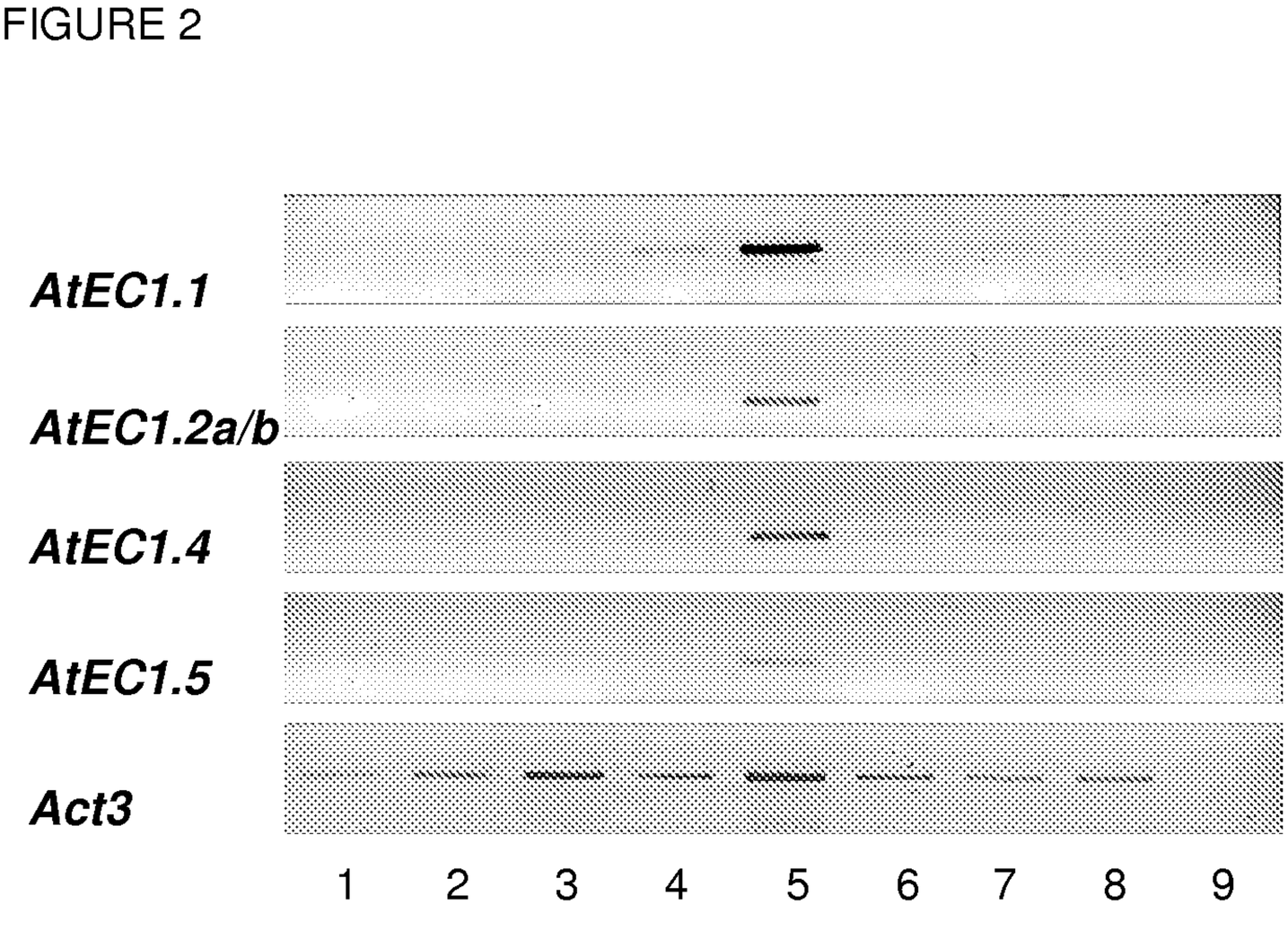


FIGURE 2



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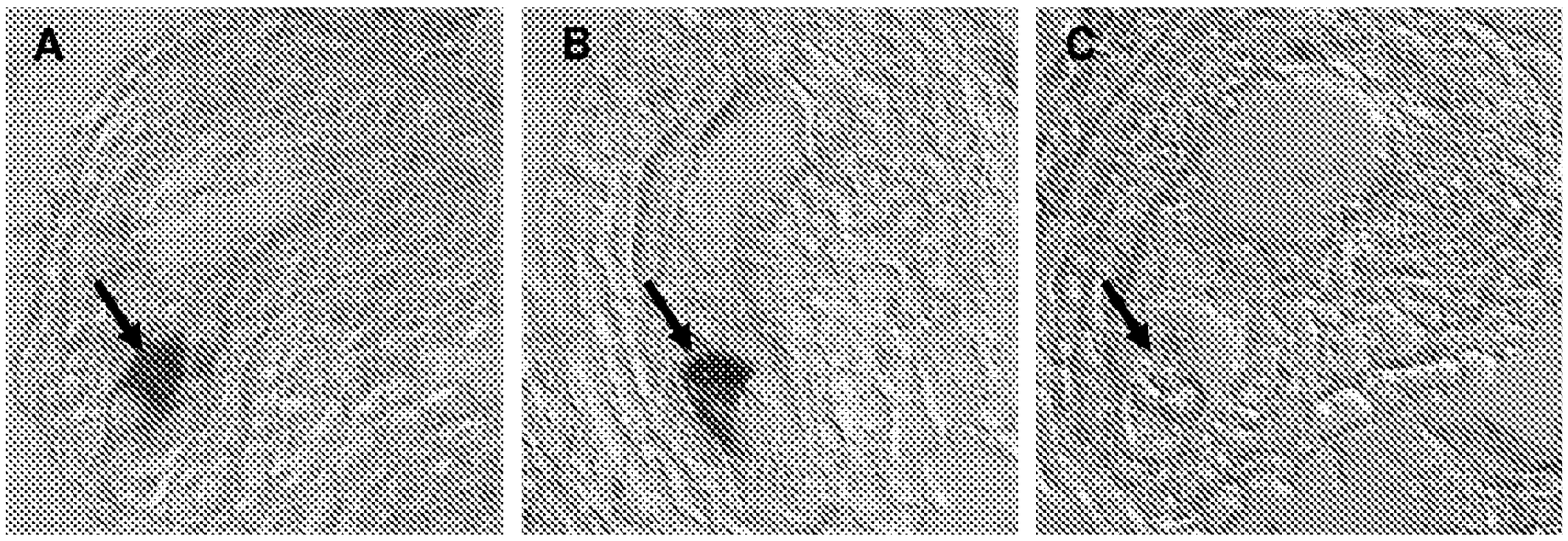

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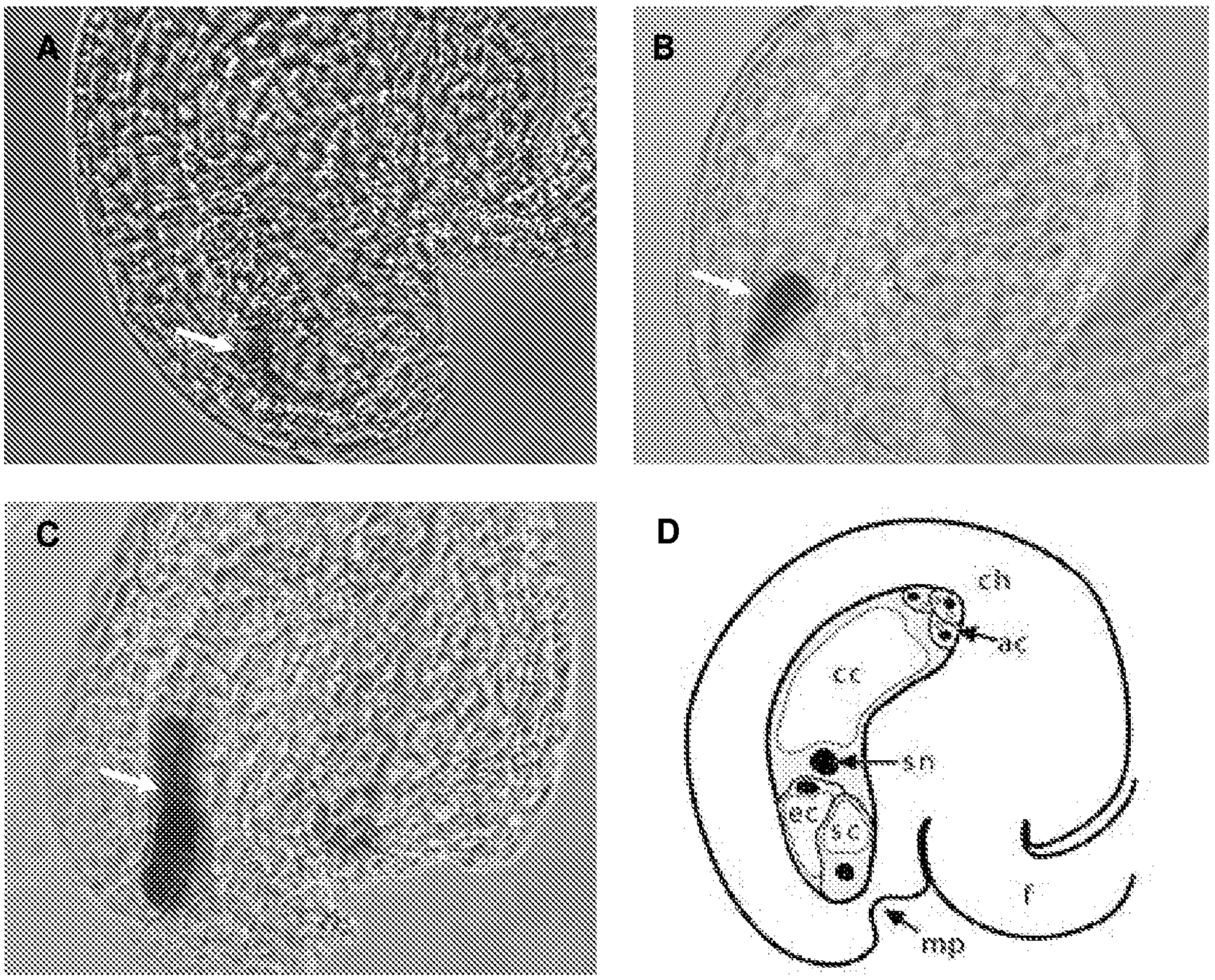
FIGURE 4



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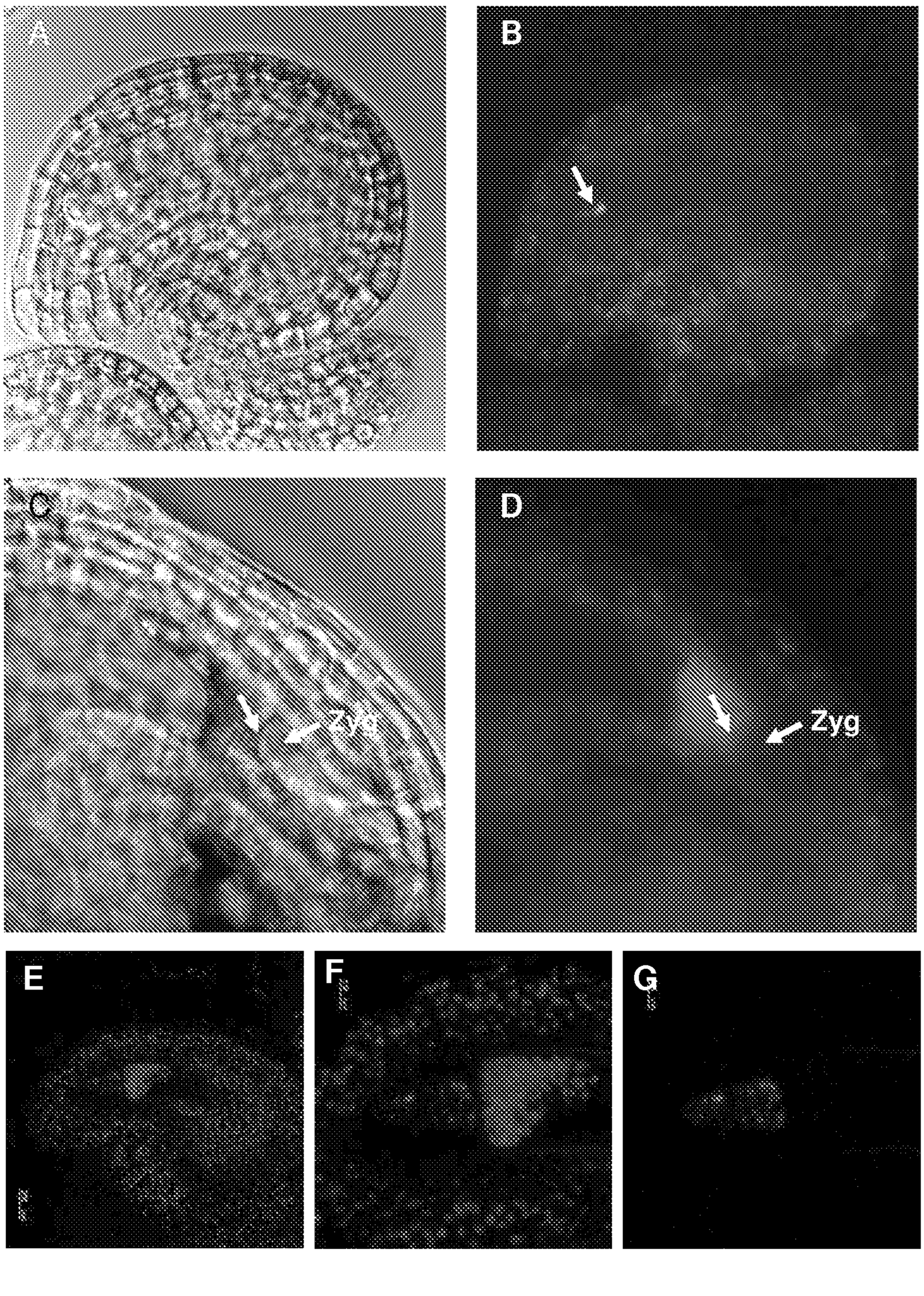

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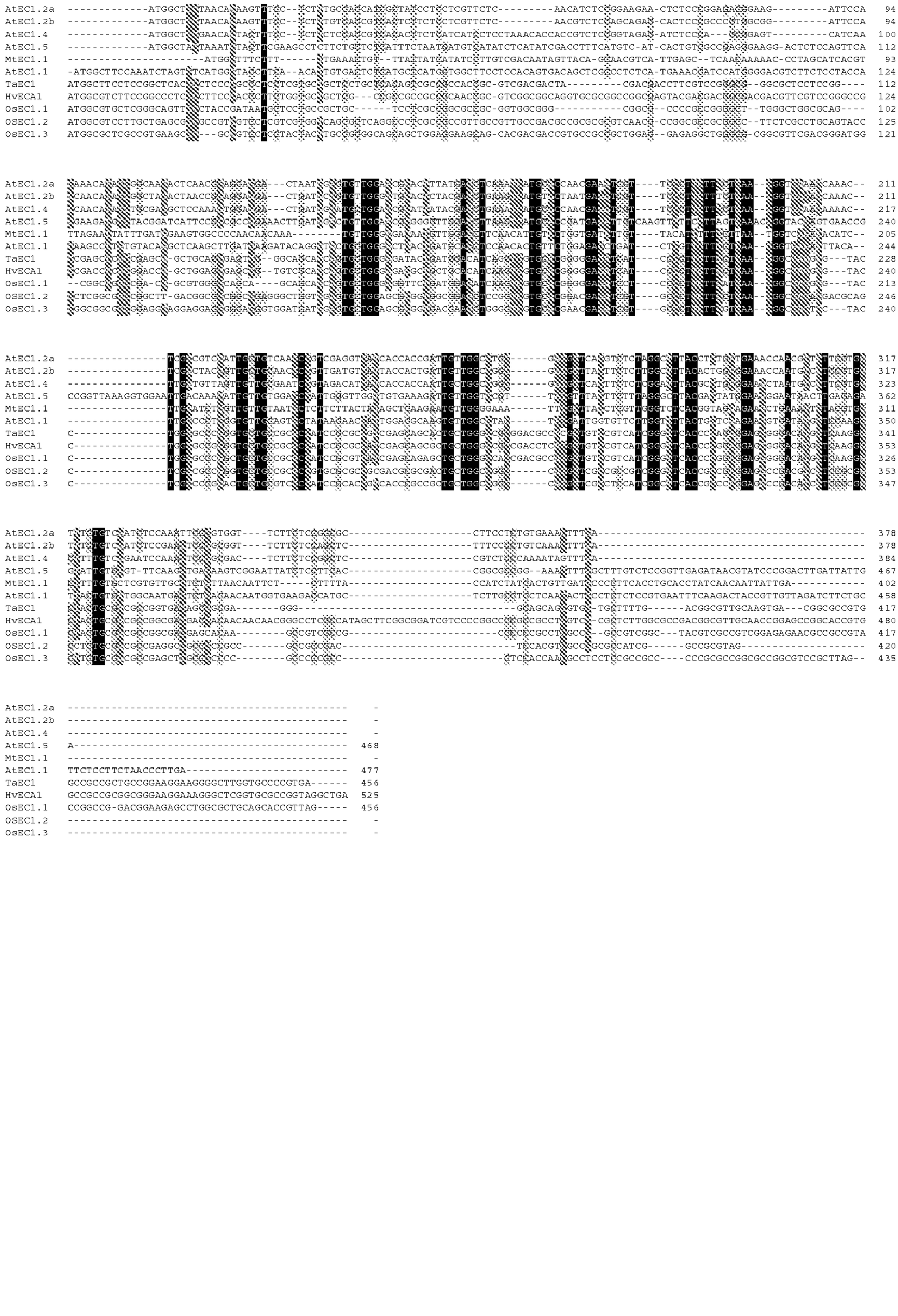
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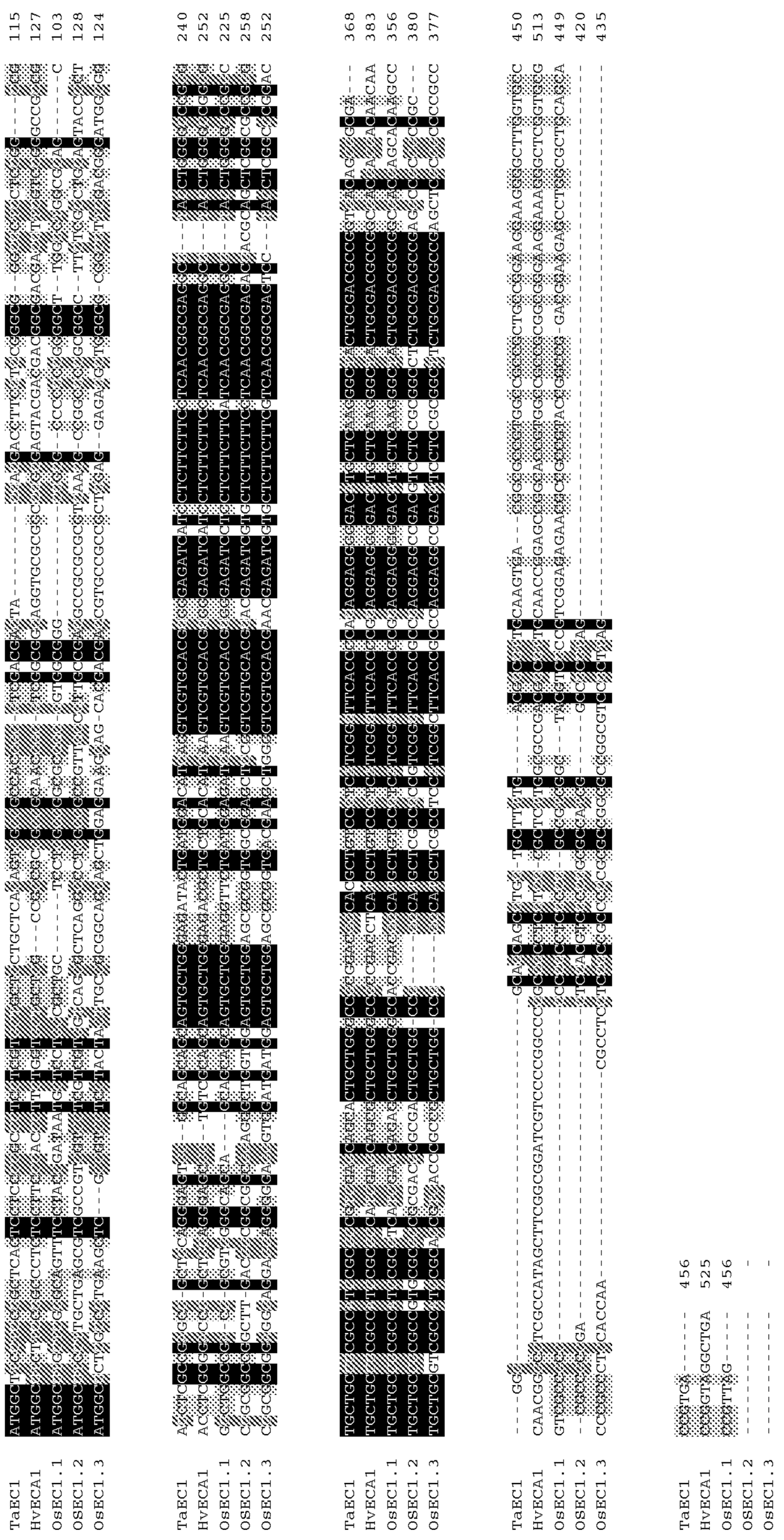
OsEC1.1
OSEC1.2
OsEC1.3

FIGURE 7



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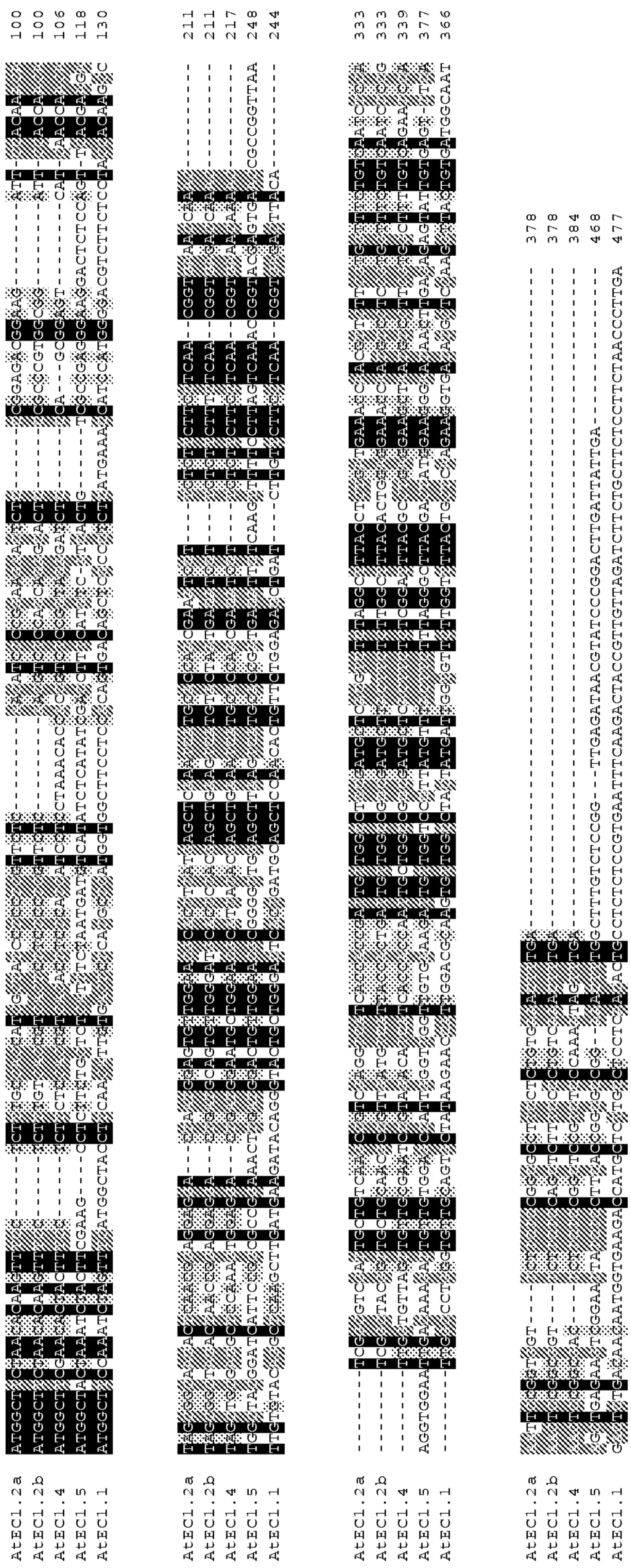
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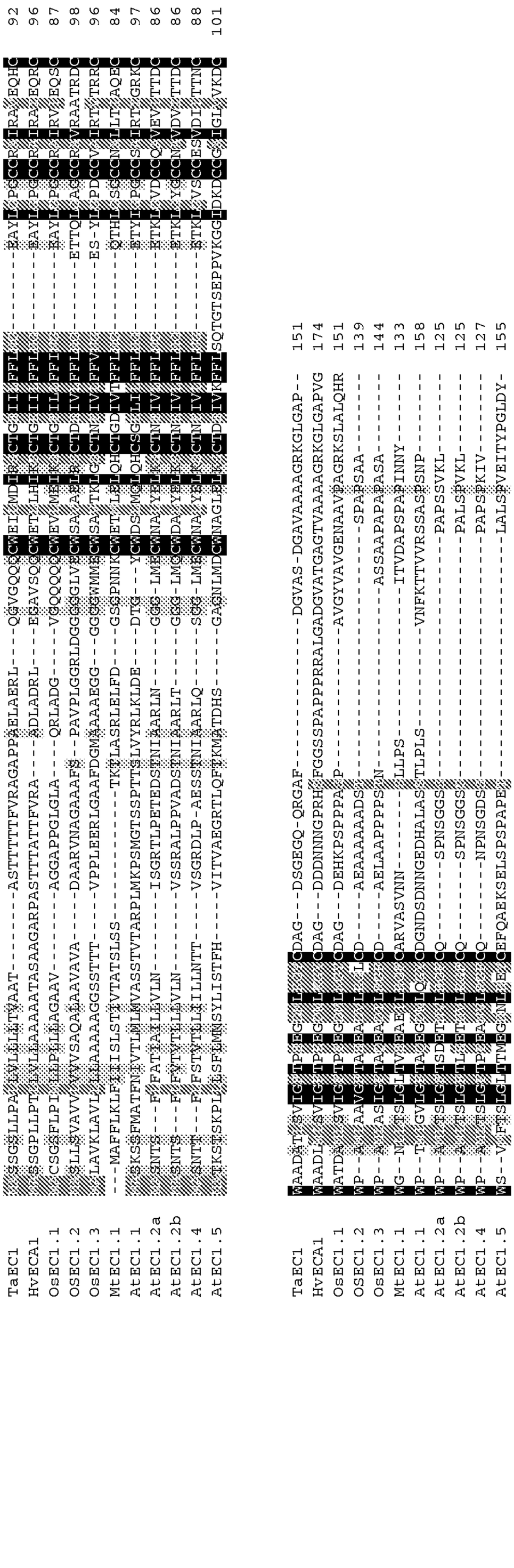
FIGURE 9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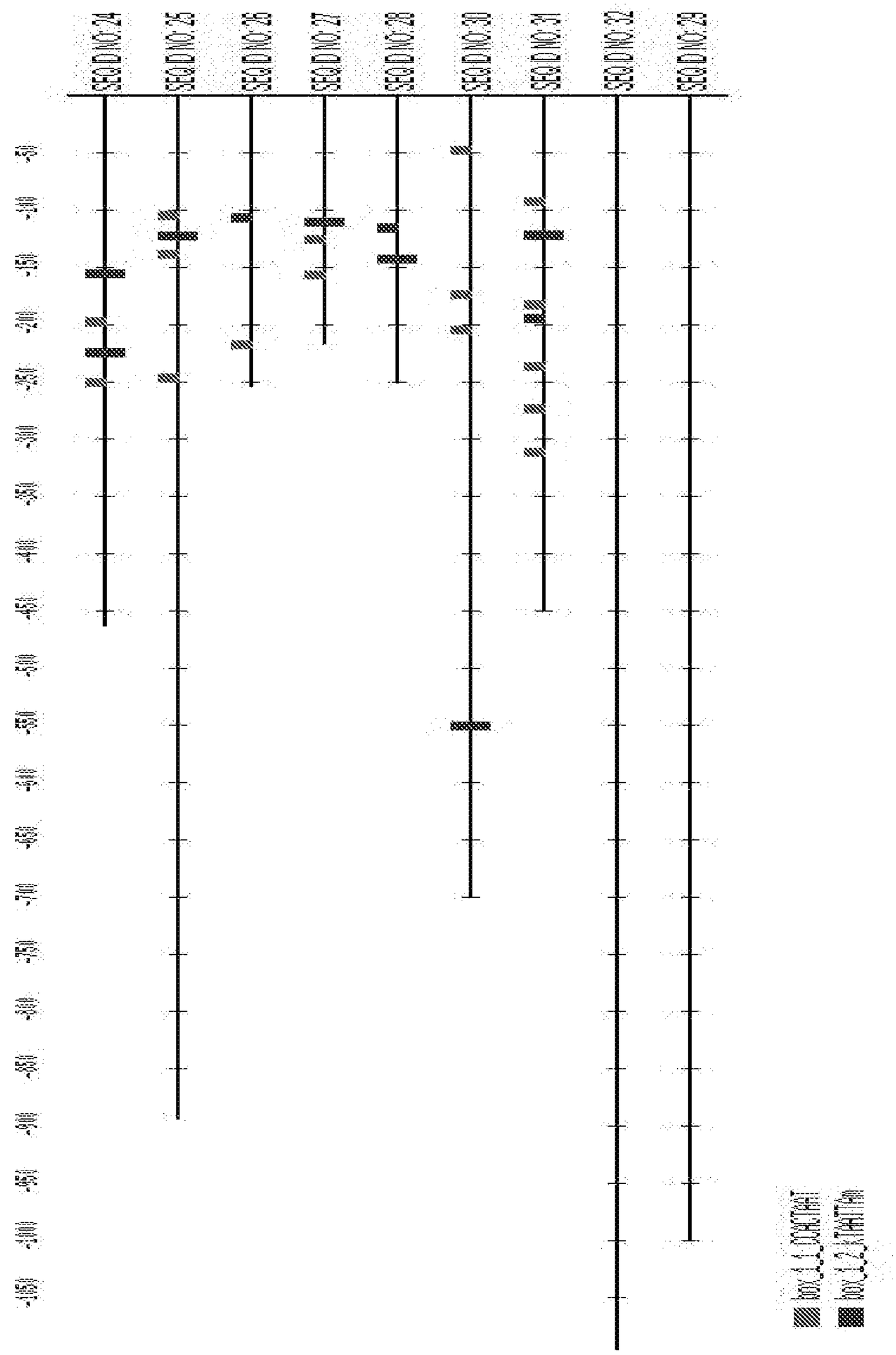
AtEC1.4
AtEC1.5

FIGURE 10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box 1 contrast
box 1 & 2 contrast