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(54) Title: METHOD OF PLASTID TRANSFORMATION IN *ASTERACEAE*, VECTOR FOR USE THEREIN AND PLANTS THUS OBTAINED

(57) Abstract: The present invention relates to a method for the transformation of plastid genomes of plant species, in particular *Asteraceae* plant species, comprising the steps of providing a transformation vector carrying a DNA sequence of interest; subjecting a plant material, which comprises plastids, to a transformation treatment in order to allow the plastids to receive the transformation vector; placing the thus treated plant material for a period of time into contact with a culture medium without selection agent; subsequently placing the plant material into contact with a culture medium comprising a selection agent; and refreshing the culture medium comprising a selection agent to allow plant material comprising plastids that have acquired the DNA of interest to grow into transformants.



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**METHOD OF PLASTID TRANSFORMATION IN *ASTERACEAE*,
VECTOR FOR USE THEREIN AND PLANTS THUS OBTAINED**

Field of the invention

5 The invention relates to methods of genetically transforming plant plastids, and more specifically to genetically transforming the plastid genomes of *Asteraceae* plant species. The invention further relates to vectors for use in the transformation of plastid genomes and to
10 transplastomic plants thus obtained and their progeny.

Background of the invention

 Plastids are self-replicating organelles containing their own DNA in a single circular chromosome, called their
15 genome. Plastids are found in all plant cells. They are inherited maternally in most plants just like mitochondria in animals and plants. This is also called cytoplasmic inheritance since these organelles are present in the cytosol of the ova.

20 Plant plastids (e.g. chloroplasts, amyloplasts, elaioplasts, etioplasts, chromoplasts, leucoplasts and proplastids) are the organelles in which major biochemical processes (i.e. photosynthesis) take place. In general, plant cells contain between 100-10,000 copies of the small 120-160
25 kb circular plastid genome. Since each molecule has one inverted repeat it is theoretically possible to obtain plant cells with 20,000 copies of (a) gene(s) of interest, after plastid transformation.

 The genetic transformation of the plastid genome
30 (plastome) has major advantages over nuclear transformation. Firstly, because in most plant species, plastids are maternally inherited, out-crossing of transgenes to weeds or other crops is minimized. Thus, this form of genetic

engineering of plants lowers the risk of dissemination of the transgene in the environment through pollen dispersal.

Furthermore, the plastid genome is highly polyploid, enabling the introduction of many copies per cell which can lead to
5 high accumulation levels of the desired protein(s). The fact that plastids are able to form disulfide bonds and to fold proteins, makes this technique in theory ready for the production of biopharmaceuticals in plants.

The principle of plastid transformation is insertion
10 of sequences through homologous recombination. Plastid transformation vectors use two targeting DNA segments that flank the gene or genes of interest. By means of homologous recombination these segments can insert the foreign gene or genes at a precise, predetermined position in the plastid
15 genome. Position effects and gene silencing, major problems in nuclear transformation experiments, have not as yet been observed in plastid transformation events.

However, successful chloroplast transformation of crop plants is described thus far only for Solanaceous crops
20 like potato, tomato, tobacco (US-5,451,513; Svab et al. (1990), Proc. Natl. Acad. Sci. USA 87:8526-8530) and Brassicaceae, like *Arabidopsis thaliana* (US-6,376,744). It is not obvious that the techniques used for these species can be readily used for other species such as Asteraceae, in
25 particular lettuce.

It is therefore the object of the invention to provide an alternative plastid transformation method that is in particular useful for transforming Asteraceae plant species, such as lettuce (*Lactuca sativa*). Lettuce is an
30 agronomical important crop and a useful transformation method therefore is thus highly desirable.

Summary of the invention

The invention thus provides a method for the transformation of plastid genomes of plant species, in particular *Asteraceae* plant species, comprising the steps of:

- 5 a) providing a transformation vector;
- b) subjecting a plant material, which comprises plastids, to a transformation treatment in order to allow the plastids to receive the transformation vector;
- c) placing the thus treated plant material for a
10 period of time into contact with a culture medium without selection agent;
- d) subsequently placing the plant material into contact with a culture medium comprising a selection agent; and
- 15 e) refreshing the culture medium comprising a selection agent to allow plant material comprising plastids that have acquired the DNA of interest to grow into transformants, in particular transplastomic plants or plant parts (i.e. plants or plant parts carrying one or more
20 transgenes in their plastids).

The transformation vector may comprise:

- an expression cassette which comprises optionally a promoter active in the plastids of the plant species to be transformed, a DNA insertion site for receiving the
25 transforming DNA of interest, optionally one or more selection markers conferring a selectable phenotype on cells having plastids that are transformed with the expression cassette, and optionally a DNA sequence encoding a transcription termination region active in the plastids of
30 the plant species to be transformed,
- optionally a set of DNA targeting segments located on either side of the expression cassette that allow double

homologous recombination of the expression cassette with the plastid genome of interest, and

- a DNA sequence of interest inserted into the insertion site of the expression cassette.

5 Preferably the vector comprises a promoter, a set of targeting segments and one or more selection markers. However, these elements may also be provided in another way. For example, the DNA of interest can be inserted at such a position in the plastome that it can use an already present
10 promoter, such as in an operon. If no targeting segments are present the DNA of interest can integrate at a random position. The DNA of interest is preferably integrated in the plastid genome but can also exist outside the plastome.

15 The DNA of interest can be either stably integrated or transiently expressed.

 It is surprising that when using the method of the invention no escapes are found in the transformation of plastids of lettuce. The results of plastid transformations thus far, mention the occurrence of escapes (due to nuclear
20 or spontaneous mutants; Kofer et al. (1998) In Vitro Cell. Dev. Biol. Plant 34:303-309).

 It was surprisingly found that not immediately starting the selection process but keeping the treated plant material in or on a culture medium for a few days highly
25 improved the efficiency of transformation. In addition, the selection procedure should not be started too late in the culture process. Preferably, selection is started after a maximum of 2-5 days. The moment to start the selection process depends on the transformation method. Another
30 important aspect of the invention is to keep the transformed cells into close contact with the selective agent for a period of time, preferably until regeneration. In addition, it is preferred to retain the concentration of the selective

agent at an efficient level, such as 500 mg/l spectinomycin dihydrochloride. This is preferably achieved by using a liquid medium containing the selective agent.

5 *Detailed description of the invention*

The invention provides methods and vectors for efficient and stable transformation of plastids of an Asteraceae plant species, in particular chloroplasts of a lettuce plant, and the plants thus obtained.

10 Other plastids that can be transformed by the method of the invention are selected from the group consisting of amyloplasts, elaioplasts, etioplasts, chromoplasts, leucoplasts and proplastids.

The vector that is used in the method of the
15 invention has a vector backbone and in addition a DNA construct that optionally comprises one or more sets of targeting DNA segments that are homologous to a sequence in the plastid genome, optionally a promoter sequence, optionally a DNA sequence encoding the transforming gene
20 inserted in an insertion site, optionally a terminator sequence, and optionally at least one DNA sequence encoding a selectable marker.

Preferably, the vector comprises the targeting DNA segments, the DNA sequence encoding the transforming gene, a
25 promoter and a selectable marker.

The promoter is any promoter that is active in the plastids of the plant species to be transformed and for lettuce for example selected from the group of (lettuce or other plant species) chloroplast specific ribosomal RNA
30 operon promoter *rrn* (16S rRNA), *psbA*, *rbcL*, *trnV*, or *rps16*. However, additional promoter regions, to enhance transcription, translation or both processes, can also be used for obtaining expression of the selectable marker and

gene of interest in lettuce plastids. Also, bacterial promoters can be used for expressing genes in the plastids.

The terminator is any terminator that is active in the plant species to be transformed and for lettuce for example selected from the group consisting of the *psb A* termination sequence, *rrn*, *rbcL*, *trnV*, or *rps16*. These and other terminators may be specific for lettuce or other plant species. A terminator sequence need not always be present in bicistronic constructs, being two open reading frames behind one promoter. Additional UTR (untranslated region) sequences, fused to coding sequences of desired gene(s), can be used as leader and/or trailer, to minimize unwanted recombination.

The selection marker is for example selected from the group consisting of spectinomycin, streptomycin, kanamycin, hygromycin and chloramphenicol, or to plant herbicides like glyphosate or bialaphos. Of these markers the *aadA* gene is preferred because it is a non-lethal marker.

Alternatively a visual marker can be used, such as *gfp* (green fluorescence protein). In that case the selective agent is not a compound or composition but the means that is used to visualize the visual marker, such as the source of blue light that leads to fluorescence of the *gfp*.

When only such visual marker is used for selecting the transformants, steps d) and e) of the method can be performed without selective agent. The selection is then made visually by illuminating the putative transformants with an appropriate source of light and selecting the transformants that show fluorescence.

The DNA segments that allow double homologous recombination of the DNA of interest with the plastid genome of interest have a DNA sequence that is homologous to a part of the plastid genome. The segments are selected such that integration of the transforming gene takes place in a desired

position in the genome. For lettuce, for instance, the set of DNA segments is selected from the *trnI(oriA)/trnA* region and the 16S/*trnV*/ORF70B region of the lettuce chloroplast genome. Preferably, the set of DNA segments is selected from LCV1 A-B and LCV1 C-D, and LCV2 A-B and LCV2 C-D as disclosed in the Examples. The advantage of these segments is that they were found to be particularly useful for lettuce.

The method of the invention can be used for the preparation of plants that can express any gene of interest. The inventive technology can be used for the transformation of plastids from any plant, but in particular for plants of the *Asteraceae* family, more in particular for lettuce. The invention can thus be used for the production of polypeptides that can be isolated from the plant or of polypeptides that are useful for the plant itself. An example of production of products that can be isolated from the plant lies for example in the field of biopharmaceuticals, i.e. pharmaceuticals produced in living organisms such as plants. The production in plants has high potential because it can lead to lower production costs as compared to production in animals or in microorganisms using Bioreactors.

A promising new field in which this invention can be used is the production of edible vaccines, but other pharmaceuticals, either therapeutic or prophylactic, can be envisaged as well as (poly)peptides that can be used in other fields.

In addition to using the plant as a factory for the production of peptides or polypeptides, the product expressed can also be of agronomical importance. Examples are herbicide resistance, insect resistance, fungal resistance, bacterial resistance, stress tolerance for instance to cold, high salt or minerals, yield, starch accumulation, fatty acid accumulation, photosynthesis.

According to the invention, the transformation treatment is selected from the group consisting of electroporation, particle gun transformation, polyethylene glycol transformation and whiskers technology. Polyethylene glycol transformation and particle gun are very advantageous since a high number of cells can be transformed simultaneously and an efficient selection of the transformed plastids within the cells can take place.

The essence of the whiskers technology is the microscopic needle-like silicon-carbide "whiskers" which are approximately 0.6 microns in diameter and vary from 5-80 microns in length. The process begins with the provision of a "transformation cocktail" consisting of DNA, silicon carbide "whiskers", and the appropriate plant target tissue. This cocktail is then stirred or mixed or shaken in a robust fashion by a variety of means (such as a Vortex Machine, a Dental Amalgam Mixer, or a Commercial Paint Shaker). The resulting collisions between plant cells and "whiskers" are hypothesized to result in the creation of very small openings in the plant cell wall and membrane. As a consequence, DNA can move into the targeted plant cells, followed by integration of the transforming DNA into the plastome. Ultimately, transplastomic plant material can be recovered.

The period of time during which the treated plant material is placed into contact with a culture medium without selection agent depends on the transformation treatment. For polyethylene glycol transformation the period of time is 1 to 14 days, preferably 3 to 7 days, more preferably about 6 days. For particle gun transformation, the period of time during which the treated plant material is placed into contact with a culture medium without selection agent is 1 to 14 days, preferably 1-5 days, more preferably about 2 days. "Without selection agent" is intended to mean "without an

effective amount of the selection agent". During this period a low, i.e. ineffective amount of selective agent may be present.

The step of placing the treated plant material into
5 contact with a culture medium without selection agent was found to be important for the transformation efficiency. In addition it is preferred for chloroplast transformation to keep the treated plant material in the dark during this step. This way no new and thus not transformed chloroplasts are
10 produced thus leading to a higher efficiency.

The treated plant material is preferably kept into contact with a culture medium with the selection agent until regeneration of the plant or plant part from the transformed material.

15 The method of the invention is suitable for plant materials selected from plant tissue, separate cells, protoplasts, separate plastids.

It was surprisingly found that the transformation efficiency can be increased when the culture medium
20 comprising the selection agent is a liquid medium. This way the cells to be transformed are in close contact with the selective agent. It was furthermore surprisingly found that no escapes were detected in the transformation experiments.

When the culture medium is refreshed after the
25 selection procedure this may mean that fresh medium with selective agent is added (i.e. so that the selection medium is not diluted) or that the selection medium is changed for medium with selective agent.

The invention further relates to an expression vector
30 for the transformation of plastid genomes of plant species, in particular *Asteraceae* plant species, which vector comprises:

- an expression cassette which comprises optionally a promoter active in the plastids of the plant species to be transformed, a DNA insertion site for receiving the transforming DNA of interest, optionally one or more selection markers conferring a selectable phenotype on cells having plastids that are transformed with the expression cassette, and optionally a DNA sequence encoding a transcription termination region active in the plastids of the plant species to be transformed,
- 10 - optionally a set of DNA targeting segments located on either side of the expression cassette that allow double homologous recombination of the expression cassette with the plastid genome of interest, and
- optionally a DNA sequence of interest inserted into
- 15 the insertion site of the expression cassette.

In a preferred embodiment, the vector comprises the promoter, the one or more selection markers and the set of DNA targeting segments. Such vector comprises:

- an expression cassette which comprises a promoter
- 20 active in the plastids of the plant species to be transformed, a DNA insertion site for receiving the transforming DNA of interest, one or more selection markers conferring a selectable phenotype on cells having plastids that are transformed with the expression cassette, and
- 25 optionally a DNA sequence transcription termination region active in the plastids of the plant species to be transformed, and
- a set of DNA targeting segments located on either side of the expression cassette that allow double homologous
- 30 recombination of the expression cassette with the plastid genome of interest.

The various elements of the vector are preferably as described above for the method. The invention relates both to

the vector in which no gene to be transformed is incorporated as well as to the vector comprising any transformable gene.

The vectors of the invention provide stable transformation of plastids of multicellular structures, such as plants of lettuce.

The invention further relates to plants carrying in their cells plastids that are transformed, in particular to plants carrying plastids transformed by means of the method of the invention. In addition, the invention relates to progeny of these plants in which at least part of the transformed plastids are still present.

The invention will be further illustrated in the Examples that follows. In these examples, as explant material, lettuce plant mesophyll protoplasts are used and via PEG transformation transplastomic protoplast-derived colonies and regeneration of plants were obtained. Alternatively, transplastomic callus was obtained using particle bombardment of excised cotyledons of lettuce. The DNA constructs comprise an expression cassette containing the transforming DNA which is targeted to a pre-determined location in the plastid genome and inserted into the plastid genome by homologous recombination. The targeting segments in the cassette comprise preferred sequences of the lettuce DNA chloroplast genome, i.e. the *trnI(oriA)/trnA* region or the 16S/*trnV*/ORF70B region of the lettuce chloroplast genome. The DNA used for transformation further contains a non-lethal selectable marker gene which confers a selectable phenotype on cells having the plastids with the transforming DNA, in this case spectinomycin. The non-lethal selectable coding sequence preferred, is the coding region of *aadA* from *E. coli*, which encodes aminoglycoside-3'-adenylyltransferase to confer spectinomycin and streptomycin resistance. Furthermore, the DNA expression cassette comprises at least one additional DNA

sequence, which is the DNA sequence of interest, such as a gene encoding a green fluorescent protein (gfp) (as a model system) or the influenza virus haemagglutinin gene (HA). The constructs furthermore are provided with a promoter and a terminator sequence functional in plant plastids.

In the Examples that follow reference is made to the following figures:

Figure 1. LCV1 lettuce chloroplast genome target sequence (not including backbone vector) (**SEQ ID NO:1**).

10 **Figure 2.** Map of LCV1 (7,545 bp).

Figure 3. LCV1 lettuce chloroplast genome target sequence (**SEQ ID NO:2**) aligned with tobacco chloroplast genome (GI Z00044) (**SEQ ID NO:3**). **SEQ ID NO:4** and **5** are the hypothetical proteins. **SEQ ID NO:41** is the ribosomal protein.

15 **Figure 4.** Cloning steps and primers (**SEQ ID NOS:6-9**) for construction of LCV1. TCG = tobacco chloroplast genome.

Figure 5. LCV2 lettuce chloroplast genome target sequence (not including backbone vector) (**SEQ ID NO:10**).

Figure 6. Map of LCV2 (6,182 bp).

20 **Figure 7.** LCV2 lettuce chloroplast genome target sequence (**SEQ ID NO:11**) aligned with tobacco chloroplast genome (GI Z00044) (**SEQ ID NO:12**).

Figure 8. Cloning steps and primers (**SEQ ID NOS:13-16**) for construction of LCV2. TCG= tobacco chloroplast genome.

Figure 9. Map of LCV1 MSK18 (9,682 bp).

Figure 10. Map of LCV2-MSK18 (8,329 bp).

Figure 11. Diploid Transplastomic lettuce pLCV2-LEC1 plants at stages of flowering (left upper panel), microspores (right upper panel) and seed set (right upper and lower panel)

Figure 12. Primer combinations (**SEQ ID NOS:17-20**) used in PCR analysis of transplastomic lettuce callus.

Figure 13. Molecular analysis of spectinomycin resistant lettuce calli.

Panel A: PCR products of the ATPase gene.

Lane 1. Marker,

- 5 2. TRSL5-01016 pLCV2-MSK18-1,
 3. TRSL5-01016 pLCV2-MSK18-1
 4. TRSL5-02002 pLCV2-MSK18-1-1,
 5. TRSL5-02002 pLCV2-MSK18-1-2,
 6. TRSL5-02002 pLCV2-MSK18-2-1,
10 7. TRSL5-02002 pLCV2-MSK18-2-1,
 8. TRSL5-02002 pLCV2-MSK18-2-2,
 9 and 10 untransformed callus,
 11 and 12 pLCV2-MSK18

Panel B: PCR products of the AadA gene.

- 15 Lane 1. Marker,
 2. TRSL5-01016 pLCV2-MSK18-1,
 3. TRSL5-01016 pLCV2-MSK18-1
 4. TRSL5-02002 pLCV2-MSK18-1-1,
 5. TRSL5-02002 pLCV2-MSK18-1-2,
20 6. TRSL5-02002 pLCV2-MSK18-2-1,
 7. TRSL5-02002 pLCV2-MSK18-2-1,
 8. TRSL5-02002 pLCV2-MSK18-2-2,
 9 and 10 untransformed callus,
 11 and 12 pLCV2-MSK18

25 Panel C: PCR products of the trnI junction.

Lane 1. Marker,

2. TRSL5-01016 pLCV2-MSK18-1,
 3. TRSL5-01016 pLCV2-MSK18-1
 4. TRSL5-02002 pLCV2-MSK18-1-1,
30 5. TRSL5-02002 pLCV2-MSK18-1-2,
 6. TRSL5-02002 pLCV2-MSK18-2-1,
 7. TRSL5-02002 pLCV2-MSK18-2-1,
 8. TRSL5-02002 pLCV2-MSK18-2-2,

9 untransformed callus

Panel D: PCR products of the trnA junction.

Lane 1. Marker,

2. TRSL5-01016 pLCV2-MSK18-1,

5 3. TRSL5-01016 pLCV2-MSK18-1

4. TRSL5-02002 pLCV2-MSK18-1-1,

5. TRSL5-02002 pLCV2-MSK18-1-2,

6. TRSL5-02002 pLCV2-MSK18-2-1,

7. TRSL5-02002 pLCV2-MSK18-2-1,

10 8. TRSL5-02002 pLCV2-MSK18-2-2,

9 untransformed callus

Figure 14. Sequence of left border (P1-P2) (**SEQ ID NO:21**) and right border (P3-P6) (**SEQ ID NO:22**) integration junction fragments amplified by PCR from transplastomic lettuce DNA. Sequence in lower case is lettuce chloroplast DNA external to the LCV2 vector target region. Upper panel: P1-P2 left border fragment consensus sequence; Lower panel: P3-P6 left border fragment consensus sequence.

Figure 15. Agarose gel electrophoresis of PCR products from reactions with primer pairs P1+P2, P3+P4 and P1+P4 and template DNA from spectinomycin resistant putative transplastomic callus sample B (TP) and non-transformed wild-type callus (WT).

Figure 16. PCR analysis on insert integration of pLCV2-MSK18 transformed calli. Lane 1: marker DNA, lanes 2-7: TRSL05-02002 pLCV2-MSK18-1-1, TRSL05-02002 pLCV2-MSK18-1-2, TRSL05-02002 pLCV2-MSK18-1-3, TRSL05-02002 pLCV2-MSK18-2-1, TRSL05-02002 pLCV2-MSK18-2-2, TRSL05-02001 pLCV2-MSK18-1-1, respectively; lane 8 and 9: control lettuce DNA, lane 10: plasmid DNA pLCV2-MSK18

Figure 17. PCR analysis of left and right border integration junction from callus, derived after particle bombardment transformation with plasmid pLCV2-MSK18. Panel A:

trnI junction (left integration junction). Panel B: *trnA* junction (right border insertion. Lane 1: lambda marker, lane 2: spectinomycin resistant callus pLCV2-MSK18, lane 3; control lettuce, lane 4: plasmid pLCV2-MSK18.

5 **Figure 18.** PCR analysis of pLCV2-LEC1 callus lines and controls.

A1: PCR products of the ATPase gene.

Lane 1; marker

2: pLCV2-LEC1 1.1

10 3: pLCV2-LEC1 2.1

4: pLCV2-LEC1 2.2

5: pLCV2-LEC1 3.1

6: pLCV2-LEC1 3.2

7: control non-treated lettuce callus

15 8: control untransformed callus

A2: PCR products of ATPase gene

Lane 1: marker

2: plasmid pLCV2-LEC1

3: water

20 B1: PCR products of the *AadA* gene.

Lane 1; marker

2: pLCV2-LEC1 1.1

3: pLCV2-LEC1 2.1

4: pLCV2-LEC1 2.2

25 5: pLCV2-LEC1 3.1

6: pLCV2-LEC1 3.2

B2: PCR products of *AadA* gene

Lane 1: marker

2: plasmid pLCV2-LEC1

30 3: water

C: PCR products of *trnI* junction (left border)

Lane 1; marker

2: pLCV2-LEC1 1.1

- 3: pLCV2-LEC1 2.1
 4: pLCV2-LEC1 2.2
 5: pLCV2-LEC1 3.1
 6: pLCV2-LEC1 3.2
 5 7: control non-treated lettuce DNA
 8: plasmid pLCV2-LEC1
 D: PCR products of *trnA* junction (right border)
 Lane 1; marker
 2: pLCV2-LEC1 1.1
 10 3: pLCV2-LEC1 2.1
 4: pLCV2-LEC1 2.2
 5: pLCV2-LEC1 3.1
 6: pLCV2-LEC1 3.2
 7: control non-treated lettuce DNA
 15 8: plasmid pLCV2-LEC1
 E: PCR products of insert
 Lane 1; marker
 2: pLCV2-LEC1 1.1
 3: pLCV2-LEC1 2.1
 20 4: pLCV2-LEC1 2.2
 5: pLCV2-LEC1 3.1
 6: pLCV2-LEC1 3.2
 7: control non-treated lettuce callus
 8: control untransformed callus
 25 **Figure 19.** PCR analysis on insert integration in 24
 different transplastomic regenerants, originated from 1
 transplastomic callus TRSL05-02002 pLCV2-MSK18 1-2 (Lanes A-L
 and M-X) and 2 control lettuce plants (control lettuce)
 30 **Figure 20.** PCR analysis on insert integration in 7
 different transplastomic regenerants, originated from 1
 transplastomic callus number pLCV2-LEC1 2.2. Lane 1: marker,
 lanes 2-8: pLCV2-LEC1 2.2 regenerated plants, lane 9: plasmid
 DNA pLCV2-LEC1, lane 10: control lettuce DNA.

Figure 21. Lettuce expression cassette LEC1. LPrrn - lettuce specific RNA operon promoter; L3' *psbA* - lettuce specific *psbA* terminator sequence.

Figure 22. Schematic representation of the PCR and cloning strategy used for LEC1 construction together with primer sequences (**SEQ ID NOS:23-30**).

EXAMPLES

EXAMPLE 1

10 Vector constructions

2. Construction of LCV1

The lettuce chloroplast vector LCV1 consists of 4571 bp of lettuce chloroplast genome sequence with a unique 16 bp *PacI/AscI* site added (**Figure 1**), cloned into *SacI/KpnI* restriction sites on the polylinker of a pBluescript SK+ backbone vector (**Figure 2**). The lettuce sequence spans from the *rps7/3'-rps12* intergenic region to the 16SrRNA/*trnI* intergenic region and corresponds to nucleotide positions 100021-104387 in the tobacco chloroplast genome (GI accession number Z00044). An alignment of this lettuce sequence with the tobacco chloroplast genome sequence is given in **Figure 3**. The following description of the construction of LCV1 is outlined in **Figure 4**.

Four primers LCV1A, LCV1B, LCV1C and LCV1D were used to amplify this region in two halves (LCV1A-B and LCV1C-D) and to introduce a unique *PacI/AscI* restriction site in the ORF70B/*trnV* intergenic region at the position corresponding to nt 102367 in the tobacco chloroplast genome sequence. DNA from clone 6 of the *SacI* library of the lettuce chloroplast genome (Jansen and Palmer, Current Genetics 11: 553-564 (1987)) was used as a template for the LCV1 vector. LCV1A and LCV1B amplified a 2575 bp fragment (2551 bp lettuce sequence + 24 bp extension) LCV1A-B spanning from the *rps7/3'-rps12*

intergenic to the ORF70B/*trnV* intergenic region (corresponding to 100021-102367 in the tobacco chloroplast genome). Primer LCV1A contains a *SacI* site and LCV1B contains *PacI*/*AscI* sites so that *SacI* and *PacI*/*AscI* sites are
5 incorporated at the 5' and 3' end, respectively, of the LCV1A-B fragment.

The LCV1 A-B fragment was cloned into the *E.coli* plasmid vector PCR2.1 to create PCR2.1 LCV1A-B. These clones were screened for orientation using *SacI* and *SacI*+*XbaI*. The
10 *SacI*/*XbaI* insert was cloned into the polylinker of pBluescript to create pBSLCV1 A-B.

Primers LCV1C and LCV1D amplified a 2042 bp fragment (2020 bp lettuce sequence + 22 bp extension) LCV1 C-D. The LCV1C primer contains *PacI*/*AscI* sites and the LCV1D primer
15 contains a *KpnI* site so that a *PacI*/*AscI* and a *KpnI* site are added to the 5' and 3' end, respectively, of the LCV1 C-D fragment. The LCV1 C-D fragment was cloned into PCR2.1 to create PCR2.1 LCV1 C-D. For the final cloning step, PCR2.1 LCV1 C-D was restricted with *AscI*+*KpnI* to release a 2031 base
20 pair insert that was ligated to pBS A-B, which was linearised with *AscI*+*KpnI*, creating LCV1.

2. Construction of LCV2

LCV2 consists of a 2253 bp lettuce chloroplast genome
25 sequence (**Figure 5**) spanning from the 16S rRNA/*trnI* intergenic region to the *trnA*/23S rRNA intergenic region, cloned into the PCR2.1 (Invitrogen) backbone vector (**Figure 6**). This sequence corresponds to nucleotide positions 104366-106260 in the tobacco chloroplast genome (GI accession number
30 Z00044). An alignment of this lettuce sequence with the tobacco chloroplast genome sequence is given in **Figure 7**. The following description of the construction of LCV2 is outlined in **Figure 8**.

Four primers LCV2A, LCV2B, LCV2C and LCV2D were used to amplify this region in two halves (LCV2A-B and LCV2C-D) and to introduce unique *PacI*/*AscI* restriction sites in the intergenic region between the *trnI* and *trnA* genes at the
5 position corresponding to nucleotide 105370 in the tobacco chloroplast genome.

For the first half (A-B) of the vector, DNA from clone 6 of the *SacI* library of the lettuce chloroplast genome (Jansen and Palmer, Current Genetics 11: 553-564 (1987)) was
10 used as a template. Primers LCV2A and LCV2B amplified a 1258 bp fragment (1242 bp lettuce sequence + 16 bp extension) (LCV2A-B) spanning from 16SrRNA/*trnI* intergenic region to the *trnI*/*trnA* intergenic region. This fragment was cloned into the *E. coli* plasmid cloning vector PCR2.1 (Invitrogen) to
15 create PCR2.1 LCV2A-B. Primer LCV2B contains *PacI*/*AscI* sites so that the LCV2A-B fragment has *PacI*/*AscI* sites at the 3' end. PCR2.1 LCV2 A-B clones were screened for orientation by digestion with *KpnI*/*AscI*, which releases a fragment of approximately 1300 bp, and *XbaI*/*AscI* which linearised clones
20 with the correct orientation for subsequent cloning.

For the second half of the vector chloroplast DNA from lettuce cultivar Evola (Leen de Moss seeds) was used as a template because the entire *trnA* gene was not contained in a single clone in the lettuce chloroplast genome library.
25 Primers LCV2C and LCV2D amplified a 1011 bp fragment (995 bp lettuce sequence + 16 bp extension) LCV2C-D. This sequence spans from the *trnI*/*trnA* intergenic region to the *trnA*/23S rRNA intergenic region. Primer LCV2C contains *PacI*/*AscI* sites so the fragment LCV2C-D has *PacI*/*AscI* sites at its 5' end.
30 This fragment was cloned into PCR2.1 to create PCR2.1 LCV2 C-D. These clones were screened for orientation using *KpnI*+*AscI*, which linearises clones with required orientation and *XbaI*+*AscI*, which releases a fragment of approximately

1000 bp in clones with the required orientation. To generate LCV2, the 1.3 kb *Asc*I+*Xba*I insert from PCR2.1 LCV2C-D was subcloned into PCR2.1 LCV2A-B linearised with *Asc*I+*Xba*I.

5 3. Construction of LCV1-MSK18 and LCV2-MSK18

MSK18 is an expression cassette adapted from pMSK18 (Hibberd et al., The Plant Journal 16, 627-632 (1998)). Plasmid MSK18 was a gift from John Gray (Dept. Plant Sciences, University of Cambridge, Downing Street, Cambridge
10 CB2 3EA, UK). Full details of the construction of pMSK18 have been described previously (Hibberd et al. 1998, *supra*). The MSK18 expression cassette consists of the mGFP coding region (Haselhoff et al., Trends in Genetics 11, 328-329 (1997)) fused to a bacterial *trc* promoter (Amman and Brosius, Gene
15 40, 183-190 (1985)), and an *aadA* coding region, derived from pUC-atpX-AAD (Goldschmidt-Clermont, Nucleic Acids Research 19, 4083-4089 (1991)) fused to a tobacco *rrn* promoter derived from pZS197 (Svab and Maliga, Proc. Natl. Acad. Sci USA 90, 913-917 (1993)). A tobacco *psbA* 3' UTR derived from pSZ197
20 (Svab and Maliga, 1993 *supra*) is fused to the 3' end of the *aadA* gene (**Figure 9**).

Using pMSK18 as a template, *Pac*I and *Asc*I sites were added by PCR amplifying the cassette with primers containing *Pac*I (5') and *Asc*I (3') restriction sites to 5' and 3' ends
25 of the of the MSK18 expression cassette. The primers used for this were MSK18 A (Forward)
5'-tagttaattaaTTGACAATTAATCATCCGGCTCGT-3' (**SEQ ID NO:31**) and MSK18 B (Reverse) 5'-tagggcgcgccTCGAATATAGCTCTTCTTTCTTA-3' (**SEQ ID NO:32**). The MSK18 A-B PCR product was cloned into
30 PCR2.1 to create PCR2.1 MSK18. PCR2.1 MSK18 was restricted with *Pac*I/*Asc*I to release the MSK18 insert that was cloned into the *Pac*I/*Asc*I sites in LCV1 and LCV2 to create LCV1-MSK18 (**Figure 9**) and LCV2-MSK18 (**Figure 10**).

EXAMPLE 2Construction of LCV2-LEC1

Lettuce expression cassette 1 (LEC1; **Figure 21**) contains the *aadA* gene, which confers spectinomycin and streptomycin resistance in plants, and the influenza virus haemagglutinin gene (HA) that codes for a potential influenza sub-unit vaccine. Both genes are under the control of a single lettuce specific promoter (*Prrn*) and terminator sequence (3' *psbA*). A chloroplast ribosome-binding site also precedes both genes. The expression cassette was assembled in three pieces using a combination of PCR amplification and overlap extension (**Figure 22**).

The lettuce chloroplast specific ribosomal RNA operon promoter (*Prrn*) was amplified from lettuce chloroplast DNA (SacI fragment 6 from the Jansen cpDNA library; Jansen and Palmer, (Current Genetics 11: 553-564 (1987)) using PCR primers A and B. The *aadA* gene and upstream ribosome-binding site (rbs) was amplified from the tobacco chloroplast transformation vector pZS197 using PCR primers C and D. The HA gene and upstream rbs was amplified from an in-house HA gene construct (HA con3) using PCR primers E and F. A lettuce specific *psbA* termination sequence (3' *psbA*) was amplified from lettuce chloroplast DNA (cv. Evola) using PCR primers G and H. PCR products A+B and C+D were fused by overlap extension using PCR primers A and D.

The resulting PCR product A+D was cloned into the SacI/NotI sites of pBS SK+ to create pBS A+D. PCR product E+F was cloned into the NotI/BamHI sites of pBS SK+ to create pBS E+F. PCR product G+H was cloned into the BamHI/PstI sites of pBS E+F to create pBS E+H. The complete insert (E+H) was excised by restriction with NotI/PstI and cloned into the NotI/PstI sites on pBS A+D to create pBS SK+ LEC1.

Expression of *aadA* and HA in pBS SK+ LEC1 was tested in *E.coli*. Transformed *E.coli* cells were resistant to streptomycin indicating that the *aadA* gene was expressed. Western analysis of HA expression with anti-HA sera showed
5 expression of HA in *E.coli*. The entire expression cassette (*Prrn/aadA/HA/psbA*) was excised from pBS SK+ LEC1 using the restriction enzymes PacI and AscI and cloned into the PacI/AscI sites on the lettuce chloroplast transformation vector LCV2 to create LCV2-LEC1.

10

EXAMPLE 3Obtaining seedlings and an *in vitro* stock of plants

Protoplasts of plants are isolated from leaf material of donor plants. In this example the obtaining of leaf shoot
15 cultures is given.

Seeds are sterilized by subsequent washing in 70% ethanol, 0.7% NaOCl solution during 20 minutes and three times washing with sterile demineralized water. Seeds are sown on Murashige and Skoog (Murashige and Skoog, *Physiol.*
20 *Plant.*, 15: 473-497 (1962)) medium with saccharose 2%, without hormones. Preferably, seeds can be cultured at 15°C for 2 days in the dark, after which the seeds are transferred to 25°C in the light (approximately 3000 lux, photo period 16
hr light/8 hr dark TL FTD 840). When first true leaves
25 appear, shoot tips are transferred to Murashige and Skoog based medium with 3% saccharose, without hormones. These sterile shoot cultures are grown under similar growth conditions.

30

EXAMPLE 4Isolation of protoplasts

Three week old shoot cultures are used for isolation of protoplasts. Leaves are cut into small pieces and
5 preplasmolysed during 1 hr in the dark in PG solution (54.66 g/l sorbitol and 7.35 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$). The PG solution is then replaced by an enzyme solution with 1% cellulase and 0.25% macerozym. Incubation takes place during 16 hrs in the dark at 25°C.

10 Subsequently, the suspension is filtered through a nylon mesh filter (41µm) and washed with a third of a volume of CPW16S solution (Frearson et al., Developmental Biology 33:130-137 (1973)) by centrifugation at 700 rpm during 8 minutes. In this way, intact protoplasts are collected on the
15 surface of the supernatant. Protoplasts are washed in W5 solution (9 g/l NaCl, 18.38 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.37 g/l KCl, 0.99 g/l glucose, 0.1 g/l Morpholinoethanesulfonide buffer (MES)) by centrifugation at 600 rpm during 5 minutes. With the procedure described, a protoplast yield of approximately
20 $10\text{--}15 \times 10^6$ protoplasts per gram leaf material can be obtained.

EXAMPLE 5Selection of protoplast derived calli on spectinomycin25 resistance

Protoplasts of lettuce, derived as described in example 4, are diluted in culture medium $\frac{1}{2}$ B5 (Gamborg et al. Exp. Cell Res. 50:151 (1968)): 375 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 18.35 mg/l NaFeEDTA, 270 mg/l sodium succinate, 103 g/l saccharose,
30 0.1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D), 0.3 mg/l 6-benzylaminopurin (BAP) and 0.1 g/l MES and set to a culture density of 6×10^4 protoplasts per ml.

The protoplast suspension is mixed 1:1 with $\frac{1}{2}$ B5 culture medium with agarose. The agarose beads are plated in

larger petri dishes with liquid $\frac{1}{2}$ B5 culture medium on top of it.

The petri dishes are taped with parafilm and cultured at 25°C in the dark. One week after initiation of culture the culture medium is diluted with fresh liquid $\frac{1}{2}$ B5 culture medium and 0.1 g/l MES. The cultures are transferred to the light (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840).

When calli are about 0.5 mm in size they are transferred to callus growth medium SH2 (Schenk & Hildebrandt, Can. J. Bot. 50:199-204 (1972)) with 30 g/l saccharose, 5 g/l agarose, 0.1 mg/l 1-naphtalene acetic acid (NAA) and 0.1 mg/l benzylaminopurin (BAP), and the selecting agent spectinomycin dihydrochloride at concentrations of 0-1000 mg/l. It was found that the optimal concentration of selection is 500 mg/l. The non-resistant calli appear as white calli. They also grow slower as compared to the control calli. The culture conditions are as described above for the above protoplast calli.

20

EXAMPLE 6

Transformation of protoplasts with polyethylene glycol and selection for *aadA* encoded antibiotic resistance

Protoplasts of lettuce, derived as described in example 4, are set to a density of approximately $1-1.5 \times 10^6$ protoplasts/0.4-0.6 ml in transformation buffer (0.4 M mannitol, 15 mM $MgCl_2$, 1% (w/v) MES, pH 5.8). Subsequently, 10 μ l of plasmid suspension (1 μ g DNA/ μ l sterile H_2O) is added to the protoplasts as well as 0.4-0.6 ml PEG solution (40% w/v PEG 6000, 2.36 g/l $Ca(NO_3)_2 \cdot 4H_2O$ and 7.28 g/100ml mannitol). Incubation is performed at room temperature for 5-30 minutes. Protoplasts are washed and resuspended in culture medium $\frac{1}{2}$ B5 (Gamborg et al., Exp. Cell Res. 50:151 (1968)): 375 mg/l $CaCl_2 \cdot 2H_2O$, 18.35 mg/l NaFeEDTA, 270 mg/l sodium

succinate, 103 g/l saccharose, 0.1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) and 0.3 mg/l 6-benzyl aminopurin (BAP).

The protoplast suspension is mixed 1:1 with $\frac{1}{2}$ B5
5 culture medium with agarose. The agarose beads are plated in larger petri dishes with liquid $\frac{1}{2}$ B5 culture medium on top of it.

The petri dishes are taped with parafilm and cultured at 25°C. After 6 days selection of the microcalli is
10 performed by adding 500 mg/l of the selective agent spectinomycin dihydrochloride (final concentration). One week after initiation of culture the culture medium is diluted with fresh liquid $\frac{1}{2}$ B5 culture medium, with addition of spectinomycin dihydrochloride and cultured in the light
15 (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840).

When calli are about 0.5 mm in size they are transferred to callus growth medium SH2 (Schenk & Hildebrandt, 1972, supra) with 30 g/l saccharose, 5 g/l
20 agarose, 0.1 mg/l 1-naphtalene acetic acid (NAA) and 0.1 mg/l benzylaminopurin (BAP), and the selecting agent spectinomycin dihydrochloride at concentrations described above. Culture conditions are as described above.

After 2 weeks calli are transferred to regeneration
25 medium SHreg (Schenk and Hildebrandt, 1972, supra) with 15 g/l saccharose, 15 g/l maltose, 0.1 mg/l NAA and 0.1 mg/l BAP and spectinomycin dihydrochloride in concentrations described above. Spectinomycin resistant calli appear as green calli amongst white (non-resistant) calli.

30 Regenerating plants appear after approximately 6 weeks and furtheron, and are transferred to rooting medium (Schenk and Hildebrandt, supra) with 30 g/l saccharose and 8 g/l agar with the concentrations of spectinomycin dihydrochloride mentioned above. Alternatively, in

transformation vectors where gfp (green fluorescent protein) is added as the gene of interest, gfp fluorescence is detected using an inverted microscope with the proper filter combinations. Green calli were detected 4-5 weeks after
 5 initiation of each experiment.

Table 1 gives an overview of the results obtained in protoplast transformation experiments with three different plasmids. Spectinomycin resistant calli were obtained after transformation of protoplasts with the plasmids PLCV2-MSK18
 10 and PLCV2-LEC1. Approximately 40-50% of the protoplasts did survive the PEG treatment. Callus lines of each individual event are maintained on medium SHreg with the selective agent spectinomycin dihydrochloride and yielded regenerated plants from plasmids pLCV2-MSK18 and pLCV2-LEC1 (**Table 1**). Also,
 15 ploidy differences were observed between individual calli.

Table 1. Selection of plastid transformants

	Treatment/ Experiment	# pps treated	# green calli	# regenerating calli
20	control	none	0	
	control + PEG	1,26 x 10 ⁶	0	
	pLCV1-MSK18	1,26 x 10 ⁶	0	
	pLCV2-MSK18/exp 1	1,26 x 10 ⁶	1	0
	pLCV2-MSK18/exp 2	2,40 x 10 ⁶	1	0
25	pLCV2-MSK18/exp 3	4,80 x 10 ⁶	5	2 (1 ++, 1+/-)
	pLCV2-LEC1/exp 1	3,60 x 10 ⁶	5	3 (1 ++, 2 +/-)

The transgenic callus has been obtained using vectors with specific lettuce chloroplast DNA homologous sequences.
 30 Selection of transformed cells with the non-lethal selective agent spectinomycin has been successful. The optimal transformation frequency for lettuce, determined as the

number of green calli to the number of surviving protoplasts is about 1 in $3-6 \cdot 10^5$ protoplasts (Table 1).

The plants obtained from transformation experiments with pLCV2-LEC1 were found to have a normal, diploid ploidy level and showed a normal growth. Seed-set after selfing was obtained from these plants (**Figure 11**).

EXAMPLE 7

Transformation of protoplasts via electroporation and selection on *aadA* encoded antibiotic resistance

Protoplasts, derived as described in example 4, are suspended in transformation buffer HBS (150 mM KCl, 4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 10 mM HEPES (pH 7.2)), and enough mannitol to osmotically balance the protoplasts. This is dependent on the genotype but it can easily be found out experimentally. Aliquots of 1×10^6 protoplasts/0.5 ml HBS buffer and mannitol are put into a conical centrifuge tube, and plasmid DNA solution is added. Plasmid DNA concentrations in the transformation buffer should preferably be in the range of 10-100 $\mu\text{g/ml}$. The protoplast-DNA suspension is transferred to the electroporation chamber and electroporated using a single electric pulse (e.g. 325 μF , 300 V) The optimal setting can vary with species and cell type, and should be determined in preliminary experiments. The most efficient parameters are set by finding the pulse settings that result in 50% protoplast death by 24h after the shocks. More details of the method are described by G.W. Bates (Plant transformation via protoplast electroporation. From: Methods in Molecular Biology Vol 111: Plant cell Culture Protocols, Pp 359-366 (1999)).

After electroporation, protoplasts culture and selection is performed as described in example 6.

EXAMPLE 8Adjustment of spectinomycin threshold levels in cotyledons

For the adjustment of the optimal concentration of spectinomycin, for selection of cells with chloroplasts/
5 plastids, which are transformed with constructs having the *aadA* gene as selectable marker, 4-10 day old cotyledons were plated with the abaxial side onto MS medium (Murashige and Skoog, supra) with 0.8% agar, 30 g/l saccharose, 100-200 mg/l carbenicillin, 0.1 mg/l benzylaminopurin (BAP), 0.1 mg/l 1-
10 naphthalene acetic acid (1-NAA) at pH 5.8, and with various concentrations of spectinomycin dihydrochloride. The cotyledons were obtained as described in Example 3, and cultured at 25°C in the light (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840). It was found that a
15 concentration of 0.5-1 g/l spectinomycin dihydrochloride was sufficient for efficient selection, leading to complete bleaching and loss of growth and regeneration of control cotyledons.

20 EXAMPLE 9Transformation of plant material via biolistics and selection for *aadA* encoded antibiotic resistance

For bombardment of cotyledons, seeds were sown as described in example 3. Alternatively, leaf pieces can be
25 used as explant material for shooting, under similar conditions. Cotyledons (3 to 12 days old) or leaf pieces from 10-14 days old seedlings are placed with the abaxial side onto MS medium (Murashige and Skoog, supra) with 0.8% agar, 0.3 mg/l BAP and 0.1 mg/l 2,4-D (pH 5.8) and preincubated for
30 1-6 days before transformation with a particle gun.

The cotyledons are cultured at 25°C in the light (approx. 3000 lux, photo period 16 hours light/8 hours dark, TL FTD 840).

Gold particles (0.6 to 1.6 μm) were prepared for transformation by mixing 50 μl of suspension (60 mg/ml 50% glycerol) with 5 μg DNA (1 $\mu\text{g}/\mu\text{l}$ H_2O), 50 μl CaCl_2 (2.5 M) and 20 μl spermidine (0.1 M base). The particle-DNA mixture was
5 incubated at room temperature for 1-3 minutes and centrifuged for 3-10 sec. in an Eppendorf centrifuge. After removal of the supernatant, the coated particles are washed and diluted in 48-60 μl ethanol. The particles (6-8 μl per carrier) are applied to the macrocarrier holders and the bombardment is
10 performed with PDS-1000/He Biolistic particle delivery system (BioRad).

The explants are placed at approximately 6 cm target distance and bombarded using a 1100 p.s.i rupture disc. Details of the procedure has been described by Klein et al.
15 (Bio/Technology 6: 559-563 (1988)).

Two to fourteen days after bombardment, the cotyledons are transferred to MS1 liquid medium (Murashige and Skoog, supra) with 30 g/l saccharose and supplemented with 100-200 mg/l carbenicillin, 0.1 mg/l benzylaminopurin
20 (BAP) and 0.1 mg/l 1-naphtalene acetic acid (1-NAA) at pH 5.8 as described above with the addition of a selective agent (e.g. spectinomycin dihydrochloride at concentration of 500 mg/l). They are incubated in liquid medium at 25°C in the light (approx. 3000 lux, photo period 16 hours light/8 hours
25 dark, TL FTD 840) for about 1-8 days, after which they are transferred to solid MS1 medium (see above with the addition of 8 g/l agar). Cultures are transferred onto fresh medium every 2 weeks.

When green callus or shoots appear, they are
30 transferred to medium MS1 without carbenicillin, but including the selective agent spectinomycin dihydrochloride.

Table 2 presents results from transformation experiments with pLCV2-MSK18. It was found that green,

spectinomycin resistant callus was formed on bombarded cotyledons, approximately 2.5 months after initiation of the experiment. The spectinomycin resistant callus was maintained on MS1 medium with the selective agent.

5

Table 2. Results of particle bombardment experiments with pLCV2-MSK18 using cotyledons or leaf pieces.

10	Explant type/treatment	Number of bombarded explants	Number of explants with spectinomycin resistant callus
	Cotyledon, bombarded selection	180	1
	Cotyledon control selection	30	0
15	Leaf bombarded selection	96	0
	Leaf control selection	16	0

20 **EXAMPLE 10**

Molecular analysis of spectinomycin resistant calli of lettuce

Spectinomycin resistance of plant cells may be the result, apart from transformation with the vector LCV2-MSK18, of spontaneous mutation of chloroplast DNA or insertion of the DNA into the nuclear genome. Therefore, the callus and regenerated plants were screened for the integration of the right and left homologous border segment as is described in this Example. Additionally, it was determined whether the *aadA* gene, the *gfp* and *HA* gene were correctly integrated in the chloroplast DNA.

1. *Analysis of calli derived from PEG protoplast transformations with pLCV2-MSK18*

Spectinomycin resistant callus of lettuce was analysed by PCR using different primer combinations to confirm the integration of the plasmid pLCV2-MSK18 in the genome of the chloroplast.

As an endogenous control for chloroplast DNA amplification, PCR analysis of the ATPase gene (Accession: AF162208) was carried out using the forward primer 5'-ACTAATAGTGGACAAATTGGC-3' (SEQ ID NO:33) and the reverse primer 5'-TTGCTTGATTGTATTTACTCG-3' (SEQ ID NO:34). To detect the presence of the selectable marker gene *AadA*, the following primer combination was used: forward 5'-AAGTCACCATTGTTGTGCACG-3' (SEQ ID NO:35) and reverse 5'-TATGACGGGCTGATACTGGGC-3' (SEQ ID NO:36). In order to demonstrate the physical integration of the plasmid into the chloroplast genome 2 primer combinations were developed which amplify hybrid regions of the plasmid and the chloroplast genome (see **Figure 12**). The first primer combination consisting of P1 and P2 amplifies the junction containing the *trnI* sequence of the chloroplast genome (left border integration). The second primer combination consisting of P3 and P4 amplifies the junction containing the *trnA* sequence of the chloroplast genome (right border integration).

Total DNA was isolated from spectinomycin resistant callus using a commercially available DNA isolation kit from Sigma (Genelute Plant Genome DNA Kit). The PCR reaction was carried out using a total amount of 30 ng DNA after which the reaction products were analysed on a 1% agarose gel.

The result of the analysis of 5 independent spectinomycin resistant calli derived from PEG protoplast transformations is shown in **Figure 13** (data of 2 calli not shown but identical to the other 5). The ATPase fragment of

about 424 bp is only present in callus material and leaf material of lettuce, and as expected not visible for the pLCV MSK18 DNA (**Figure 13A**). PCR amplification of the *aadA* gene gave the expected fragment of approximately 413 bp for the
5 transgenic callus and the plasmid pLCV2-MSK18 (**Figure 13B**).

To confirm the integration of the pLCV2-MSK18 vector into the lettuce chloroplast genome, the two primer combinations were used which specifically detect either one of the two junctions which emerge after integration of the
10 plasmid by homologous recombination. The integration on *trnI* junction was investigated using the PCR primers indicated above, which resulted in an expected band of approximately 2000 bp as well (**Figure 13C**). **Figure 13D** shows the amplification of the *trnA* junction which results in an
15 expected band of approx. 1500 bp in the spectinomycin resistant callus. The results of this analysis confirm the transplastomic nature of the obtained spectinomycin resistant pLCV2-MSK18 lettuce calli, and no escapes were found.

For further confirmation of integration, the left and
20 right integration junctions were amplified by PCR using primer pairs P1+P2 and P3+P4. The PCR products from one spectinomycin resistant callus sample were cloned into PCR2.1 and sequenced using M13 forward and M13 reverse primers. These sequences confirmed that LCV2-MSK18 was integrated in
25 the lettuce chloroplast genome (**Figure 14**).

To eliminate the possibility of amplification of unintegrated LCV2-MSK18 plasmid DNA, primers P1 and P4 were designed from lettuce chloroplast sequences external to the vector target region (**Figure 12**). PCR analysis was carried
30 out on DNA isolated from 6 putatively transformed calli. In all cases, P1 and P4 give two PCR products, a 2476 bp band corresponding to the expected size of a product amplified from an untransformed wild-type chloroplast genome, and a 4623 bp band corresponding to the size of a PCR product

expected from a transformed chloroplast genome. **Figure 15** shows the results in detail for one callus, and **Figure 16** shows the PCR results on insert integration for 6 independent calli.

5

2. Molecular analysis of spectinomycin resistant callus, derived after biolistic transformation with pLCV2-MSK18

Similar primer combinations, as used for the spectinomycin resistant callus out of PEG protoplast experiments were used to evaluate the transplastomic nature of the callus derived from bombarded tissue. **Figure 17** shows the products of the *trnI* and *trnA* junction, respectively. It was verified that the callus was of a transplastomic nature.

15 *3. Molecular analysis of putative transplastomic callus, derived from PEG protoplast transformation experiments with pLCV2-LEC1.*

For the analysis of the calli, obtained by protoplast transformation experiments with pLCV2-LEC1, similar primer combinations as for the pLCV2-MSK18 plasmid transformations could be used for the *aadA* gene, the endogenous control and the insert integration P1 + P4 (**See Figure 13**). Furthermore, PCR analysis on left border integration was performed by using the forward primer 5'-ACTGGAAGGTGCGGCTGGAT-3' (**SEQ ID NO:37**) and the reverse primer 5'TATGACGGGCTGATACTGGGC-3' (**SEQ ID NO:38**). Right border integration was performed by using the forward primer 5'-ATGCAAAAACCTCCCGGAAAT-3' (**SEQ ID NO:39**) and reverse primer 5'-CTCGCCCTTAATTTTAAGGC-3' (**SEQ ID NO:40**).

Results of these analyses are shown in **Figure 18**. It is clear that all 5 independent calli are true transplastomic ones, and no escapes were found.

30

4. Molecular analysis of regenerated plants from transplastomic callus, derived from PEG protoplast transformation experiments with pLCV2-MSK18 and LEC1

Figure 19 shows the PCR results from DNA derived from
5 several plants regenerated from one transplastomic pLCV2-MSK18 callus. **Figure 20** shows PCR analysis of pLCV2-LEC1 regenerated plants. It is clear that both types of plants are truly transplastomic.

CLAIMS

1. Method for the transformation of plastid genomes of plant species, in particular Asteraceae plant species,
5 comprising the steps of:

a) providing a transformation vector carrying a DNA sequence of interest;

b) subjecting a plant material, which comprises plastids, to a transformation treatment in order to allow the
10 plastids to receive the transformation vector;

c) placing the thus treated plant material for a period of time into contact with a culture medium without selection agent;

d) subsequently placing the plant material into
15 contact with a culture medium comprising a selection agent;
and

e) refreshing the culture medium comprising a selection agent to allow plant material comprising plastids that have acquired the DNA of interest to grow into
20 transformants.

2. Method as claimed in claim 1, wherein the expression vector comprises:

- an expression cassette which comprises optionally a promoter active in the plant species to be transformed, a DNA
25 insertion site for receiving the transforming DNA of interest, optionally one or more selection markers conferring a selectable phenotype on cells having plastids that are transformed with the expression cassette, and optionally a DNA sequence encoding a transcription termination region
30 active in the plant species to be transformed,

- optionally a set of DNA targeting segments located on either side of the expression cassette that allow double homologous recombination of the expression cassette with the plastid genome of interest, and

- optionally a DNA sequence encoding a gene of interest inserted into the insertion site of the expression cassette.

3. Method as claimed in claim 2, wherein the vector
5 comprises the promoter, the DNA sequence encoding the gene of interest the one or more selection markers and the set of DNA targeting segments.

4. Method as claimed in any one of the claims 1-3,
wherein the transformants carry the DNA of interest in their
10 genome.

5. Method as claimed in any of the claims 1-4,
wherein the plastids to be transformed are selected from the group consisting of chloroplasts, amyloplasts, elaioplasts, etioplasts, chromoplasts, leucoplasts and proplastids.

15 6. Method as claimed in any one of the claims 2-5,
wherein the promoter is selected from the group consisting of the chloroplast specific ribosomal RNA operon promoter rrn (16S rRNA), psbA, rbcL, trnV, or rps16.

7. Method as claimed in any one of the claims 2-6,
20 wherein the DNA of interest is a gene encoding a therapeutic or prophylactic (bio)pharmaceutical (poly)peptide, such as an edible vaccine.

8. Method as claimed in any one of the claims 2-6,
wherein the DNA of interest is selected from the group
25 consisting of genes encoding herbicide resistance, insect resistance, fungal resistance, bacterial resistance, genes that lead to stress tolerance, for instance to cold, high salt or minerals, genes that improve yield, starch accumulation, fatty acid accumulation, photosynthesis.

30 9. Method as claimed in any one of the claims 2-8,
wherein the terminator is selected from the group consisting of the psb A termination sequence, rrn, rbcL, trnV, or rps16.

10. Method as claimed in any one of the claims 1-9,
wherein the selection marker is selected from the group

consisting of genes conferring resistance against spectinomycin, streptomycin, kanamycin, hygromycin and chloramphenicol, glyphosate, bialaphos.

11. Method as claimed in any one of the claims 1-9,
5 wherein the selection marker is a visual markers, such as a fluorescent marker like gfp (green fluorescence protein).

12. Method as claimed in claim 11, wherein the steps
d) and e) of the transformation method are omitted and the
transformants are selected by illuminating the putative
10 transformants with an appropriate light source corresponding
to the visual marker and selecting the plant material that
shows fluorescence.

13. Method as claimed in any one of the claims 2-12,
wherein the DNA segments that allow double homologous
15 recombination of the DNA of interest with the plastid genome
of interest have a DNA sequence that is homologous to a part
of the plastid genome.

14. Method as claimed in claim 13, wherein the set of
DNA segments is selected from the group consisting of the
20 *trnI(oriA)/trnA* region and the 16S/*trnV*/ORF70B region of the
lettuce chloroplast genome.

15. Method as claimed in claim 13, wherein the set of
DNA segments is selected from LCV1 A-B and LCV1 C-D, and LCV2
A-B and LCV2 C-D.

25 16. Method as claimed in any one of the claims 1-15,
wherein the transformation treatment is selected from the
group consisting of electroporation, particle gun
transformation, polyethylene glycol transformation and
whiskers technology.

30 17. Method as claimed in any one of the claims 1-16,
wherein the transformation treatment is polyethylene glycol
transformation and the period of time during which the
treated plant material is placed into contact with a culture

medium without selection agent is 1 to 14 days, preferably 3-7 days, more preferably about 6 days.

18. Method as claimed in any one of the claims 1-16, wherein the transformation treatment is particle gun transformation and the period of time during which the treated plant material is placed into contact with a culture medium without selection agent is 1 to 14 days, preferably 1-5 days, more preferably about 2 days.

19. Method as claimed in any one of the claims 1-18, wherein the plant material to be treated is selected from the group consisting of plant tissue, separate cells, protoplasts, separate plastids.

20. Method as claimed in any one of the claims 1-19, wherein the culture medium comprising the selection agent is a liquid medium.

21. Method as claimed in any one of the claims 1-20, wherein step c) is performed in the dark.

22. Vector for the transformation of plastid genomes of plant species, in particular *Asteraceae* plant species, which vector comprises:

- an expression cassette which comprises optionally a promoter active in the plastids of the plant species to be transformed, a DNA insertion site for receiving the transforming DNA of interest, optionally one or more selection markers conferring a selectable phenotype on cells having plastids that are transformed with the expression cassette, and optionally a DNA sequence encoding a transcription termination region active in the plastids of the plant species to be transformed, and
- optionally a set of DNA targeting segments located on either side of the expression cassette that allow double homologous recombination of the expression cassette with the plastid genome of interest.

23. Vector as claimed in claim 22, wherein the vector comprises the promoter, the one or more selection markers and the set of DNA targeting segments.

24. Vector as claimed in claim 22 or 23, which vector
5 comprises:

- an expression cassette which comprises a promoter active in the plant species to be transformed, a DNA insertion site for receiving the transforming DNA of interest, one or more selection markers conferring a
10 selectable phenotype on cells having plastids that are transformed with the expression cassette, and optionally a terminator active in the plant species to be transformed, and
- a set of DNA targeting segments located on either side of the expression cassette that allow double homologous
15 recombination of the expression cassette with the plastid genome of interest.

25. Vector as claimed in any one of the claims 22-24, further comprising a DNA sequence of interest inserted into the insertion site of the expression cassette.

20 26. Vector as claimed in any one of the claims 22-25 for use in the method as claimed in any one of the claims 1-20.

27. Vector as claimed in any one of the claims 22-26, wherein the promoter is selected from the group consisting of
25 the chloroplast specific ribosomal RNA operon promoter *rrn* (16S rRNA), *psbA*, *rbcL*, *trnV*, or *rps16*.

28. Vector as claimed in any one of the claims 21-26, wherein the DNA of interest is a gene encoding a therapeutic or prophylactic (bio)pharmaceutical (poly)peptide, such as an
30 edible vaccine.

29. Vector as claimed in any one of the claims 22-27, wherein the DNA of interest is selected from the group consisting of genes encoding herbicide resistance, insect resistance, fungal resistance, bacterial resistance, genes

that lead to stress tolerance, for instance to cold, high salt or minerals, genes that improve yield, starch accumulation, fatty acid accumulation, photosynthesis.

30. Vector as claimed in any one of the claims 22-29,
5 wherein the terminator is selected from the group consisting of the *psb A* termination sequence, *rrn*, *rbcL*, *trnV*, or *rps16*.

31. Vector as claimed in any one of the claims 22-30,
wherein the selection marker is selected from the group consisting of genes conferring resistance against
10 spectinomycin, streptomycin, kanamycin, hygromycin and chloramphenicol, glyphosate, bialaphos.

32. Vector as claimed in any one of the claims 22-30,
wherein the selection marker is a visual marker, such as fluorescent markers like *gfp* (green fluorescence protein).

15 33. Vector as claimed in any one of the claims 22-32,
wherein the DNA segments that allow double homologous recombination of the DNA of interest with the plastid genome of interest have a DNA sequence that is homologous to a part of the plastid genome.

20 34. Vector as claimed in claim 33, wherein the set of DNA segments is selected from the group consisting of the *trnI(oriA)/trnA* region and the 16S/*trnV*/ORF70B region of the lettuce chloroplast genome.

35. Vector as claimed in claim 34, wherein the set of
25 DNA segments is selected from LCV1 A-B and LCV1 C-D, and LCV2 A-B and LCV2 C-D.

36. Transplastomic plant or plant part obtainable by the method as claimed in any one of the claims 1-21.

37. Transplastomic plant or plant part as claimed in
30 claim 33 obtained by the method as claimed in any one of the claims 1-21.

38. Transplastomic plant or plant part as claimed in claim 36 or 37, wherein the plant is a lettuce plant.

39. Progeny of a plant or plant part as claimed in any one of the claims 36-38 carrying plastids at least part of which have the gene of interest in their genome.

40. Plant parts as claimed in any one of the claims
5 36-39, which plant parts are selected from the group consisting of tissues, cells, meristems, calli, protoplasts, plastids, proplastids, plastid DNA.

Fig. 1-1

SEQ ID NO:1

GTTCAAGAATCAGTTTTCTTTTTATAAGGGCTAAAATCACTTATTTTGGCTTTTTTACCCCATATTGTAGGGTG
GATCTCGAAAGATATGAAAGATCTCCCTCCAAGCCGTACATACGACTTTCATCGAATACGGCTTTCGCAGAAAT
TCTATATGTATCTATGAGATCGAGTATGGAATTTCTGTTTACTCACTTTAAATTGAGTATCCGTTTCCCTCCTTT
TCCTGCTAGGATTGGAAATCCTGTATTTTACATATCCATACGATTGAGTCCTTGGGTTTCCGAAATAGTGTA
AAGAAGTGCTTCAAATCATTGCTATTTGACTCGGACCTGTTCTAAAAAGTCGAGGTATTTTCAATTGTTTGTG
ACACGGACAAAGTCAGGGAACCTCTGAAATTTTTTCAATATTGAACCTTGACATATAATAGTTCGGAATCG
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TTAGCCATACACTTCACATGTTTCTAGCGATTACATGGCATCATCAAATGATACAAGTCTTGGATAAGAATCT
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ACCGGGTATATAAGCAGTGATTTCAAATCCAGAGGTTAATCGTACTCTGGCACTTTACGTAAGGCAGATTTG
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ATTTTCACCTCATACGGCTCCTCGTTCAATTCTTTCGAAGTTATTGGATCCTTTTCCGCGTTCGAGAATCCCT
CCCTTCTTCCACTCCGTCCCGAAGAGTAAGTACGAGCAATTTAGTCACGTTTTCATGTTCCAATTGAACACTTT
CCGTTTTTGTATTCTCTTTACCAAACATATGCGGATCCAATCACGATCTTATAATAAGAACAAGAGATCTTT
CTCGATCAATCCCTTGGCCCTCATTTCTCGAGAATCAGAAAGATCCTTTTCAAGTTTGAATTTGTTTCAATTTG
AATCTGAGTTCTTCTACTTCATTATTTATTTAATATCAATATTTTTGCCTCTCTTTTTTTTATATTATTCCTTA
AGTCCCATAGGTTTGATCCTTTAGAAATTGGACTCATTTTCTCATTGAGCGAAGGGTACGAAATAAATCAGATTG
ATTAAGCACTATGTGAAATATTCGGTTTTTCTCTCTCTATCCCATAGGTACAGTGTTTGAATCAATCG
AGAACCTTTCTCTGTCTGAATCGATATTATCCATTCCAATTCCTTCCCGATACCTCTCAAGGAAAATCTCG
AATTGGATCCTAAATTGACGGGTAGTGTGAGCTTATCCATGCGGTTATGCACTCTTGAATAGGAATCCATTT
TCTGAAAGATCCTGGCTTTTCGTGCTTTGGTGGGTCTCCGAGATCCTTTTCGATGACCTATGTTGTGTTTGTGAA
GGGATATCTATATAATACGATCGATTGCGTAAAGCCCGCGGTAGCAGTGGAACCGGGGAAAGTATACAGAAAAG
ACAGTTCTTTTCTATTATATATATATAGTCTTTTCTATTTAATTCATATTAGATTAGTCTTAGTTAGTGATC
CCGGCTTAGTGAGTCCCTTTCTTCCGTGATGAACGTGTTGGCGCCAGTCTACATTTTGTCTGTGGACAGAGGA
GAAAGGGGCTCCGCGGGAAGAGGATTGTACCGTGAGAGAAGCAAGGAGGTCAACCTCTTCAAATATACAACA
TGGATTCTGGCAATGCAATGTACTTGGACTCTCATGTGATCCGAATGAATCATCCTTCCACGGAGGCAATC
TTTGCCTGTTAGGTAACAGGATAGCAAGTTACAACTCTGTCTCGGTAGGACATGGATCTCTATTACTATGAAT
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CACGCTGAGCCAAATCTTCTCATGTAAAACCTGCTTGAATTTAGATCGGGAAATCGTGTGGTTTTATGAAACC
ATGTGCTATGGCTCGAATCCGTAGTCAATCCTATTTCCGATAGGGACAGTTGACAACCTGAATCCTATTTTCCCA
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TGCCGCGTGAGGAGTAGAAGGCCACGGGTGATGAACCTCTTTTCCCGGAGAAGAAGCAATGACGGTATCTGGGG
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CGTAAAGCGTCTGTAGGTGGCTTTTAAAGTCCGCCGTCAAATCCAGGGCTCAACTCTGGACAGGCGGTGGAAA
CTACCAAGCTGGAGTACGGTAGGGGCAGAGGGAATTTCCGGTGGAGCGGTGAAATGCGTAGAGATCGGAAAGAA
CACCAACGGCCAAAGCACTCTGCTGGGCCCCACTGACACTGAGAGACGAAAGCTAGGGGAGCGAATGGGATTA

Fig. 1-2

(continued)

GATACCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGCTGTGCGTATCGACCCGTGCAGTGCTGTAGCT
AACGCGTTAAGTATCCCGCCTGGGGAGTACGTTTCGCAAGAATGAACTCAAAGGAATTGACGGGGGCCCCGACA
AGCGGTGGAGCATGTGGTTTAATTCGATGCAAAGCGAAGAACCCTTACCAGGGCTTGACATGCCGCGAATCCTCT
TGAAAGAGAGGGGTGCCTTCGGGAACGCGGACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGCCGTAAAGGTGT
TGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTGTTTAGTTGCCATCATTGAGTTTGGAACCCTGAACAGACTG
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TACAAATGGCCGGGACAAAGGGTCGCGATCCCGCGAGGGTGAGCTAACCCCAAAAACCCGTCTCAGTTCGGATT
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CCCGGGCCTTGTTACACACCGCCCGTCACACTATGGGAGCTGGCCATGCCCGAAGTCGTTACCTTAACCGCAAGG
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GATCACCTCCTTTTCAGGGAGAGCTAATGCTTGTTGGGTATTTTGGTTTGACACTGCTTCACACCCAAAAAGA
AGGGAGCTACGTCTGAGTTAACTTGAGATGGAAGTCTTCATTTCTCGACAGTGAAGTAAGACCAAG

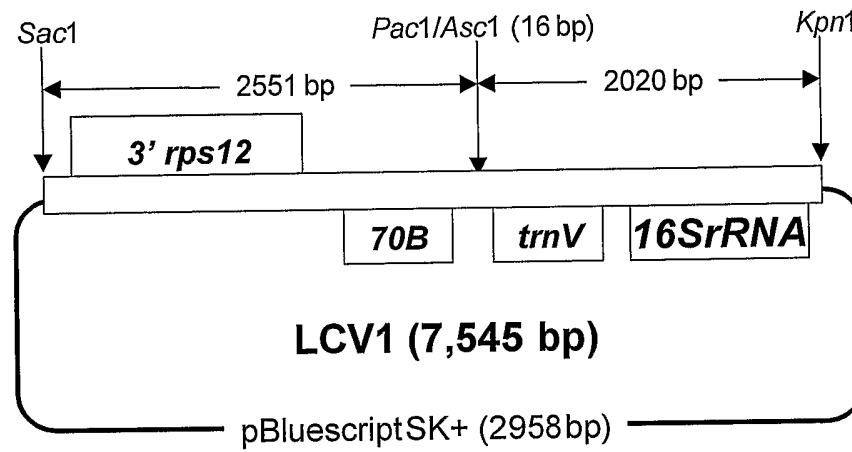


Fig. 2

Fig. 3-1

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LCV1 (SEQ ID NO:2): 1      gttcaagaatcagttttctttttataagggtctaaatcacttattttggctttttaccc 60
|||||
tobac (SEQ ID NO:3): 100021 gttcaagaatcagttttctttttataagggtctaaatcacttattttggctttttaccc 100080
ribosomal protein S12 80      ^^^ K P K K V G
(SEQ ID NO:41)

LCV1: 61      catattgtagggtggatctcgaaagatatgaaagatctccctccaagccgtacatacgac 120
|||||
tobac: 100081 catattgtagggtggatctcgaaagatatgaaagatctccctccaagccgtacatacgac 100140
ribosomal protein S12 78      Y K~~~~~

LCV1: 121      tttcatcgaatacggctttccgcagaattctatatgtatctatgagatcgagtatggaat 180
|||||
tobac: 100141 tttcatcgaatacggctttccgcagaattctatatgtatctatgagatcgagtatggaat 100200
ribosomal protein S12 1      ~~~~~

LCV1: 181      tctgtttactcacttttaaattgagtatccgtttccctccttttctgctaggttggaat 240
|||||
tobac: 100201 tctgtttactcacttttaaattgagtatccgtttccctccttttctgctaggttggaat 100260
ribosomal protein S12 1      ~~~~~

LCV1: 241      tcctgtattttacatatccatacgattgagtccttgggtttccgaaatagtgtaaaaaga 300
|||||
tobac: 100261 tcctgtattttacatatccatacgattgagtccttgggtttccgaaatagtgtaaaaaga 100320
ribosomal protein S12 1      ~~~~~

LCV1: 301      agtgcttcaaatcattgctattttgactcggacctgttctaaaaa-gtcgaggtatttcga 359
|||||
tobac: 100321 agtgcttcaaatcattgctattttgactcggacctgttctaaaaaagtcgaggtatttcga 100380
ribosomal protein S12 1      ~~~~~

LCV1: 360      attgtttgttgacacggacaaagtcagggaaaacctctgaaatttttcaatattgaacc 419
|||||
tobac: 100381 attgtttgttgacacggacaaagtcagggaaaacctctgaaatttttcaatattgaacc 100440
ribosomal protein S12 1      ~~~~~

LCV1: 420      ttggacatataatagttccgaatcgaatctctttgaaagaagatctttgtctcatggt 479
|||||
tobac: 100441 ttggacatataagagttccgaatcgaatctctttgaaagaagatctttgtctcatggt 100500
ribosomal protein S12 1      ~~~~~

LCV1: 480      agcctgctccagtcaccttacgaaactttcgttattgggttagccatacacttcacatgt 539
|||||
tobac: 100501 agcctgctccagtcaccttacgaaactttcgttattgggttagccatacacttcacatgt 100560
ribosomal protein S12 1      ~~~~~

LCV1: 540      ttctagcgattcacatggcatcatcaaatgatgacaagtcttgataagaatctacaacgc 599
|||||
tobac: 100561 ttctagcgattcacatggcatcatcaaatgatgacaagtcttgataagaatctacaacgc 100620
ribosomal protein S12 1      ~~~~~

LCV1: 600      actagaacgcccttgttgacgatcctttactccgacagcatctagggttcctcgaacaat 659
|||||
tobac: 100621 actagaacgcccttgttgacgatcctttactccgacagcatctagggttcctcgaacaat 100680
ribosomal protein S12 59      ~~ S R G Q Q R D K V G V A D L T G R V I

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Fig. 3-2

(continued)

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LCV1:          660      gtgatatctcacaccgggtaaatccttaaccctccccctcttactaagactacagaatg 719
                  |||
tobac:         100681  gtgatatctcacaccgggtaaatccttaaccctccccctcttactaagactacagaatg 100740
ribosomal protein S12 39      H Y R V G P L D K V R G G R V L V V S H

LCV1:          720      ttcttgtgaattatggccaataccgggtatataagcagtgatttcaaaccagaggttaa 779
                  |||
tobac:         100741  ttcttgtgaattatggccaataccgggtatataagcagtgatttcaaaccagaggttaa 100800
ribosomal protein S12 19      E Q L N H G I G P I Y A T I E F G S T L

LCV1:          780      tcgtactctggcaactttacgtaaggcagagtttggttttttggggtgatagtggaataa 839
                  |||
tobac:         100801  tcgtactctggcaactttacgtaaggcagagtttggttttttggggtgatagtggaataa 100860
ribosomal protein S12 1      R V R A V K R L A S N P K K P T I T

LCV1:          840      gttgacagataagtcacccttactgccactctacagaaccgtacatgagattttcacctc 899
                  |||
tobac:         100861  gttgacagataagtcacccttactgccactctacagaaccgtacatgagattttcacctc 100920

LCV1:          900      atacggctcctcgttcaattctttcgaagttattggatccttttccgcgttcgagaatcc 959
                  |||
tobac:         100921  atacggctcctcgttcaattctttcgaagttattggatccttttccgcgttcgagaatcc 100979

LCV1:          960      cctcccttcttccactccgtcccgaagagtaactaggaccaatttagtcacgttttcatg 1019
                  ||
tobac:         100980  cc-cccttcttccactccgtcccgaagagtaactaggaccaatttagtcacgttttcatg 101038

LCV1:          1020     ttccaattgaacactttccggtttt-----
                  |||
tobac:         101039  ttccaattgaacactgtccatttttgattatttctcaaaggataa 101082

LCV1:  1045      gattattctctttaccaaacatatgcggatccaatcacgatcttata---ataagaaca 1100
                  |||
tobac:  101083  gattattctctttaccaaacatatgcggatccaatcacgatcttataataagaagaaca 101142

LCV1:  1101      agagatctttctcgatcaatccccttgcccctcattcttcgagaatcagaagatocctt 1160
                  ||
tobac:  101143  aaagatctttcttgatcaatccccttgcccctcattcttcaagaataaggaagatcctt 101202

LCV1:  1161      tcaagtttgaatttggttcatttggaatctgagttcttctacttcattatttatttaatat 1220
                  |||
tobac:  101203  tcaagtttgaatttggttcatttggaatctgggttcttctacttcat-atttatttaatat 101261

LCV1:  1221      caatatatttgcctctcttttttttatattattccttaagtcctataggtttgatccttt 1280
                  |||
tobac:  101262  gaatatatttc-cctctcttttttttatatcattccttaagtcctataggtttgatcctgt 101320

LCV1:  1281      agaattggactcattttctcattgagcgaagggtacgaaataaatcagattgattaaaag 1340
                  |||
tobac:  101321  agaatttgaccattttctcattgaaacgaagggtacgaaataaatcagattgat-aaaag 101379

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Fig. 3-3

(continued)

LCV1:	1341	cactatgtgaaatattcgggttttt-----tcctcttctctatcccataaggt-----aca	1390
tobac:	101380	taccatgtgaaatcttcgggtttttcccttctctcgatcccatcccataggttaggtaca	101439
LCV1:	1391	gtgtttgaatcaatcgagaacctttttcttctgtctgaatcgatattattccattccaatt	1450
tobac:	101440	gtgtttgaatcaatagagaacctttttcttctgtatgaatcgatattattccattccaaat	101499
LCV1:	1451	ccttcccgatacctctcaaggaaaaatctcgaatt-ggatcctaattgacggggttagtgt	1509
tobac:	101500	ccttcccgatacctcccaaggaaaaatctcgaatttgatcccaaattgacggggttagtgt	101559
LCV1:	1510	gagcttatccatgcgggttatgcactcttcgaataggaatccattttctgaaagatcctgg	1569
tobac:	101560	gagcttatccatgcgggttatgcactcttgaataggaatccgttttctgaaagatcctgg	101619
LCV1:	1570	ctttcgtgctttgggtgggtctccgagatcctttcgatgacctatgttgtgtttgtgaag	1629
tobac:	101620	ctttcgtactttgggtgggtctccgagatcctttcgatgacctatg-----ttgaag	101670
LCV1:	1630	ggatatctatataatacgcgattgcgtaaagcccgcggtagcagtggaaccggggaaa	1689
tobac:	101671	ggatatctatctaataccgcgattgcgtaaagcccgcggtagcaacggaaccggggaaa	101730
LCV1:	1690	gtatacagaaaagacagttcttttctattatat	1722
tobac:	101731	gtatacagaaaagacagttcttttctattatat	101763
LCV1:	1723	attatatagtccttttctatttaattc	1749
tobac:	101764	tagta ttttctattatattaagatatattagactatt	101799
LCV1:	1750	atattagattagtccttagttagtgatcccggttagtgagtcctttcttccgtgatgaac	1809
tobac:	101800	atattagattagttattagttagtgatcccgacttagtgagtc-----tgatgaat	101849
LCV1:	1810	tgttgggccagtccttacattttgtctctgtgtggacagaggagaaaaggggtccgcggga	1869
tobac:	101850	tgttggccaccagtccttacattttgtctctgtgtggaccagaggagaaaaggggtccgcggga	101909
LCV1:	1870	agaggattgtaccgtgagagaagcaaggagggtcaacctctttcaaatacataaacatggat	1929
tobac:	101910	agaggagtgtaccatgagagaagcaaggagggtcaacctctttcaaatacataaacatggat	101969
hypothetical protein	127	^ ^ I Y L M S	
(SEQ ID NO:4)			
LCV1:	1930	tctggcaatgcaatgtacttggactctcatgtcgatccgaatgaatcatcctttccacgg	1989
tobac:	101970	tctggcaatg-----tagttggactctcatgtcgatccgaatgaatcatcctttccacgg	102024
hypothetical protein	123	E P L T T P S E H R D S H I M R E V S	

Fig. 3-4

(continued)

LCV1: 1990 aggc aaatc tttg cctgt taggtaacaggatagcaagttacaaactctgtctcggttagga 2049
||| |||||||||||| | ||| || |||||||||||| ||| ||||||||||||
tobac: 102025 aggtaaatc tttg cctgctagggcaagaggatagcaagttccaaattctgtctcggttagga 102084
hypothetical protein 88 T F R Q R S P L L I A L E L N Q R P L V

LCV1: 2050 catggatctctattactatgaatttcataaatgaagttagtgaaatgggtgggtaccatta 2109
||||| |||||||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
tobac: 102085 catgtatttctattactatgaatttcataaatgaagttagttaatggtaggtaccatta 102144
hypothetical protein 1 M K F I N E V V N G R V T I
(SEQ ID NO:5)
hypothetical protein 68 H I E I V I F N M F S T T L P L T V M I

LCV1: 2110 tcctttttgtagtgacgaatcctgtatgtgttcctaagaaaaggaatttgtacatttttc 2169
||||| |||||||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
tobac: 102145 tcctttttgtagtgacgaatcctgtatgtgttcctaagaaaaggaatttgtccatttttc 102204
hypothetical protein 15 I L F V V T N L V C V P K K R N L S I F
hypothetical protein 48 R K T T V F R T H T G L F L F K D M K R

LCV1: 2170 gggatctcaaaggagcgtggaacacataagaactcttgaatggaaatggaaaagagatg 2229
||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
tobac: 102205 ggggtctcaaagggcgtggaacgcataagaactcttg-----aatggaaaagagatg 102258
hypothetical protein 35 R G L K G A W K R I R T L E W K R D
hypothetical protein 35 P R L P A H F R M L V R S H F L S T

LCV1: 2230 gaactccagttccttcggaaatggtaagatctttggcgcaaaaaaggggtgatccgta 2289
||||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
tobac: 102259 taactccagttccttcg----- 102275
hypothetical protein 24 V G T G E
hypothetical protein 53 V T P V P S

LCV1 2290 tcactcttgacttggttctgcttctctatttttttaataataccgggtcggttcttctc 2349
Tobac: -----

LCV1 2350 ctaccgcatcgaatagaacacgctgagccaaatcttcttcatgtaaaacctgcttgatt 2409
Tobac: -----

LCV1 2410 tagatcgggaaaatcgtgtggttttatgaaacatgtgctatggctc 2456
Tobac: -----

LCV1: 2457 gaatccgtagtcaatcctatttccgatagggacagttgacaactgaatcctatttt-ccc 2515
||||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
tobac: 102276 gaatccgtagtcaatcctatttccgataggggcagttgacaattgaatccgattttgacc 102335
hypothetical protein 6 S D T T L G I E S L P L Q C N F G I K V
hypothetical protein 59 E S V V N P I S D R G S ^^

Pac1/AscI

LCV1: 2516 attattttcatatccgtaatagtgcgaaaaaaaaagttaaattaaaggcgcgcc 2567
||||| ||||||| ||||||| ||||||| ||||||| ||||||| ||||||| |||||||
tobac: 102336 attattttcatatccgtaatagtgcgaaaaga----- 102367
hypothetical protein 1 M I K M

Fig. 3-5

(continued)

```
LCV1: 2568 aggcccgcccccaagttgttcaagaatagtgctggtgagtttctcgaccctttgccttag 2627
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102368 aggcccgctccaagttgttcaagaatagtggtggtgagtttctcgaccctttgacttag 102427

LCV1: 2628 gattaatcagttctatttctcgatgggggcaggggaaggatataactcacggtagagtg 2687
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102428 gattagtcagttctatttctcgatgggg-cggggaaggatataactcagcggtagagtg 102486

LCV1: 2688 tcacccttgacgtggtggaagtcacagttcgagcctgattatccctaaacccaatgtga 2747
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102487 tcacc-ttgacgtggtggaagtcacagttcgagcctgattatccctaagcccaatgtga 102545

LCV1: 2748 gttttgataatttgatttgctaccccgccgtgattgaatgagaatggataagaggctcgt 2807
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102546 gtttttctagttggatttgctcccccgccgtcgttcaatgagaatggataagaggctcgt 102605

LCV1: 2808 gggattgacgtgagggggcagggatggctatatttctgggagcgaactccgggcgaatat 2867
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102606 gggattgacgtgagggggcagggatggctatatttctgggagcgaactccgggcgaatat 102665

LCV1: 2868 gaagcgcacatggatacaagtttaggccttggaatgaaagacaattccgaatccgctttgtct 2927
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102666 gaagcgcacatggatacaagtttaggccttggaatgaaagacaattccgaatccgctttgtct 102725

LCV1: 2928 acgaacaaggaagctataagtaatgcaactatgaatctcatggagagttcgatcctggct 2987
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102726 acgaacaaggaagctataagtaatgcaactatgaatctcatggagagttcgatcctggct 102785

LCV1: 2988 caggatgaacgctggcgccatgcttaacacatgcaagtcggacgggaagtgggtgttcca 3047
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102786 caggatgaacgctggcgccatgcttaacacatgcaagtcggacgggaagtgggtgttcca 102845

LCV1: 3048 gtggcgacgggtgagtaacgcgtaagaacctgcccttgggaggggaacaacagctggaa 3107
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102846 gtggcgacgggtgagtaacgcgtaagaacctgcccttgggaggggaacaacagctggaa 102905

LCV1: 3108 acggctgctaataccccgtaggctgaggagcaaaaggaggaatccgccgaggaggggct 3167
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102906 acggctgctaataccccgtaggctgaggagcaaaaggaggaatccgccgaggaggggct 102965

LCV1: 3168 cgcgtctgattagctagttggtgaggtaatagcttaccaaggcgatgatcagtagctggt 3227
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 102966 cgcgtctgattagctagttggtgaggcaatagcttaccaaggcgatgatcagtagctggt 103025

LCV1: 3228 ccgagaggatgatcagccacactgggactgagacacggcccagactcctacgggaggcag 3287
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 103026 ccgagaggatgatcagccacactgggactgagacacggcccagactcctacgggaggcag 103085

LCV1: 3288 cagtggggaattttccgcaatgggcgaaagcctgacggagcaatgccgcgtggaggtaga 3347
          ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
tobac: 103086 cagtggggaattttccgcaatgggcgaaagc-tgacggagcaatgccgcgtggaggtaga 103144
```

Fig. 3-6

(continued)

(continued)

LCV1:	3348	aggccacggtcatgaacttcttttcgccggagaagaagcaatgacggatatctggggaat	3407
tobac:	103145	aggccccacgggtcgtgaacttcttttcgccggagaagaagcaatgacggatatctggggaat	103204
LCV1:	3408	aagcatcggtctaactctgtgccagcagccgcggtaatacacaggatgcaagcggttatccg	3467
tobac:	103205	aagcatcggtctaactctgtgccagcagccgcggtaatacacaggatgcaagcggttatccg	103264
LCV1:	3468	gaatgattgggcgtaaagcgtctgttaggtggctttttaagtccgccgtcaaatcccagg	3527
tobac:	103265	gaatgattgggcgtaaagcgtctgttaggtggctttttaagtccgccgtcaaatcccagg	103324
LCV1:	3528	ctcaactctggacaggcggtggaactaccaagctggagtacggtaggggcagagggaa	3587
tobac:	103325	ctcaaccctggacaggcggtggaactaccaagctggagtacggtaggggcagagggaa	103384
LCV1:	3588	ttccggtggagcggtgaaatgcgtagagatcggaaagaacaccaacggccaagcactct	3647
tobac:	103385	ttccggtggagcggtgaaatgcgtagagatcggaaagaacaccaacggccaagcactct	103444
LCV1:	3648	gctggggccacactgacactgagagacgaaagctaggggagcgaatgggattagatacc	3707
tobac:	103445	gctggggccacactgacactgagagacgaaagctaggggagcgaatgggattagatacc	103504
LCV1:	3708	cagtagtcctagccgtaaacgatggatactaggcgctgtgcgtatcgacccgtgcagtgc	3767
tobac:	103505	cagtagtcctagccgtaaacgatggatactaggcgctgtgcgtatcgacccgtgcagtgc	103564
LCV1:	3768	tgtagctaacgcgttaagtatcccgctggggagtagcttcgcaagaatgaaactcaaag	3827
tobac:	103565	tgtagctaacgcgttaagtatcccgctggggagtagcttcgcaagaatgaaactcaaag	103624
LCV1:	3828	gaattgacgggggcccgcacaagcggttgagcatgtggtttaattcgatgcaaagcgaag	3887
tobac:	103625	gaattgacgggggcccgcacaagcggttgagcatgtggtttaattcgatgcaaagcgaag	103684
LCV1:	3888	aaccttaccagggcttgacatgccgcgaatcctcttgaaagagaggggtgccttcgggaa	3947
tobac:	103685	aaccttaccagggcttgacatgccgcgaatcctcttgaaagagaggggtgccttcgggaa	103744
LCV1:	3948	cgcggaacacaggtggtgcatggctgtcgtcagctcgtgccgtaaggtgttggttaagtc	4007
tobac:	103745	cgcggaacacaggtggtgcatggctgtcgtcagctcgtgccgtaaggtgttggttaagtc	103804
LCV1:	4008	ccgcaacgagcgcaaacctcgtgttagttgccatcattgagtttgaaccctgaacaga	4067
tobac:	103805	ccgcaacgagcgcaaacctcgtgttagttgccatcattgagtttgaaccctgaacaga	103864
LCV1:	4068	ctgccggtgataagccggaggaaggtgaggtacgtcaagtcacatgcccccttatgcc	4127
tobac:	103865	ctgccggtgataagccggaggaaggtgaggtacgtcaagtcacatgcccccttatgcc	103924

Fig. 3-7

(continued)

```
LCV1: 4128 ctgggcgacacacgtgctacaatggccgggacaaaggtcgcgatcccgcgaggggtgagc 4187
|||||
tobac: 103925 ctgggcgacacacgtgctacaatggccgggacaaaggtcgcgatcccgcgaggggtgagc 103984

LCV1: 4188 taacccccaaaaacccgtcctcagttcggattgcaggctgcaactcgctgcatgaagccg 4247
|||||
tobac: 103985 taacccccaaaaacccgtcctcagttcggattgcaggctgcaactcgctgcatgaagccg 104044

LCV1: 4248 gaatcgctagtaatcgccggtcagccatacggcgggtaatccggtcccgggccttgtaga 4307
|||||
tobac: 104045 gaatcgctagtaatcgccggtcagccatacggcgggtaatccggtcccgggccttgtaga 104104

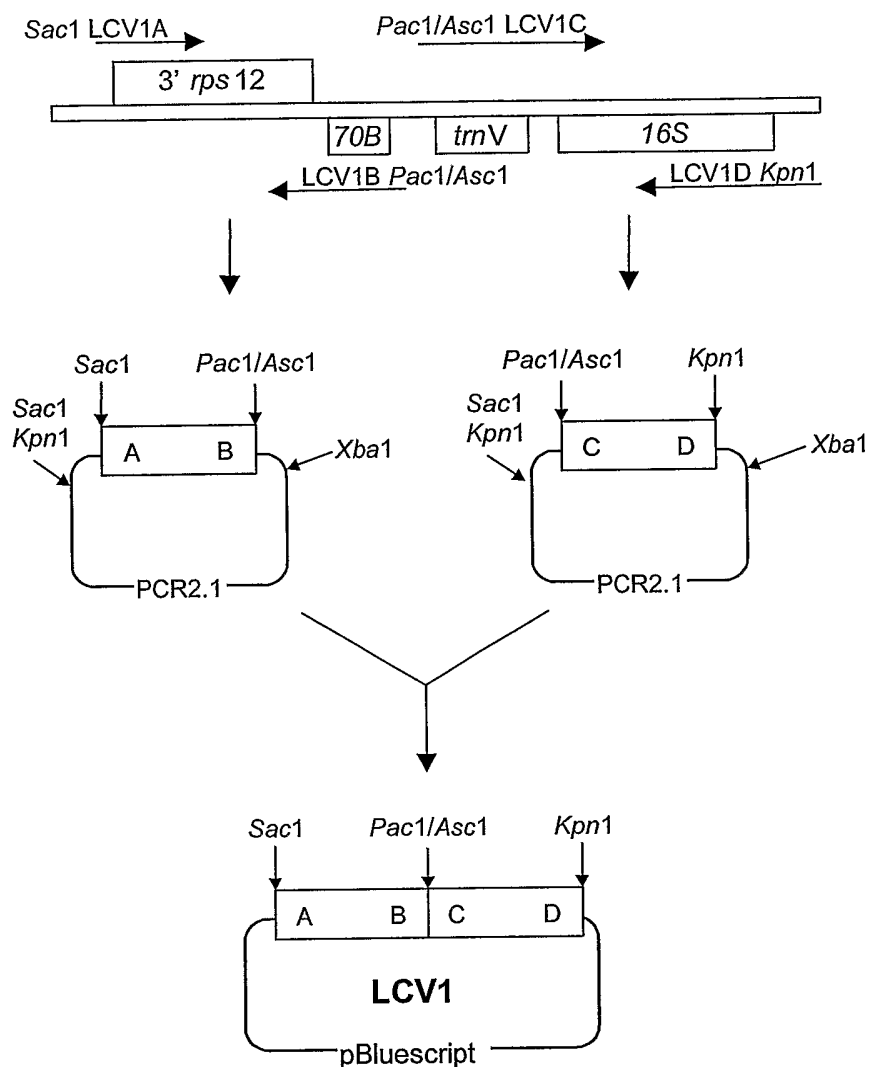
LCV1: 4308 caccgcccgtcacactatgggagctggccatgcccgaagtcgttaccttaaccgcaagga 4367
|||||
tobac: 104105 caccgcccgtcacactatgggagctggccatgcccgaagtcgttaccttaaccgcaagga 104164

LCV1: 4368 gggggatgccgaaggcagggctagtactggagtgaagtcgtaacaaggtagccgtactg 4427
|||||
tobac: 104165 gggggatgccgaaggcagggctagtactggagtgaagtcgtaacaaggtagccgtactg 104224

LCV1: 4428 gaagggtcggctggatcacctccttttcagggagagctaatactgttgggtattttgg 4487
|||||
tobac: 104225 gaagggtcggctggatcacctccttttcagggagagctaatactgttgggtattttgg 104284

LCV1: 4488 ttgacactgcttcacaccc----aaaaaagaaggagctacgtctgagttaaacttgag 4543
|||||
tobac: 104285 ttgacactgcttcacacccccaaaaaagaaggagctacgtctgagttaaacttgag 104344

LCV1: 4544 atggaagtcttcatttcgtttctcgacagtgaagtaagaccaag 4587
|||||
tobac: 104345 atggaagtcttc-tttcctttctcgacgggtgaagtaagaccaag 104387
```



LCV1A-5' ATGAGCTCGTTCAAGAATCAGTTTTCTT3' (100021-100040 in TCG) (SEQ ID NO:6)
 LCV1B-5' GGCGCGCCTTAATTAACTTTTTTTTCGCACTATTACGGATAT3' (102345-102367 in TCG)
 (SEQ ID NO:7)
 LCV1C-5' TTAATTAAGGCGCGCCAGGCCCGGCCCAAGTT3' (102368-102384 in TCG) (SEQ ID NO:8)
 LCV1D-5' ATGGTACCCTTGGTCTTACTTCACTGTCTGA3' (104366-104387 in TCG) (SEQ ID NO:9)

Fig. 4

Fig. 5

SEQ ID NO:10

TCGACAGTGAAGTAAGACCAAGCTCATGAGCTTATTATCTCAGGTCGGAACAAGTTGATAGGATCCCCCTTTTT
ACGTCCCCATGCCCCCTGTGTGGCGACATGGGGGCGAAAAAAGGAAAGAGAGAGATGGGGTTTCTCTCGCTTTT
GGCATAGTGGGCCCCAGTGGGGGGCTCGCACGACGGGCTATTAGCTCAGTGGGTAGAGCGCGCCCCGTGATAAT
TGCGTCGTTGTGCGTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCATCGGCGCCTGACCCT
GAGATGTGGATCATCCAAGGCACATTAGCATGGCGTACTCCTCCTGTTTGAACCGGGGTTTGAAACCAAACTTC
TCCTCAGGAGGATAGATGGGGCGATTAGGTGAGATCCAATGTAGATCCAACCTTCGATTCACTCGTGGGATCC
GGGCGGTCCGGGGGGGACCACCATGGCTCCTCTCTTCTCGAGAATCCATACATCCCTTATCAGTGTATGGACAG
CTATCTCTCGAGCACAGGTTTAGGTTTCGGCCTCAATGGGAAAAATAAAATGGAGCACCTAACACGCATCTTCAC
AGACCAAGAAGTACGAGATCACCCCTTTTATTCTGGGGTGACGGAGGGATCATACCATTCGAGCCTTTTTTTTTT
CATGCTTTTCCCCGAGGCTGTGGAGAAAGCTGAAATCAATAGGATTTCCCTAATCCTCCCTTACCGAAAGGAAGA
GCGTGAAATTCTTTTTCTTTCCGCGAGGGACCAGGAGATTGGATCTAGCCGTAAGAAGATGCTTGGTATAAAT
AACTCACTTCTTGGTCTTCGACCCCCGCAGTCACTACGAACGCCCCCGATCAGTGAATGGGATGTGTCTATTT
ATCTATCTCTTGACTCGAAATGGGAGCAGGTTTGAAGAAAGGATCTTAGAGTGTCTAGGGTTGGGCCAGGAGGGT
CTCTTAACGCCTTCTTTTTCTTCTCATCGGAGTTATTTACAAAGACTTGCCATGGTAAGGAAGAAGGGGGGA
ACAGGCACACTTGGAGAGCGCAGTACAACGGAGAGTTGTATGCTGCGTTTCGGAAGGATGAATCGCTCCCGAAA
AGGAATCTATTGATTCTCTCCAATTGGTTGGACCGTAGGTGCGATGATTTACTTCACGGGCGAGGTCTCTGGT
TCAAGTCCAGGATGGCCAGCTGCGCCAGGGAAAAGAATAGAAGAAGCGTCAGACTATTAATTAAGGCGCGCCC
ATGCATGCTCCACTTGGCTCGGGGGGATATAGCTCAGTTGGTAGAGCTCCGCTCTTGCAATTGGGTCGTTGCGA
TTACGGGTTGGATGTCTAATTGTCCAGGCGGTAATGATAGTATCTTGTACCTGAACCGGTGGCTCACTTTTTCT
AAGTAATGGGGAAGAGGACCGAAACATGCCACTGAAAGACTCTACTGAGACAAAGATGGGCTGTCAAGAACGTC
AAGAACGTAGAGGAGGTAGGATGGGCAGTTGGTCAGATCTAGTATGGATCGTACATGGACGGTAGTTGGAGTCG
GCGGCTCTCCTAGGGTTCCCTTATCGGGGATCCCTGGGGAAGAGGATCAAGTTGGCCCTTGCGAACAGCTTGAT
GCACTATCTCCCTTCAACCCCTTGAGCGAAATGCGGCAAAAGGAAGGAAAATCCATGGACCGACCCATCATCT
CCACCCCGTAGGAACACGAGATTACCCCAAGGACGCCTTCGGCATCCAGGGGTCACGGACCGACCATAGAACC
CTGTTCAATAAGTGAACGCATTAGCTGTCCGCTCTCAGGTTGGGCAGTAAGGGTCGGAGAAGGGCAATCACTC
ATTCTTAAAACAGCGTTCTTAAGGCCAAAGAGTCGGCGGAAAAGGGGGGAAAGCTCTCCGTTCTGGTTTCCT
GTAGCTGGATCCTCCGGAACCAACAAGAATCCTTAGTTAGAATGGGATTCCAACCTCAGCACCTTTTGTAGTGAGAT
TTTGAGAAGAGTTGCTCTTTGGAGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTTCGGGGGGGAGTTATTGTC
TATCGTTGGCCTCTATGGTAGAATCAGTCGGGGGACCTGAGAGGCGGTGGTTTACCCTGCGGCGGATGTCAGCG
GTTTCGAGTCCGCTTATCTCCAACCTCGTGAACCTTAGCCGATACAAAGCTATATGACAGCACCCAATTTTCCGAT
TTGGCGGTTTCGATCTATGATTTATCATTCATG

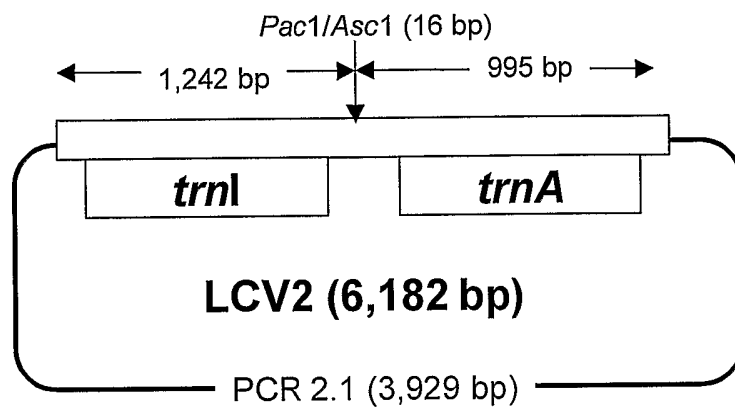


Fig. 6

LCV2 : 1 tgcacagtgaaagtaagaccaagctcatgagcttattatctcagggtcggaacaagttagata 60
SEQ ID NO:11 ||||| |||||||||||||||||||||||||||| |
tobac: 104366 tcgacgggtgaagtaagaccaagctcatgagcttattatctcagggtcggaacaagttagata 104425
SEQ ID NO:12

LCV2 : 61 ggatcccccttttttacgtcccccattg--ccccctgtgtggcgacatgggggcgaaaaaagg 118
||| ||| |||||||||||||||| |||| |
tobac: 104426 ggaccccccttttttacgtcccccattgtccccccgtgtggcgacatgggggcgaaaaaagg 104485

LCV2 : 119 aaagagagagatgggggtttctctcgcttttggcatagtggggccccagtggggggctcgc 178
||||| ||||| |||||||||||||||| ||||| |
tobac: 104486 aaagagaggggatgggggtttctctcgcttttggcatagcggggccccagtgggaggctcgc 104545

LCV2 : 179 acgacgggctattagctcagtggggtagagcgcgccccctgataattgcgtcgttgtgcctg 238
||||| ||||| |||||||||||||||| ||||||| |
tobac: 104546 acgacgggctattagctcagtggt-tagagcgcgccccctgataattgcgtcgttgtgcctg 104604

LCV2 : 239 ggctgtgagggctctcagccacatggatagttcaatgtgctcatcggcgcctgacctga 298
||||| ||||| |||||||||||||||| ||||||| |
tobac: 104605 ggctgtgagggctctcagccacatggatagttcaatgtgctcatcggcgcctgacctga 104664

LCV2 : 299 gatgtggatcatccaaggcacattagcatggcgactactcctcgttcgaaccgggggttg 358
||||| ||||| |||||||||||||||| ||||||| |
tobac: 104665 gatgtggatcatccaaggcacattagcatggcgactactcctcgttcgaaccgggggttg 104724

LCV2 : 359 aaaccaaacttctcctcaggaggatagatggggcgattcagggtgagatccaatgtagatc 418
||||| ||||| |||||||||||||||| ||||||| |
tobac: 104725 aaaccaaactcctcctcaggaggatagatggggcgattcgggtgagatccaatgtagatc 104784

LCV2 : 419 caactttcgattcactcgtgggatccggggcggtccgggggggaccaccatggctcctctc 478
||||| ||||| |||||||||||||||| ||||||| |
tobac: 104785 caactttcgattcactcgtgggatccggggcggtccgggggggaccaccacggctcctctc 104844

LCV2 : 479 ttctcgagaatccatacatcccttatcagtgatatggacagctatctctcgagcacaggtt 538
||||| ||||| |||||||||||||||| ||||||| |
tobac: 104845 ttctcgagaatccatacatcccttatcagtgatatggacagctatctctcgagcacaggtt 104904

LCV2 : 539 taggttcggcctcaatgggaaaataaaatggagcacctaacaacgcattcttcacagacca 598
||| ||||| |||||||||||||||| ||||||| |
tobac: 104905 tag-----caatgggaaaataaaatggagcacctaacaacgcattcttcacagacca 104955

LCV2 : 599 agaactacgagatcacccctttcattctgggggtgacggagggatcataccattcgagcc 657
||||| ||||| |||||||||||||||| ||||||| |
tobac: 104956 agaactacgagatcgcccctttcattctgggggtgacggagggatcgtagcattcgagcc 105014

LCV2 : 658 tttttttttcatgcttttccccgagggtctggagaaagctgaaatcaataggatttcccta 717
||||| |
tobac: 105015 gttttttt----- 105021

atcctcccttacggaaaggaagagcgtgaaattcttttcccttccgcagggaccaggagattggatctagccgtaagaagaatgcttg
gtataaataactcacttcttgggtcttcgacccccgcagtcactacgaacgccccgatcagtgcaatgggatgtgtctatttatctatc
895
(231 bp present in lettuce maize, rice and soybean but not tobacco)

Fig. 7-2

(continued)

```

LCV2 : 896      tcttgactcgaaatgggagcagggttgaaaaaggatcttagagtgtctaggggtgggcca 955
                |||||||
tobac: 105022   tcttgactcgaaatgggagcagggttgaaaaaggatcttagagtgtctaggggtgggcca 105081

LCV2 : 956      ggaggggtctcttaacgccttctttttcttctcatcgaggtatttcacaaagacttgcc 1015
                |||||||
tobac: 105082   ggaggggtctcttaacgccttctttttcttctcatcgaggtatttcacaaagacttgcc 105141

LCV2 : 1016     atggtaaggaagaaggggggaacaggcacacttgagagcgagtcacaacggagagttgt 1075
                | |||||
tobac: 105142   agggtaaggaagaaggggggaacaaggcacacttgagagcgagtcacaacggagagttgt 105201

LCV2 : 1076     atgctgcgttcgggaaggatgaatcgctcccgaaaaggatctattgattctctccaat 1135
                |||||||
tobac: 105202   atgctgcgttcgggaaggatgaatcgctcccgaaaaggatctattgattctctccaat 105261

LCV2 : 1136     tggttggaccgtaggtgcatgatttacttcacgggcgaggtctctggttcaagtccagg 1195
                |||||||
tobac: 105262   tggttggaccgtaggtgcatgatttacttcacgggcgaggtctctggttcaagtccagg 105321

LCV2 : 1196     atggcccagctgcccagggaagaatagaagaagcgtcagactccttaattaaggcgccgc 1258
                |||||||
tobac: 105322   atggcccagctgcccagggaagaatagaagaagcatctgactactt-----105370
                                Pac1/Asc1

LCV2 : 1259     catgcatgctccacttggtcgggggatatagctcagttggtagagctccgctcttgca 1318
                |||||||
tobac: 105371   catgcatgctccacttggtcgggggatatagctcagttggtagagctccgctcttgca 105430

LCV2 : 1319     attgggtcgttgcgattacgggttgatgtctaattgtccaggcggtaatgatagtatct 1378
                |||||||
tobac: 105431   attgggtcgttgcgattacgggttgatgtctaattgtccaggcggtaatgatagtatct 105490

LCV2 : 1379     tgtacctgaaccggtggctcactttttctaagtaatggggaagaggaccgaaacatgcca 1438
                |||||||
tobac: 105491   tgtacctgaaccggtggctcactttttctaagtaatggggaagaggaccgaaacatgcca 105550

LCV2 : 1439     ctgaaagactctactgagacaaagatgggctgtcaagaacgtcaagaacgtagaggaggt 1498
                |||||||
tobac: 105551   ctgaaagactctactgagacaaagatgggctgtcaagaa-----cgtagaggaggt 105601

LCV2 : 1499     aggatgggcagttggtcagatctagtaggtacgtacatggacggtagttggagtcggcg 1558
                |||||||
tobac: 105602   aggatgggcagttggtcagatctagtaggtacgtacatggacggtagttggagtcggcg 105661

LCV2 : 1559     gctctcctaggggttccttatcggggatccctggggaagaggatcaagttggcccttgcg 1618
                |||||||
tobac: 105662   gctctcccagggttcctcatctgagatctctggggaagaggatcaagttggcccttgcg 105721

LCV2 : 1619     aacagcttgatgcactatctcccttcaaccctttgagcgaaatgcggc-----aaaagga 1673
                |||||||
tobac: 105722   aacagcttgatgcactatctcccttcaaccctttgagcgaaatgcggcaaaagaaaagga 105781

```

Fig. 7-3

(continued)

LCV2 : 1674 aggaaaatccatggaccgaccccatcatctccaccccgtaggaactacgagattaccca 1733
 |||||
 tobac: 105782 aggaaaatccatggaccgaccccatcatctccaccccgtaggaactacgagatcaccca 105841

LCV2 : 1734 aggagccttcggcatccaggggtcacggaccgaccatagaaccctgttcaataagtga 1793
 |||||
 tobac: 105842 aggagccttcggcatccaggggtcacggaccgaccatagaaccctgttcaataagtga 105901

LCV2 : 1794 acgcattagctgtccgctctcaggttggcgagtaagggtcggagaagggaatcactcat 1853
 |||||
 tobac: 105902 acgcattagctgtccgctctcaggttggcgagtaagggtcggagaagggaatgactcat 105961

LCV2 : 1854 tctta 1858
 |
 tobac: 105962 t----- 105962

LCV21859aaaccagcgttcttaaggccaaagagtcggcgaaaaggggggaaagctctccgttcctggttccgtgtagctggatcctc
 cggaaaccacaagaatc 1955 (97 bp sequence absent in tobacco but present in spinach, Solanum
 nigrum, Arabidopsis, Soybean, rice and wheat)

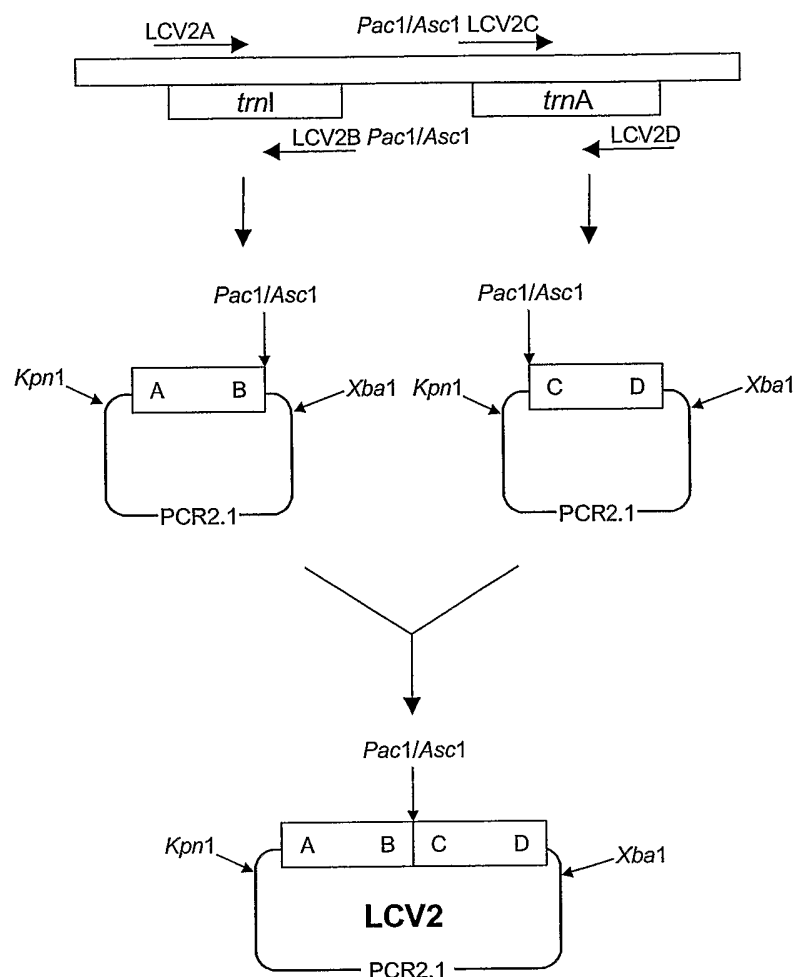
LCV2 : 1956 cttagttagaatgggattccaactcagcaccttttgagtgagattttgagaagagttgct 2015
 |||||
 tobac: 105963 cttagttagaatgggattccaactcagcaccttttgagtgagattttgagaagagttgct 106022

LCV2 : 2016 ctttgagagcacagtagcatgaaagttgtaagctgtgttcgggggggagttattgtcta 2075
 |||||
 tobac: 106023 ctttgagagcacagtagcatgaaagttgtaagctgtgttcgggggggagttattgtcta 106082

LCV2 : 2076 tcgttggcctctatggtagaatcagtcgggggacctgagaggcggtggtttaccctgcgg 2135
 |||||
 tobac: 106083 tcgttggcctctatggtagaatcagtcgggggacctgagaggcggtggtttaccctgcgg 106142

LCV2 : 2136 cggatgtcagcggttcgagtcggttatctccaactcgtgaacttagccgatacaaaagct 2195
 |||||
 tobac: 106143 cggatgtcagcggttcgagtcggttatctccaactcgtgaacttagccgatacaaaagct 106202

LCV2 : 2196 atatgacagcacccaattttccgatttggcggttcgatctatgatttatcattcatg 2253
 |||||
 tobac: 106203 ttatgatagcacccaattttccgatttggcggttcgatctatgatttatcattcatg 106260



LCV2A 5' TCGACAGTGAAGTAAGACCAAG3' (104366-104387 in TCG) (SEQ ID NO:13)
 LCV2B 5' GGCGCGCCTTAATTAAGGAGTCAGACGCTTCTTCTATTC3' (10346-105370 in TCG)
 (SEQ ID NO:14)
 LCV2C 5' TTAATTAAAGGCGCGCCCCATGCATGCTCCACTTGGCTCGG3' (105371-105393 in TCG)
 (SEQ ID NO:15)
 LCV2D 5' CATGAATGATAAATCATAGATCGAAC3' (106234-106260 in TCG) (SEQ ID NO:16)

Fig. 8

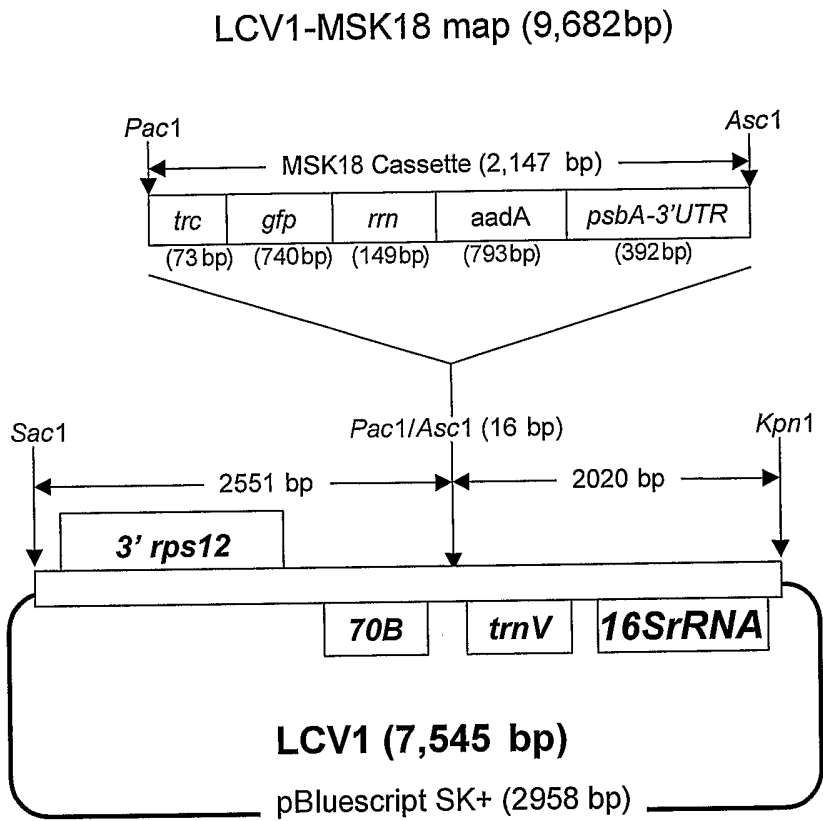


Fig. 9

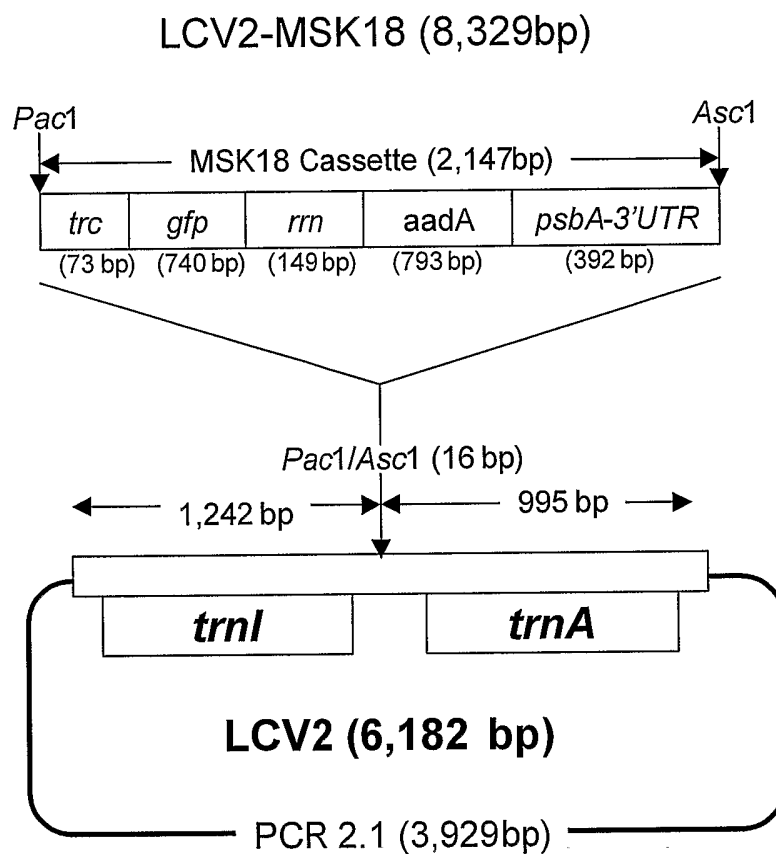
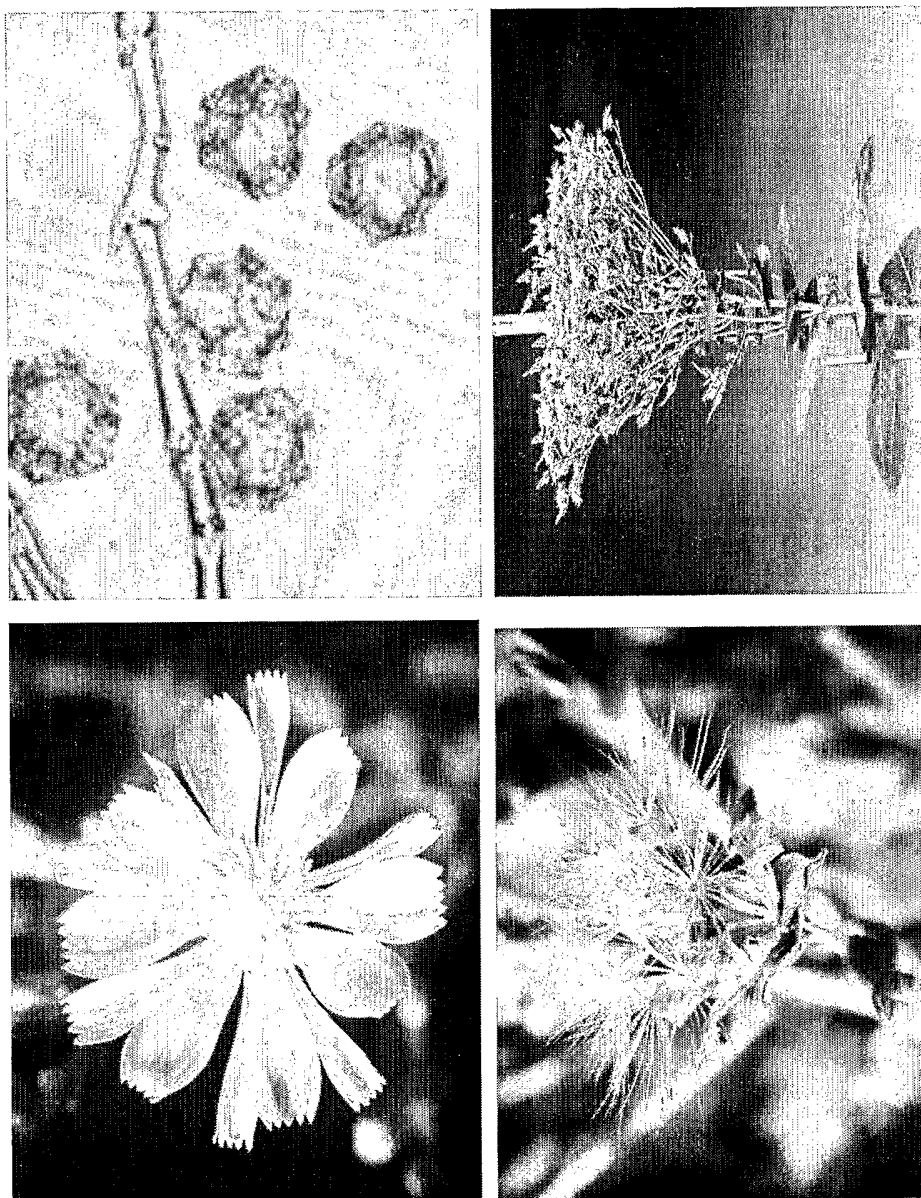
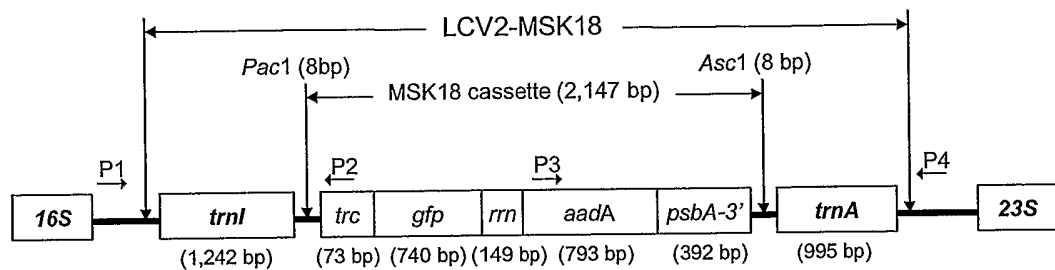


Fig. 10

Fig. 11





P1 + P2 = 1415 bp

P3 + P4 = 2006 bp

P1 + P4 = 4623 bp

P1 5'-ACTGGAAGGTGCGGCTGGAT-3' (SEQ ID NO:17)

P2 5'-ACGAGCCGGATGATTAATTGTCAATTAATTAATA-3' (MSK18A comp)- (SEQ ID NO:18)

P3 5'-AAGTCACCATTTGTTGTGCACG-3' (starts at 259 bp on aadA CDS) (SEQ ID NO:19)

P4 5'-CTCGCCCTTAATTTTAAGGC-3' (SEQ ID NO:20)

Fig. 12

Figure 13

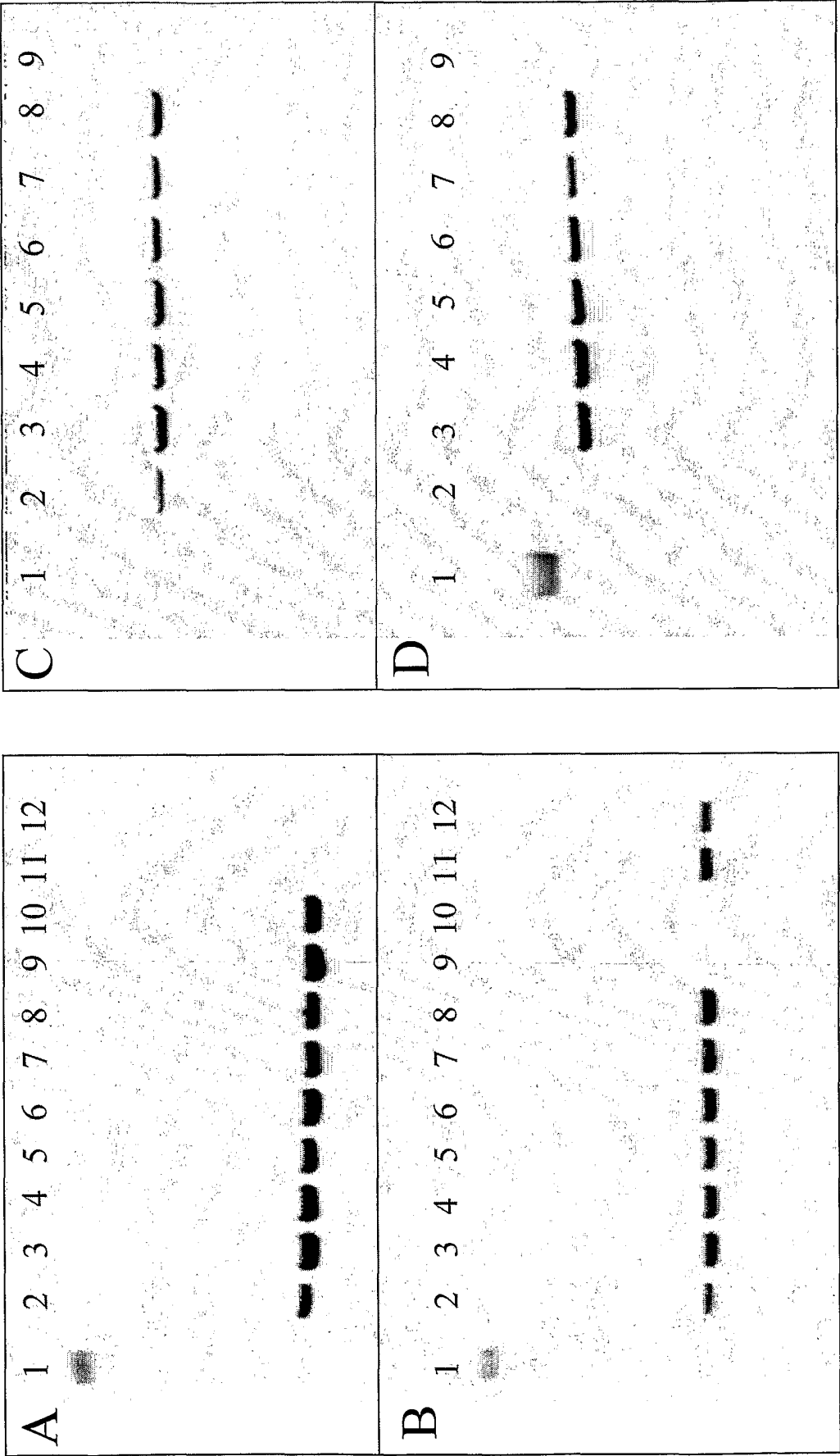


Fig. 14-1

P1-P2 left border fragment consensus sequence (SEQ ID NO:21)

Primer P1→

actggaaggtgcggtggatcacctccttttcagggagagctaattgcttggtgggtattttggttgacac
tgcttcacacccaaaaaagaagggagctacgtctgagttaaacttggagatggaagtcttcatttcggttc

Primer LCV2A→=LCV2A left border

TCGACAGTGAAGTAAGACCAAGCTCATGAGCTTATTATCTCAGGTCGGAACAAGTTGATAGGATCCCCCTT
TTTACGTCCCCATGCCCTGTGTGGCGACATGGGGGCGAAAAAGGAAAGAGAGATGGGGTTTCTCTC
GCTTTTGGCATAGTGGGCCCCAGTGGGGGGCTCGCACGACGGGCTATTAGCTCAGTGGGTAGAGCGCGCC
CCTGATAATTGCGTCGTTGTGCCTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCATCG
GCGCCTGACCCGTAGATGTGGATCATCCAAGGCACATTAGCATGGCGTACTCCTCCTGTTCGAACCGGGGT
TTGAAACCAAACCTTCTCCTCAGGAGGATAGATGGGGCGATTTCAGGTGAGATCCAATGTAGATCCAACCTTTC
GATTCACCTCGTGGGATCCGGGCGGTCCGGGGGGGACCACCATGGCTCCTCTCTTCTCGAGAATCCATACAT
CCCTTATCAGTGTATGGACAGCTATCTCTCGAGCACAGGTTTAGGTTTCGGCCTCAATGGGAAAAATAAATG
GAGCACCTAACACGCATCTTCACAGACCAAGAACTACGAGATCACCCCTTTTATTCTGGGGTGACGGAGG
GATCATACCATTTCGAGCCTTTTTTTTTTCATGCTTTTCCCGAGGCTCTGGAGAAAGCTGAAATCAATAGGAT
TTCCCTAATCCTCCCTTACCGAAAGGAAGAGCGTGAAATTTCTTTTTCCTTTCCGCAGGGACCAGGAGATTG
GATCTAGCCGTAAGAAGAAATGCTTGGTATAAATAACTCACTTCTTGGTCTTCGACCCCGCAGTCACTACG
AACGCCCCCGATCAGTGCAATGGGATGTGTCTATTTATCTATCTCTTGAATCGAAATGGGAGCAGGTTTGA
AAAAGGATCTTAGAGTGTCTAGGGTTGGGCCAGGAGGTTCTTAAACGCCTTCTTTTTTCTCTCATCGGA
GTTATTTACAAAGACTTGCCATGGTAAGGAAGAAGGGGGGAACAGGCACACTTGGAGAGCGCAGTACAAC
GGAGAGTTGTATGCTGCGTTTCGGGAAGGATGAATCGCTCCCGAAAAAGGAATCTATTGATTCTCTCCCAATT
GGTTGGACCGTAGGTGCGATGATTTACTTACGGGCGAGGTTCTCTGGTTCAAGTCCAGGATGGCCCAGCTG

Pac1 trc promoter→ ←Primer P2

CGCCAGGGAAAAGAATAGAAGAAGCGTCTGACTCC [TTAATTAA] [TTGACAATTAATCATCCGGCTCGT]

P3-P6 left border fragment consensus sequence (SEQ ID NO:22)

Primer P3→(aadA gene)

AAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGA
GAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTT
GCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACCTTTTGATCCGGTTC
CTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAACCTCGCCGCCGACTGGGCTGGC
GATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTGGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAA
GGATGTGCGCTGCCGACTGGGCAATGGAGCGCCTGCCGGCCAGTATCAGCCCGTCATACTTGAAGCTAGAC
AGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCGCGAGATCAGTTGGAAGAATTTGTCCACTAC

aadA stop/psbA 3' UTR→

GTGAAAGGCGAGATCACCAAGGTAGTCGGCAAAATAATGTCCTAGAGCGATCCTGGCCTAGTCTATAGGAGGT
TTTGAAGAAGAAAGGAGCAGTAATCATTTTCTTGTCTATCAAGAGGGTGCTATTGCTCCTTTCTTTTTTTC
TTTTTATTTATTTACTAGTATTTTACTTACATAGACTTTTTTGTTTTACATTATAGAAAAAGAAGGAGAGGT
TATTTTCTTGCAATTTATTCATGATTGAGTATTCTATTTTGATTTTGTATTGTTTAAATTTGTAGAAATAG
AACTTGTTTCTCTTCTTGCTAATGTACTATATCTTTTTGATTTTTCCTTTTCCAAAAAATAATCAATTTT
GACTTCTTCTTATCTCTTATCTTTGAATATCTCTTATCTTTGAAATAATAATATCATTGAAATAAGAAAGA

Asc1

trnA gene→

AGAGCTATATTCTGA [GGCGCGCC] CATGCATGCTCCACTTGGCTCGGGGGGATATAGCTCAGTTGGTAGA
GCTCCGCTCTTGCAATTGGGTGCTTGGGATTACGGGTGGATGTCTAATTGTCCAGGCGGTAATGATAGTA
TCTTGTACCTGAACCGGTGGCTCACTTTTTCTAAGTAATGGGGAAGAGGACCGAAACATGCCACTGAAAGA
CTCTACTGAGACAAAGATGGGCTGTCAAGAACGTCAAGAACGTAGAGGAGGTAGGATGGGCAGTTGGTCAG
ATCTAGTATGGATCGTACATGGACGCTAGTTGGAGTCGGCGGCTCTCCTAGGGTTCCCTTATCGGGGATCC

Fig. 14-2

(continued)

CTGGGGGAAGAGGATCAAGTTGGCCCTTGCGAACAGCTTGATGCACTATCTCCCTTCAACCCTTTGAGCGAA
ATGCGGGCAAAAGGAAGGAAAATCCATGGACCGACCCCATCATCTCCACCCGTAGGAACCTACGAGATTACC
CCAAGGACGCCTTCGGCATCCAGGGGTCACGGACCGACCATAGAACCCTGTTCAATAAGTGGAACGCATTA
GCTGTCCGCTCTCAGGTTGGGCAGTAAGGGTCGGAGAAGGGCAATCACTCATTCTTAAACCAGCGTTCTT
AAGGCCAAAGAGTCGGCGGAAAAGGGGGGAAAGCTCTCCGTTCCCTGGTTTCCTGTAGCTGGATCCTCCGGA
ACCACAAGAATCCTTAGTTAGAATGGGATTCCAACCTCAGCACCTTTTGAGTGAGATTTTGAGAAGAGTTGC
TCTTTGGAGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTCCGGGGGGAGTTATTGTCTATCGTTGGCCT
CTATGGTAGAATCAGTCGGGGGACCTGAGAGGCGGTGTTTACCCTGCGGCGGATGTCAGCGGTTTCGAGTC

trnA end

CGCTTATCTCCAACCTCGTGAACCTAGCCGATACAAAGCTATATGACAGCACCCAATTTTCCGATTTGGCG

←Primer LCV2D = RB of LCV2

gttcgatctatgatttatcattcatggacgttgataagatccatccatttagcagcaccttaggatggcat

←Primer P6

agccttaaaattaaggcgag

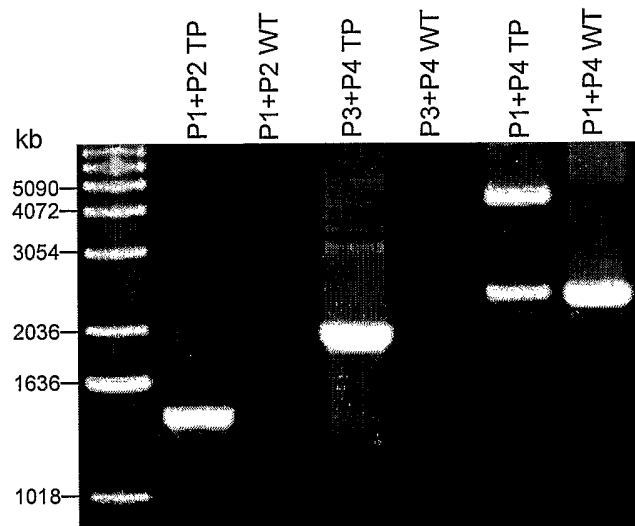


Fig. 15

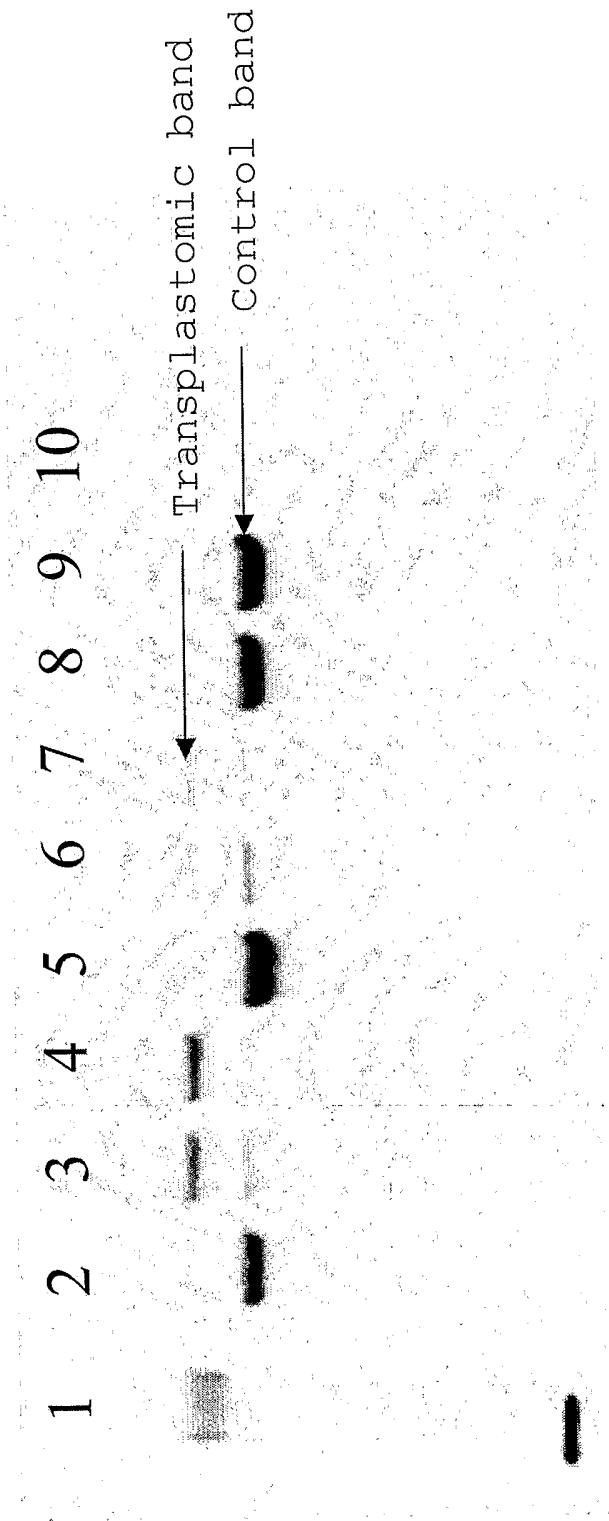


Fig. 16

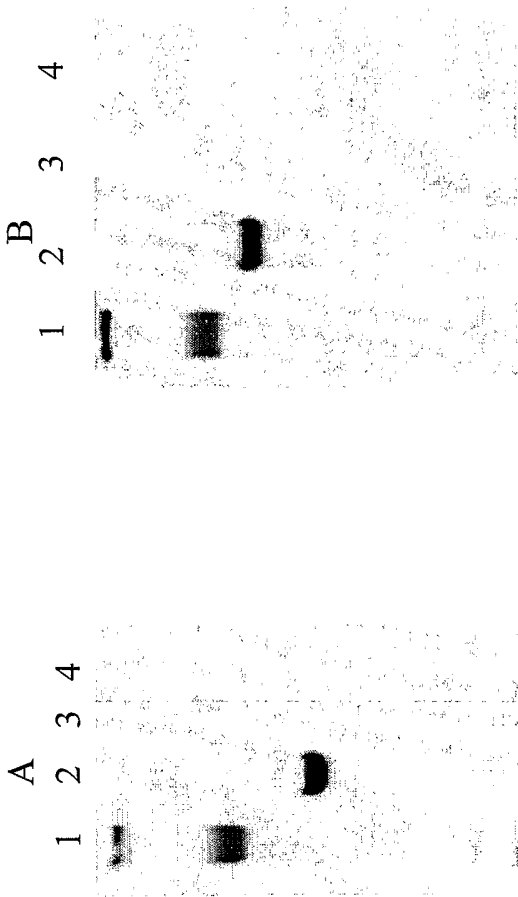
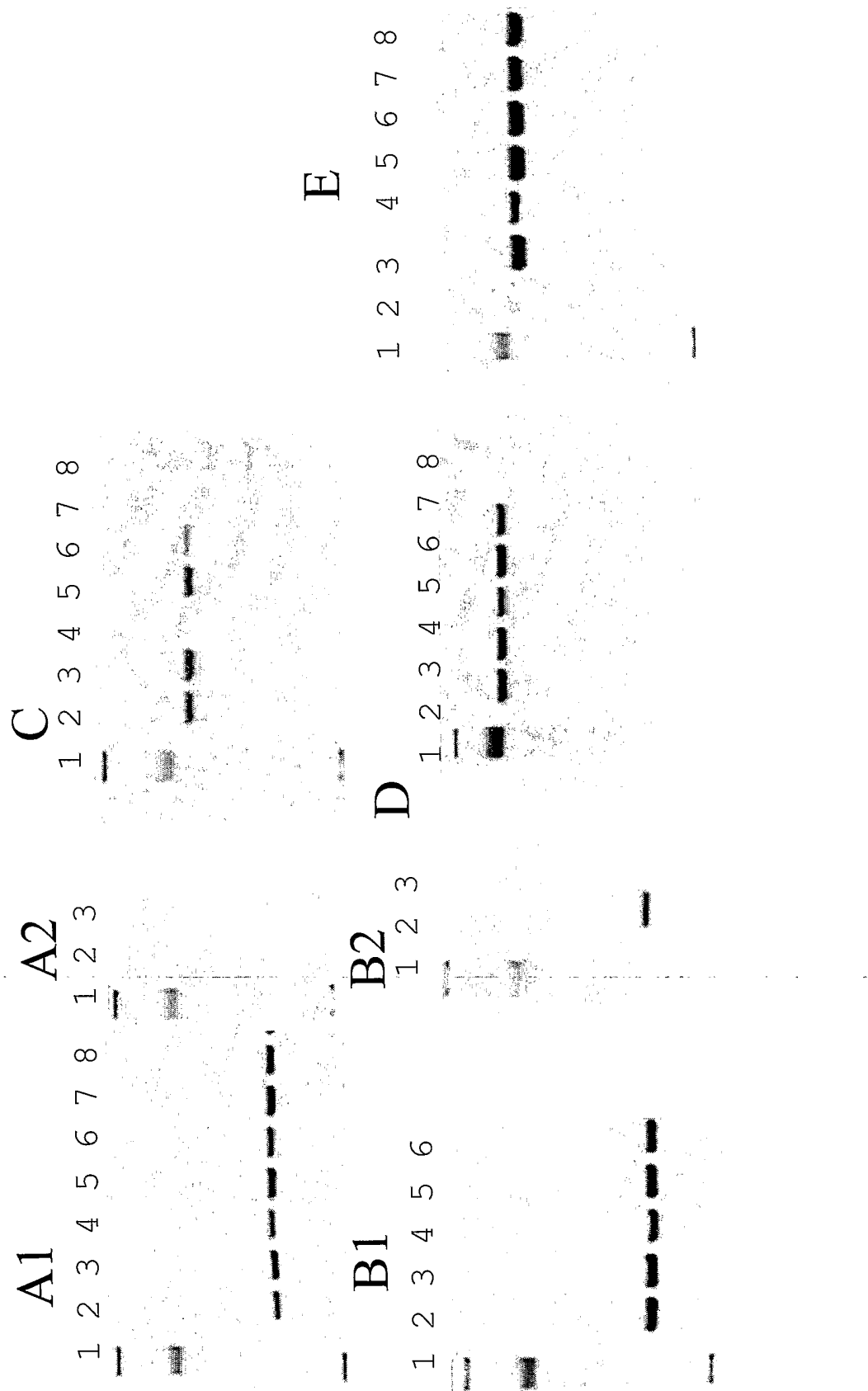


Fig. 17

Fig. 18



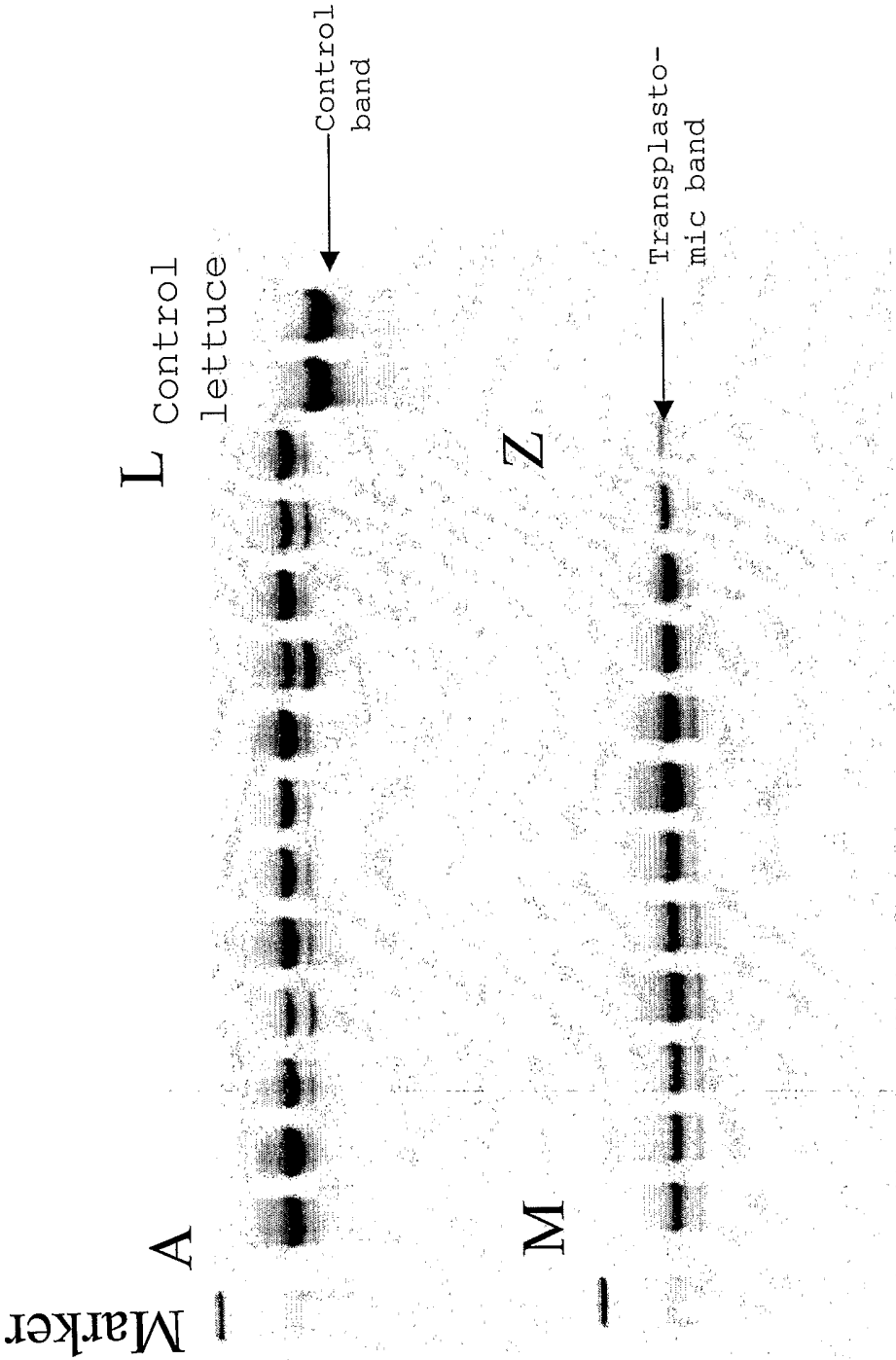


Fig. 19

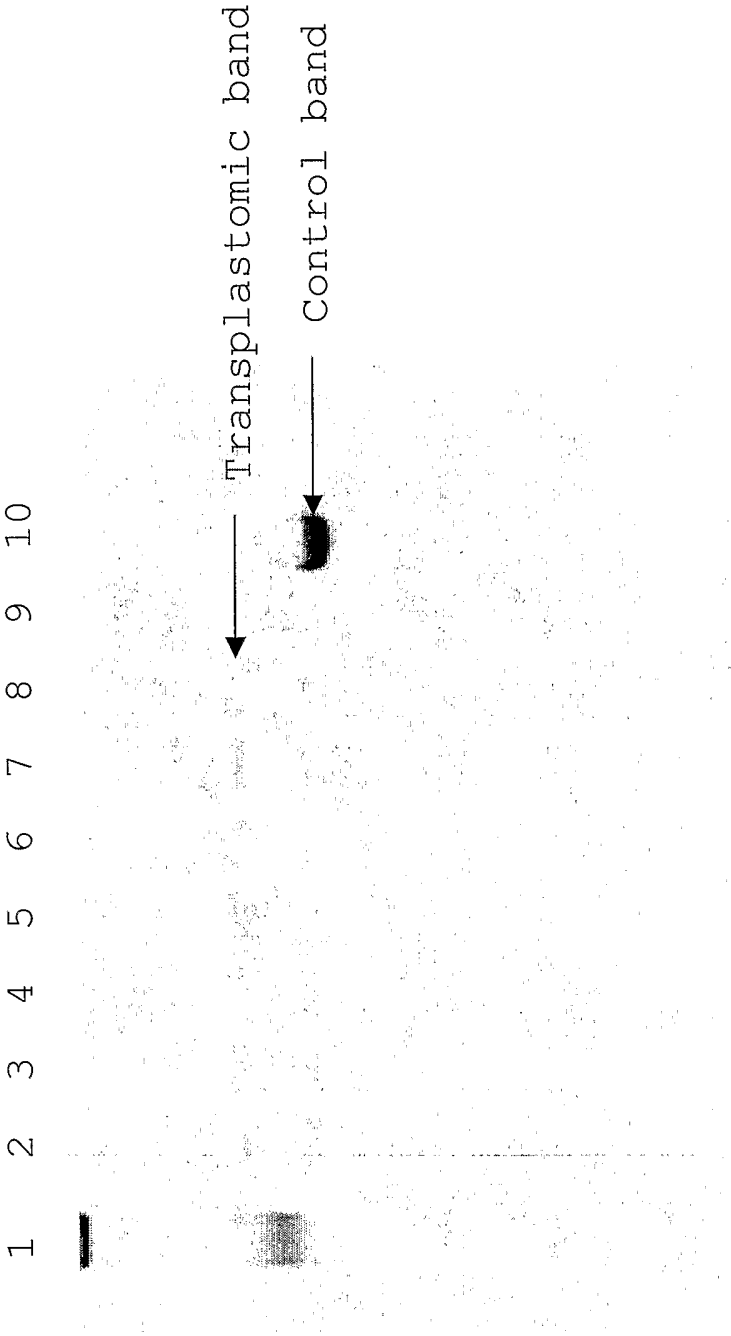
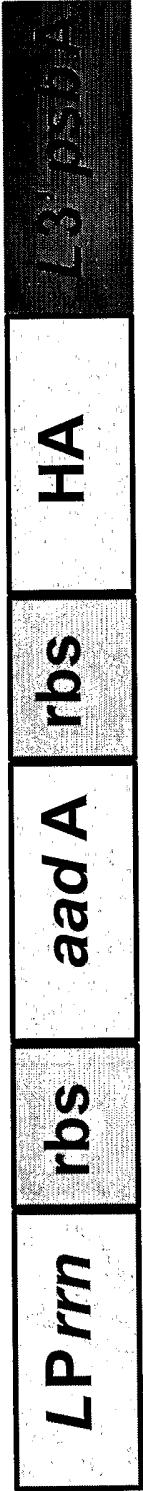


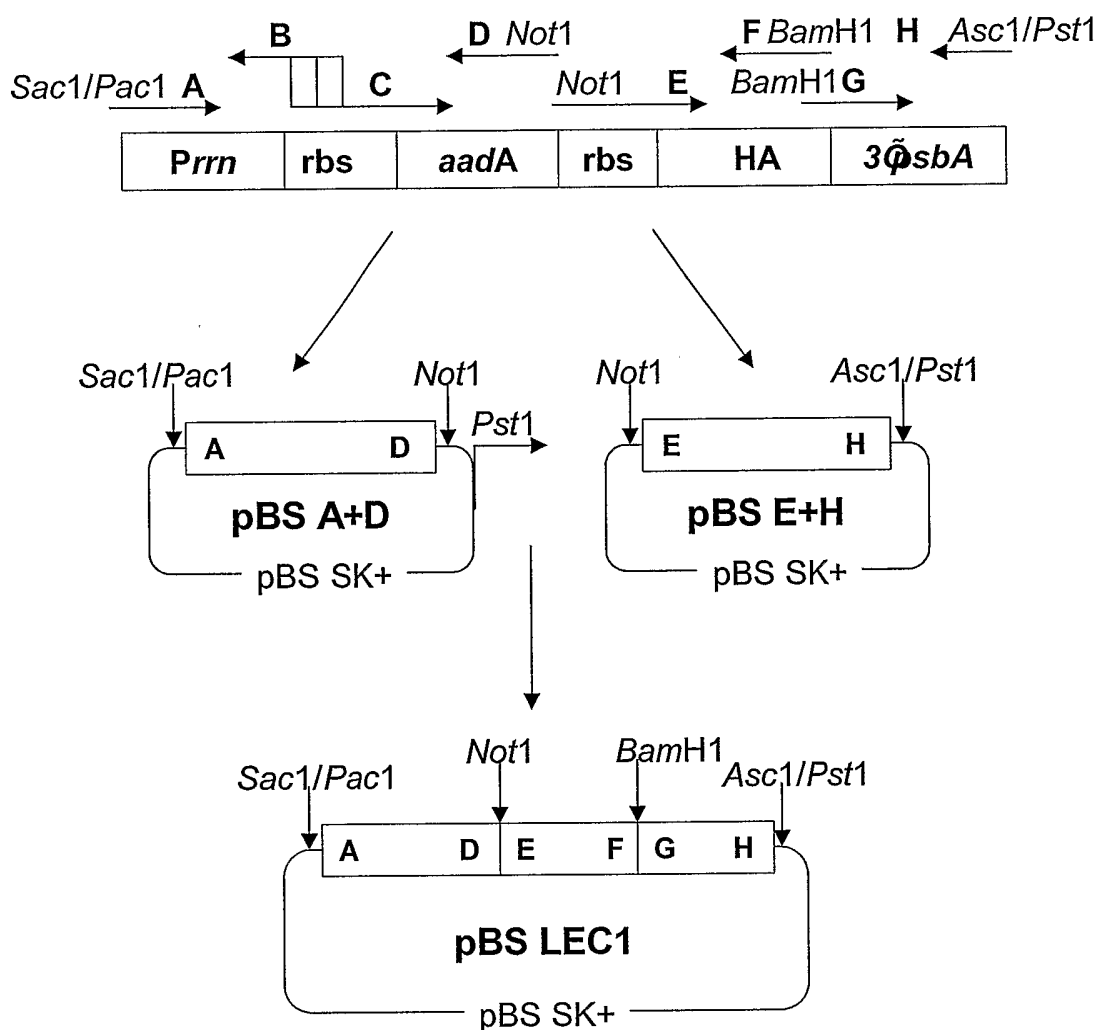
Fig. 20

Fig. 21

LEC1 (dicistronic)



LEC1 construction

List of PCR primers used in LEC1 construction:

LEC1 A	tgc agc tct taa tta agc tac ccc gcc gtg att gaa tga gaa t (SEQ ID NO:23)
LEC1 B	aaa tcc ctc cct aca act gta tcc aag cgc ttc gta ttc gc (SEQ ID NO:24)
LEC1 C	gtt gta ggg agg gat tta tgg cag aag cgg tga tgc ccg aa (SEQ ID NO:25)
LEC1 D	tgc cgg ccg ctt att tgc cga cta cct tgg tga t (SEQ ID NO:26)
LEC1 E	tgc cgg ccg cag ttg tag gga ggg att tat gca aaa act tcc cgg aaa tga caa (SEQ ID NO:27)
LEC1 F	gga tcc tta gta tcc tga ctt cag ctc aac (SEQ ID NO:28)
LEC1 G	aac att taa gga tcc gac ttt ggt ctt att gta att gta tag (SEQ ID NO:29)
LEC1 H	atc tgc agg gcg gcc atc cac ttg gct aca tcc gcc (SEQ ID NO:30)

Fig. 22