Title: TRANSGENIC CONTAINMENT SYSTEM THROUGH THE RECOVERABLE INHIBITION OF THE GERMINATION IN TRANSGENIC SEEDS

Abstract: The present invention relates in general to a transgenic containment system through the recoverable inhibition of the germination in transgenic seeds. In particular, the invention concerns a genome construct which can be inserted into the genome of a crop and which is able to block in a reversible and controllable way a determined fundamental function for the development of a new generation.
DESCRIPTION

"TRANSGENIC CONTAINMENT SYSTEM THROUGH THE RECOVERABLE INHIBITION OF THE GERMINATION IN TRANSGENIC SEEDS"

TECHNICAL FIELD OF THE INVENTION

The present invention relates in general to a transgenic containment system through the recoverable inhibition of the germination in transgenic seeds. In particular, the invention concerns a genic construct that can be inserted into the genome of a crop and which is able to block in a reversible and controllable way a determined fundamental function for the development of a new generation.

BACKGROUND

The spread of transgenes from genetically modified crops to organic crops or to wild generation remains a source of public and scientific concern. Indeed, transgenic plants which are engineered for the production of pharmaceutics, biofuel, specific chemicals are arising a great interest from a commercial point of view.

There derives that there are necessary methods that maintain these genes and their products under strict control in order to avoid contamination for instance in the food chain.

The co-existence of transgenic and non-transgenic crops has been so far possible only through the development of transgene containment strategies. Moreover, for those crops wherein seed-set is required from the market, the irreversible block of out-crossing strategies cannot be used. For these crops, however, the dispersal of seeds from genetically modified plants during harvest, transportation and planting creates a mix population.
Germination of spontaneous seeds creates the possibility for gene transfer to sexually compatible crops in the successive generations and the production of fertile hybrids.

The planning of a transgene containment system through the recoverable inhibition of germination of transgenic seeds would block spontaneous seed germination allowing a safe co-existence between genetically modified and non-genetically modified crops.

To solve the above mentioned problem, two different systems of recoverable seed sterility have been substantially proposed.

A first system named "terminator" (terminator) (US 5,723,765) is based on the seed-suicide mechanism. This system uses an exogenous stimulus (tetracycline) to control the activation of a site-specific recombinase which will in turn allow the expression of the cytotoxic ribosome-inhibitor protein (RIP) specifically in the seed. Seeds of transgenic plants not treated with tetracycline are capable of germinating under natural conditions while treated seeds dye during late embryogenesis.

Alternatively, it has been proposed the use of a method for the regulation of the transgene expression through "receptor DNA cassettes" (receptor DNA cassette) and chemical ligands of constructs activation. For example, unrecoverable blocks (arrest) of embryo development have been obtained in *Brassica napus* by the expression of the exotoxin A of *Pseudomonas aeruginosa* under the control of the napin promoter.

Another system that has been used in order to prevent spontaneous seeds germination is represented by the "Recoverable Block of Function" (RBF) (US 6,849,776). This strategy takes advantage of the presence of a
blocking sequence linked to the gene of interest and a recovering sequence, all in one transformable construct. Kuvshinov et al. (2001) reports an RBF example based on the blocking construct BARNASE and on the recovery construct BARSTAR in tobacco. When transformed seeds are dispersed the activity of the BARNASE construct causes RNA degradation following germination. In the recoverable construct, the recovery activity BARSTAR is under the control of a heat shock promoter so that fertility can be restored through temperature or osmotic shocks.

The serious problem that verifies with the use of the terminator system resides in the fact that this technique does not really prevent gene escape. In fact, seeds development is inhibited in the second generation. Without the mechanism of suppression, the 'killer' gene is activated during late stages of embryo development of the progeny and the seeds of the successive generation do not germinate. If transgenic plant carrying the tetracycline construct are spread into the environment, they are able to germinate, grow to maturity, flower and sexually reproduce. There derives that the control for which the system was created is completely lost.

A further problem linked to all kind of the unrecoverable systems is that there cannot be newly used the seeds once the system is activated.

For this reason, the terminator system has not encountered appreciation in the sector. Moreover, in this way, the companies producing seeds so modified have the complete control on the production of transgenic seeds.

The above mentioned RBF system solves the problem of unrecoverability since the blocked function is not recovered under natural
conditions, but is capable of being recovered only when an external controllable treatment or intervention is applied. Nevertheless, experimental data show that this system works well in tobacco but it does not work as efficiently in other species. Moreover, the BARNASE-BARSTAR strategy is highly dependent on a tightly controlled activity of the constructs promoters since even a minimal transcriptional level can lead to the precocious expression of the BARNASE gene and to cell death. There derives that the success of this approach depends on how well the block and control construct are regulated and on whether they show a balanced expression, as an expression from moderated to high is necessary for the BARNASE activity to be inhibited by the recover construct. A basal expression, on the contrary, of BARNASE activity, even if at very low levels, can be sufficient to cause the cell death. This is a fundamental problem especially for those species which can be transformed only passing through in vitro cultures. In this case, during calli development part of the tissue specific promoter can be activated leading to the expression of the blocking construct.

Summary of the invention

The technical problem at the base of the present invention is to provide a transgene containment system which works through the recoverable inhibition of germination in transgenic seeds in a safe way without the danger of transgene dissemination in crop of no interest and without the creation of uncontrollable fertile hybrids. Another problem is that of providing a system working in a really effective way.

Such problems are solved thorough the use of a genetic construct which can be inserted in a plant genome in a stable way so that if the embryo
carrying this construct does not undergo environmental stimuli, the deriving seeds do not complete their maturation. On the contrary, only if such embryo is stimulated with specific and selected environmental stimuli, the seed germinates originating a new progeny.

A first object of the invention is therefore to make available a genetic construct comprising a genic block element and a genic recoverable element operably associated.

A second object is to offer a method for the transgene containment in sexual reproducing genetically modified plants.

Further characteristics, objects and advantages of the present invention will be more evident in the following detailed description of a realization provided purely as a non limiting example with reference to the figures.

DESCRIPTION OF THE FIGURES

- Figure 1 represents the nucleotide sequence of the AGL23 gene from Arabidopsis thaliana;
- Figure 2 represents the nucleotide sequence of the PHERES promoter (pPHERES);
- Figure 3 represents the nucleotide sequence of the LEC2 promoter (pLEC2);
- Figure 4 represents the nucleotide sequence of the precursor of the artificial micro RNA amiR-BnAGL23;
- Figure 5 represents the sequence of the BnAGL23 gene;
- Figure 6 represents the sequence of the mutated BnAGL23 gene;
- Figure 7 represents the sequence of the Pheres promoter: amiR-BnAGL23 construct;
- Figure 8 represents the sequence of the LEC2::amiR-BnAGL23 construct;
- Figure 9 represents the nucleotide sequences of the Atp1552, Atp1553, Atp1554, Atp 1555, Atp983, Atp984, Atp1556, Atp1557, Atp1558, Atp1559, Atp1575, Atp1576 oligos;
- Figure 10 represents the pBGW,0 vector;
- Figure 11 represents the NOB1 plasmid;
- Figure 12 represents the NOB2 plasmid;
- Figure 13 represents the nucleotide sequences of the Ve49 e Ve50 oligos;
- Figure 14 represents the NOB4 plasmid;
- Figure 15 represents the pHETERS::GUS construct;
- Figure 16 represents the pLEC2::GUS construct;
- Figure 17 represents GUS sequence;
- Figure 18 represents the NOB7 plasmid;
- Figure 19 represents the NOB8 plasmid;
- Figure 20 represents the NOB9 plasmid;
- Figure 21 represents the nucleotide sequences of the Bnp1, Bnp2, Bnp3, Bnp4 oligos;
- Figure 22 represents the NOB10 plasmid;
- Figure 23 represents the recombination between NOB 419 plasmid and NOB 10 plasmid which gives the NOB11 plasmid;
- Figure 24 represents the NOB5 plasmid;
- Figure 25 represents the transformation of the NOB11 plasmid with the genic element reported in figure 7 to give the NOB12 plasmid;
- Figure 26 represents NOB 13 plasmid;
- Figure 27 represents the NOB 6 plasmid;
- Figure 28 represents the NOB14 plasmid;
- Figure 29 represents the NOB16 plasmids;
- Figure 30 represents the pPHRES::ami23 construct (of Seq. ID n. 29).

**DETAILED DESCRIPTION OF THE INVENTION**

The idea at the base of the invention is to contrive a transgene containment system of the type of a recoverable block of the function by using a function of some genes which does not involve the above mentioned problems linked to the known systems.

Studies on a group of plant transcription factors named MADS-box and identified also in even very distantly related species have been performed. These transcription factors are responsible for the control of a wide range of developmental processes, including the flowering time, the transition from the vegetative to the reproductive phase, as well as floral meristem identity, floral organs identity and ovule identity. The choice fell on MADS-box transcription factors in the light of their known role in many developmental processes.

*Arabidopsis thaliana* MADS-box genes comprise 107 identified members; among which only for about 20 of them the function is known.

In particular, there have been studied some genes of plants in order to
find new functions correlated with seed maturation. Said genes have been subjected to mutations in order to allow a permanent block of their function. In other words, a genic expression construct has been ideated and generated wherein various genic elements have been associated to each other in order to block seeds maturation in a crop.

Following many experiments, it has been found out that the AtAGL23 gene from Arabidopsis thaliana (At1G65360), having the sequence SEQ. I.D. N.1 reported in figure 1, plays a role in the regulation of megagametogenesis and in embryo development.

In particular, it has been found that the AGL23 gene plays a key role in the regulation of chloroplast biogenesis during embryo development: in fact the destruction of AGL23 provokes the formation of a seed containing an albino embryo. The agl23/agl23 homozygous embryos cannot complete the chloroplast biogenesis and dye.

On the base of these observations, it was thought to develop a construct comprising a genetic block element having a genic sequence which is able to inactivate the AGL23 gene, and a recoverable element operably associated to that genetic block element having an AGL23 sequence insensitive to said inactivation genetic sequence. The version of the AGL23 sequence insensitive to the inactivation genetic sequence is under the control of a promoter sequence which can be regulated by external factors.

The first object of the present invention is therefore a genic construct for the recoverable inhibition of the germination of transgenic seeds, comprising:

a genic element which blocks the embryo development
comprising a vegetal embryo promoter operably associated to a nucleotide sequence capable of inactivate a gene involved in such embryo development; a genetic element for the recovery of embryo development comprising an inducible promoter operably associated to a sequence of said gene involved in embryo development which is mutated in order to result insensitive to such sequence capable of inactivating a gene involved in embryo development but active in the function of embryo development; The construct of the invention can comprise one or more genes or transgenic nucleotidic sequences of interest. Such genes or sequences can be represented by genetic elements encoding for a genetic product having a desired function like a protein, an enzyme including metabolites, hormones, antibiotics or toxins. Moreover, these genetic elements are operably associated in the construct in order to be contained between its right and left ends. In particular, they will be located in the proximity of the genetic block element.

The genetic element for the block of embryo development is represented by one or more nucleotidic sequences operably associated together so that their expression might cause the death or at least the alteration of the phenotype or of the physiology of the natural host organism. This construct therefore allows the containment of seeds which have been possibly spread in the environment during harvest and/or transportation, since such seeds are not able to originate adult and vital plants.

Also the seeds produced from random crossings between engineered
plants and wild varieties (or non-engineered) would be unable. Modified crops and wild relatives (non genetically modified) would not be able to germinate and, as a consequence, also the dispersion of the pollen produced by transgenic individuals would be controlled. This results has been obtained, as it will be described in detail in the following, allowing the activation of the blocking genetic constructs under normal conditions, natural, i.e. without any external interventions. There derives that the block of the embryo development occurs automatically, planned during a phase of embryo development without interfering with female and male gametophytes maturation.

The genetic element for blocking the embryo development comprises a vegetal embryo specific promoter like, for instance, the PHERES promoter, the LEC2 promoter or the napin promoter (Josefsson, 1987).

Preferably, the promoter is pHERRER or pLEC2 which nucleotide sequences SED I.D. N.2 and SEQ I.D. N. 3 are represented in figure 2 and 3 respectively.

The promoter has been chosen on the base of its capability of activating only during the embryo development of a plant, therefore, without interfering with female and male gametophytes maturation.

Said promoter is then operably associated to a nucleotide sequence able to inactivate a gene involved in a plant embryo development. Such sequence may be any sequences designed in order to express in turn a nucleotide sequence which is able to bind to an active site of a gene involved in the embryo development to inactivate it, thus blocking the normal embryo development of a plant.

In particular, said sequence is a sequence which hybridizes with a
target sequence to be inactivated and it is able to provoke a brakeage inside it. Sequences of this kind can be designed using technologies such as the PCR. Preferably, the sequence able to inactivate said target sequences or genes is a sequence which encodes for the expression of an artificial micro RNA (amiRNA) or for a small interference RNA (siRNA).

Among the target sequences, as previously described, the plant MADS-box sequences represent those preferred for the present invention. There have been selected for the study mutations of the MADS-box sequences of the plant Arabidopsis, as previously reported, and it has been surprisingly found that among the numerous mutations of said MADS-box, a mutation of the AGL23 gene is responsible of the embryo development. In particular, it has been observed that the destroy of the AGL23 gene with the agl23-1 mutation due to the insertion of a T-DNA element (Salk line_147048, ecotype Col-0 (Alonso et al., 2003) causes the formation of an albino embryo unable to germinate and to develop an adult plant. As a consequence, the non mutated gene AGL23 is responsible for the chloroplast biogenesis.

Therefore, the sequence able to inactivate the target gene preferably is a sequence amiRNA SEQ I.D. N. 30 (Figure 4a), projected to hybridize with the AGL23 gene of Arabidopsis thaliana and to inactivate it through breakage.

It has to be noted that other variants of the SEQ I.D. N. 30 can be used. Such variants can be represented by sequences having an identity of at least 70%, preferably at least 80%, with said sequence SEQ I.D. N. 30. Examples of such variants are:

5'-'TTAATGGAGACTTACTCGGCC-3' (SEQ ID 31), and

5'-'TGAACACGTATCATACAGCTT-3' (SEQ ID 32).
There have been obtained experimental data in the model species *Arabidopsis thaliana*, which strongly support the functionality of the blocking construct proposed in the present patent. Wild-type Arabidopsis plants have been transformed with a construct composed of the seed-specific promoter (pPHERES) regulating the expression of a amiRNA sequence which recognizes and silences the AGL23 gene (Figure 30). The transgenic plants thus obtained show a phenotype identical to the mutant for insertion of a T-DNA element (Salk line_147048, ecotype Col-0. These data demonstrate that the chosen and cloned promoter works actively during the seed development and does not interfere with the preceding development of the female gametophyte, but they overall demonstrate that the AGL23 gene can be efficiently silenced by an amiRNA. Considering that Arabidopsis is a phylogenetically very closely species to Brassica, as demonstrated by the often demonstrated nucleotidic analogy between Arabidopsis and Brassica genes, the obtained results effectively support the proposed invention.

The construct of the invention comprises also a genetic element to recover the embryo development which is able to recover the blocked function, thus it is able to reactivate the normal embryo development, following a specific external stimulus.

To this end, this element has an inducible system which is able to react to said external stimulus activating the transcription of the nucleotidic sequence operably associated to it. Inducible systems are known in literature (Wang et al., 2003) and in general each of these systems can be used in the present invention.

Preferably, the inducible system is chosen among the system mediated
by glucocorticoids (positively regulated by dexamethasone), the heat shock induced system (which were already used with success in the model species *Arabidopsis thaliana* (Sablowski e Meyerowitz, 1998) and the ethanol inducible system. It is preferred the use of the ethanol inducible system, which comprises an inducible pALC promoter which is activated by the ALCR protein, which is present in the cell but in an inactive form, which is in turn activated by the external stimulus represented by the ethanol. The just described system is known in literature (Roslan et al., 2001; Laufs et al., 2003).

The genetic recovery element can comprise a constitutive promoter operably associated to a gene which synthetizes a protein which, when activated by the external stimulus, is able to bind to the inducible promoter and to activate in turn its transcription.

In particular, the constitutive promoter can be chosen among the constitutive promoters known in literature, like for instance the 35S-CaMV, the ubiquitin promoter, the actin and tubulin promoter. Preferably, the constitutive promoter is the 35S-CaMV, which regulates the production of the ALCR protein, which responds to the action of the ethanol. Treatment with ethanol induces a conformation modification in ALCR and it becomes able to bind to the pALCA promoter. pALCA regulates the transcription of the sequence of the gene involved in embryo development mutated in order to be insensitive to the action of the sequence which inactivates the genes which control the embryo development. Nevertheless, the mutated version is an active version in relation to the embryo development promoting function.

The sequence of genes involved in embryo development mutated in
order to result insensitive to the above said sequence capable to inactivate the
gen genes involved in embryo development but active in its function is downstream
associated to said inducible promoter.

It has to be noted that such sequence has been projected by creating a
mutation, alteration, deletion or other modification in nucleotides so that the
sequence of the genetic block element is no more able to bind it, hybridizing
and carrying out the in activation function. In other words, the created
modification is such to make no more recognizable the sequence of recovery
of the blocked function from the inactivation sequence.

Such modification will be made by the technician in the field by
adopting known procedures such those available in the recombinant DNA
technology, for example for obtaining a point mutation.

In any case, the modification has to be projected in order to maintain
the functionality of the original sequence thus to recover the natural function.

This result can be achieved considering the degeneracy of the genetic code,
therefore, the last nucleotidic base of a triplet encoding for an amino acid can
be substituted without altering the correct translation. Its alteration is useful in
order to provoke the non-recognition by part of the blocking sequence.

A system particularly useful to make a sequence insensitive to a
blocking sequence but maintaining its natural function is the one which uses of
the amiRNA or siRNA technology above hinted and following detailed.
In fact, if the recovery sequence is modified even through the substitution of a
sole nucleotide, the blocking sequence is no more able to hybridize, i.e. to
recognize its target.

Taking advantage of this concept, the original sequence of the AGL23
gene from Brassica napus SEQ. I.D. N. 5 (BnAGL23) (Figure 5) has been preferably modified by introducing a mutation in position 405 from A to G, as it will be described in details in the following.

The sequence of the genes involved in the embryo development mutated in order to be insensitive sequence of in activation of such genes but active in its natural function is therefore preferably represented by the mutated sequence BnAGL23 SEQ. I.D. N. 6 (figure 6).

In accordance with a further object of the present invention, the method for the preparation of a genetic construct for the recoverable inhibition of the germination in transgenic seeds as previously described, comprises the steps of:

1) isolating from of the genome of interest the nucleotidic sequence of a vegetal embryo promoter;
2) amplifying said sequence;
3) digesting the sequence with specific restriction enzymes in order to allow the insertion into a suitable cloning plasmid;
4) digesting of a suitable cloning plasmid with the same restriction enzymes used in the preceding step to allow the integration of said sequence into said plasmid;
5) inserting said embryo promoter into said plasmid;
6) isolating from the genome of interest a target sequence;
7) amplifying said sequence;
8) synthesizing a sequence capable of hybridizing and inactivating such target sequence;
9) cloning said sequence which is able to hybridize and inactivate the
target sequence in suitable plasmids;

10) Recombinating the products of phase 5) and phase 9) to obtain a genetic element for blocking the embryo development;

11) synthesizing a nucleotidic sequence of a gene involved in embryo development mutated in order to be insensitive to said inactivating sequence;

12) recombinating the product of step 11) in a suitable vector;

13) isolating from the genome of interest the nucleotide sequence of an inducible promoter;

14) amplifying said sequence;

15) digesting the sequence with suitable restriction enzymes to allow the insertion in a suitable cloning plasmid;

16) digesting a suitable cloning plasmid with the same restriction enzymes used in the previous step to allow the integration of said sequence into said plasmid;

17) integration of the product from step 15) into the plasmid of step 16);

18) ligating the insert of the vector of step 12) and the linearized plasmid from step 16) in order to obtain a plasmid containing the genetic element of recovery of the embryo development;

19) recombinating the products from step 12) and step 18) to obtain the genetic construct for the recoverable inhibition of the germination in transgenic seeds.

Each of the above described steps is realized using techniques of molecular biology known in the field and which can be adated by a technician depending on the specific type of sequence, promoters and other specific
genetic elements.

Moreover, further steps can be foreseen to insert further genetic elements and sequences previously described with reference to the construct of the invention, in the substantially same way.

In accordance with a further object of the present invention, the genetic construct previously described is inserted into a suitable host which is able, when in contact with the target organism of interest or part of it like its gametes, to infect and thus to transfer the construct.

Therefore, the method comprises the steps of:

a) providing a genetic construct for the recoverable inhibition of the germination in transgenic seeds as above described;

b) inserting said construct into an host organism which is able to transform a target organism or parts of it.

The genetic construct can be cloned and transferred into vectors for the heterologous expression, like binary vectors, using the known techniques in the field. Preferably, it can be used the Gateway system (Invitrogen) summed up in the following. Through such system, cloning plasmids containing the construct of interest are inserted for instance through electroporation, into a host among those commonly used to transfer transgenes into vegetal organisms.

In particular, it can be used Agrobacterium tumefaciens as a host to transform Brassica napus.

Hereby a specific realization of the invention will be reported as a purely non-limitative example.

PREPARATION OF THE CONSTRUCTS
The blocking construct is necessary for the degradation of the endogenous RNA messenger produced by the gene BnAGL23. It is composed of an embryo-specific promoter that drives the transcription of the artificial micro-RNA specifically directed toward BnAGL23 mRNA (the artificial micro-RNA will be from now onwards indicated as amiR-BnAGL23). Ami-RNAs are short sequences 21 nucleotides long complementary to the endogenous RNA messenger to be desiderably degraded, therefore amiR-BnAGL23 causes the specific degradation of BnAGL23 mRNA. The bases in position 10-11 are strategic for the recognition and the pairing to the endogenous messenger which is desired to be silenced, if a mis-match is introduced in the above said positions the degradative property is irremediably lost (Schwab et al., 2006).

Two blocking constructs have been prepared which differ for the use because there have been used two embryo-specific promoters active in two different phases of the embryo development. The promoter which drives the transcription of the gene PHERES (pPHERES) is active in the initial phases of the embryo development, from phases immediately successive to the fertilization up to the globular embryo stage (Khoeler et al., 2003). The promoter of the gene LEC2 (pLEC2) on the contrary is active during the final phases of the embryo development, from the globular embryo stage up to the torpedo stage (Kroj et al., 2003). The blocking construct with the amiR-BnAGL23 regulated by pPHERES will be indicated as PHERES::amiR-AGL23 (SEQ. I.D. N.7 represented in figure 7), while the construct amiR-BnAGL23 regulated by pLEC2 will be named LEC2::amiR-AGL23 (SEQ. I.D. N.8 represented in figure 8).

Amplification of the promoters
The promoters pPHERES and pLEC2 have been amplified by the genomic DNA extracted from *Arabidopsis thaliana*. pPHERES has been amplified with the primers Atp1552 of SEQ ID n. 9 and Atp1553 of SEQ ID n.10 (figure 9), while pLEC2 has been amplified with Atp1554 of SEQ ID n 11 and Atp1555 of SEQ ID n. 33 (figure 9). Atp1552 and Atp1554 contain a XbaI restriction site, while Atp 1553 and Atp 1555 possess a site recognized by AatII. The introduction of the above said restriction sites has been necessary for the cloning of the two promoters in the pBGW plasmid. The PCR reaction has been performed using the Taq phusion (Finnzymes), taq polymerase with high fidelity and high processivity, indeed the error rate of this polymerase has been determined in $4.4 \times 10^{-7}$ and also thanks to its exonucleasic activity it is able to correct possible inserted mis-matches. For the amplification 10 ng of genomic DNA of *Arabidopsis thaliana* have been used. As specifically requested for this enzyme the denaturation is performed at 98°C. The Taq phusion (Finnzymes) has a processivity of 4 kilo-bases/minute. 35 cycles of amplification have been performed. The PCR products, after having been checked on 1% agarose gel, have been purified with the employment of the "QIAquick PCR purification kit (Qiagen)" following the enclosed instructions. The PCR products have been then digested with XbaI-AatII (Roche), the double digestion has been carried out in buffer A (33 mM Tris-acetate, 66mM K-acetate, 10 mM Mg-acetate, 0.5 mM dithiothreitol, pH 7.9) following the instructions supplied by Roche. The reaction has been incubated at 37°C for 2 hours. Also the pBGW plasmid has been digested with XbaI-AatII (Roche). pBGW is a gateway vector (Karimi et al., 2002) with a pollilinker placed upstream the gateway cassette (figure 10).
Subsequently, the plasmid digested with XbaI-AatII and the two PCR fragments (pPHERES and pLEC2) digested with XbaI-AatII have been purified from gel employing the QIAquick Gel Extraction Kit (Qiagen). For each ligation reaction there have been used 10 ng of pBGW, digested with XbaI-AatII and then purified, and about 20 ng of the two inserts (pPHERES and pLEC2) they also digested and purified. For the reaction there have been used the T4 ligase produced by Promega, incubating at 16°C for 12 hours.

The vectors obtained in this way have been named NOB1 (pPHERES:pBGW) (figure 11) and NOB2 (pLEC2:pBGW) (figure 12).

amiR-AGL23

The gene BnAGL23 (the homologous of AGL23) in Brassica napus has been amplified using the primers Atp983 of SEQ ID n. 12 and Atp984 of SEQ ID n. 13 (figure 9). The PCR product to be conveniently sequenced has been cloned in the vector pGEM®-T Easy. The vector pGEM®-T Easy has been prepared by the Promega supplier by cutting the vector pGEM®-5Zf with EcoRI and adding a terminal thymine to the two 3' ends. This vector allows an easy cloning of the templates if the Taq employed for the production of the fragment of interest adds some "Adenines (A)" to the ends of the fragments. Since the gene BnAGL23 has been amplified with the enzyme Taq phusion (Finnzymes), unable to add the A to the ends of our amplified, these have been added in a subsequent phase through incubation at 72°C for 20' with the Taq polymerase supplied by Roche. The PCR product has then been purified with the "QIAquick PCR purification kit" and then ligated in pGEM®-T Easy following the indications provided by Promega. In particular the reaction has been performed in a final volume of 20 µl and incubated for 2 hours at room
temperature.

Comparative analyses between the sequences AtAGL23 and BnAGL23 have revealed a very strong conservation of the two genes in the two species of interest.

amiR-BnAGL23 has been generated following the algorithm and the protocol found in the following site: http://wmd.weigelworld.org (Schwab et al., 2006). According to such program, the best artificial micro directed against BnAGL23 is the following: 5'-GGTGGAGTAAGTCTCCATTAG-3'. This sequence is used to silence specifically BnAGL23. It is inserted with the methodology described here below inside a gene, which therefore produces the precursor of BnAGL23.

Shortly, the precursor of amiR-BnAGL23 is generated with 4 subsequent and overlapped cycles of amplifications. The first reaction forsees the use of the primers Ve50 of SEQ ID n. 21 (figure 13) with Atp 1559 of SEQ ID n. 17 (figure 9) (the amplified fragment is designated Te1); the starting template is the vector pRS300. In the second reaction the template is always the pRS300 plasmid, but it is amplified with Atp 1557 of SEQ ID n. 15 and Atp 1558 of SEQ ID n. 16 (figure 9) (the product is named Te2). Finally, in the third amplification, pBRS300 is amplified with Ve50 and Atp 1556 of SEQ ID n. 14 (the product is named Te3). 0.5 μl of the PCR products Te1-Te2-Te3 are mixed and further amplified using Ve49 of SEQ ID n. 20 and Ve50 (the final template is named Te4). For the PCR reactions here described we have employed Taq phusion (Finnzymes). All the PCR products (Te1, Te2, Te3 and Te4) have been separated on a 2% agarose gel, and extracted with the QIAquick Gel Extraction Kit (Qiagen).
Te4 has been then cloned in pENTR™/SD/D-TOPO (Invitrogen) generating the Nob4 represented in figure 14. Indeed, Ve49 possesses the extension (CACC) necessary for Te4 to enter the pTOPO. The reaction has been prepared in a final volume of 10 µl containing 10 ng of the purified PCR product. The reaction mixture has been then incubated at 22°C for 30 minutes and 2 µl have been used for the subsequent electroporation of the cells of E. coli.

The final constructs pPHERES::amiR-BnAGL23 and pLEC2::amiR-BnAGL23, have been produced through a Gateway recombination of LR type between NOB4 and the vectors previously described NOB1 (pPHERES::pBBGW) and NOB2 (pLEC2::BBGW).

The Gateway® system (Invitrogen) is a cloning system based on the bacteriophage λ site-specific recombination in Escherichia coli. When the bacteriophage λ infects E. coli bacterial cells it can undertake the lytic or the lysogenic cycle. In the lysogenic cycle, conversely, the bacteriophage integrates itself in E. coli genome thanks to a site-specific recombination process and it is transmitted to the daughter cells from generation to generation, until when there not verify suitable conditions for its excision from the genoma. At this point the bacteriophage begins the lytic stadium of its lifecycle. The processes through which the bacteriophage integrates and excises itself from E. coli genome are mediated by proteins produced both by the bacteriophage and by the guest cell. These site-specific recombination reactions, set in vitro, are at the base of the Gateway® technology and they can be summarized in the following way:

attB X attP attL X attR (X denotes the recombination
INTEGRATION EXCISION

The four att sites are recombination sites of the bacteriophage λ and they contain the recognition sites for the proteins involved in the processes of integration and excision. In particular the reaction attB X attP mediates the process of integration, while the reaction attL X attR mediates the process of excision from the genome. Through the combination of the described reactions, it is possible to clone and transfer the genes to different vectors of heterologous expression (in the binary vector to transform *Brassica*), maintaining unchanged the orientation and the reading frame. This makes the Gateway technology highly versatile and with high processivity.

To put amiR-BnAGL23 under the control of pHERES and pLEC2 a LR recombination reaction has been set in a final volume of 10 µl. The reaction has been incubated at 25°C for about 3 hours. Subsequently, 1 µl has been used for submitting *E. coli* to electroporation. The final plasmids have been checked, extracted by *E. coli* and introduced through electroporation in *Agrobacterium tumefaciens*, the microorganism that will be then used for transforming *Brassica napus* with the purpose to check the goodness of the construct. Nevertheless the generation of these plasmids is also necessary for obtaining the final construction of interest, in fact the purpose is that of insert the blocking construct and the recovery construct inside the same T-DNA so that they are transferred to the *B. napus* genome in a single genetic locus.

For the procedure of electroporation of both agrobacteria and *E. coli* the Electroporator 2510 (Eppendorf) has been used applying a potential difference of 1500 V.
Promoter activity and tissue specific control

Since pPHERES and pLEC2 are regulating sequences of *Arabidopsis thaliana*, it is necessary to check that these two promoters are able to drive the transcription of the genes also in *Brassica napus* embryos. To this end pPHERES and pLEC2 have been cloned upstream the GUS reporter gene (two constructs have been therefore produced, pPHERES::GUS (SEQ. I.D. N. 22) and pLEC2::GUS (SEQ. I.D. N. 23) (figures 15 and 16) just to verify that the product of the GUS gene SEQ. I.D. 24 (figure 17) is tracked in *Brassica* developing embryos.

The coding sequence of the GUS gene has been amplified with Atp 1575 of SEQ ID n. 18 and Atp 1576 of SEQ ID n. 19 (figure 9) and the Taq phusion (Finnzymes) has been used as polymerase for its high fidelity (see above). The amplification product obtained has been purified with the "QIAquick PCR purification kit (Qiagen)". The PCR product has then been cloned in pENTR™/SD/D-TOPO (see above); the CACC extension necessary for the reaction is brought by the primer Atp 1575, the vector thus generated has been called NOB7 (figure 18). NOB7 has been then recombined through a gateway LR recombination reaction (see above) with both NOB1 and NOB2, generating respectively pPHERES::GUS (NOB8 represented in figure 19) and pLEC2::GUS (NOB9, represented in figure 20).

These construc make use of *Agrobacterium* generating two independent strains used for infecting and therefore moving in *B. napus* genome such constructs. Plants positive to the selection have been submitted to GUS assay following the instruction reported by Vielle-Calzada et al. (2000). Therefore developing seeds of different independent lines have
been immersed for two hours in 100% acetone solution and left to -20°C. At the end of these two hours the B. napus transgenic seeds have been moved in the GUS solution and incubated in such buffer at 37°C. Pictures of stained ovules and embryos have been acquired with a microscope Zeiss Axiophot D1 microscope (Jena, Germany) equipped with phase contrast (differential interface contrast, DIC). The images have been captured with a digital camera MRc5 (Zeiss) managed by the program Axiovision (version 4.1).

Recovery genic element

The recovery genic element contains an ethanol inducible promoter which drives the expression of a BnAGL23 version insensitive to the action of amiR-BnAGL23 because, by means of site-specific mutagenesis, a point mutation has been introduced in bases 10-11 recognized by amiR-BnAGL23 and fundamental for the silencing of the gene. When induced, this construct allows to recover the damages caused to the seed by amiR-BnAGL23 action and therefore vital plants are produced only when desired and after the treatment with the ethanol of the recovery construct.

The BnAGL23 mutated version has been obtained introducing a point mutation through PCR, using a couple of primers (which introduce the mutation) complementary between them, each specific for a filament (sense and antisense). In our case there have been used the primers Bnp2 of SEQ ID n. 26 and Bnp3 (figure 21) which change the position 405 of the BnAGL23 sequence from A to G. Bnp2 is used in combination with Bnp1 of SEQ ID n. 25 (figure 21) to generate a first emi-fragment of BnAGL23 (Product1, Pr1), while in a second PCR reaction Bnp3 of SEQ ID n. 27 and Bnp4 of SEQ ID n. 28 (figure 21) generate the second emi-fragment of BnAGL23 (Pr2). The two Pr1
e Pr2 are run on 1% agarose gel and purified. Subsequently 1 µl of Pr1 and Pr2 are used as templates in a PCR reaction with Bnp1 and Bnp4 as primers, generating a unique BnAGL23 fragment containing however the desired mutation. The two Pr1 and Pr2 can anneal thanks to the fact that Bnp3 and Bnp2 are complementary. Taq Phusion enzyme (Finnzymes) has been used for the three PCR cycles.

Bnp1 and Bnp4 primers possess the gateway extensions therefore they can be cloned in pDNR through a BP recombination reaction. In the present case, as pDNR there has been used NOB10 (figure 22), also known as pDONR201 (Invitrogen). BP reaction has been performed in 10 µl incubating at 22°C for at least 12 hours. The pDNR construct containing the BnAGL23 mutated version has been named NOB10.

The inducible system

Several inducible systems are reported and extensively described in literature (Wang et al., 2003), for instance the system inducible by glucocorticoid (Sablowski and Meyerowitz, 1998) or by heat shock (Huang et al., 2005). To verify the present system of genic containment, there has been decided to use the ethanol induced system, already extensively described in literature (Roslan et al., 2001; Laufs et al., 2003).

The ethanol induced system is composed by a ALCR protein that is constitutively expressed since transcriptionally regulated by the 35S Cauliflower Mosaic Virus promoter (CaMV35S=35S). ALCR can bind to the pALCA promoter, nevertheless such binding is possible only in the presence of ethanol. Therefore, any gene which is cloned downstream of pALCA will be transcripted and translated if the plant will be submitted to an ethanol
treatment.

The recovery system contains 35S::ALCR, while pALCA regulates the transcription and therefore the translation of the BnAGL23 mutated version (35::ALCR; pALCA::BnAGL23mutated). In the laboratory of the Department of Biomolecular Sciences and Biotechnology the inventors had already available some vectors for the ethanol inducible system, so it was already available the NOB419 with 35S::ALCR and the NOB425 with pALC::Gateway cassette. NOB419, was kindly provided by T. Laufs and it is described in Roslan et al. (2001). For NOB425, the gateway cassette (frameA) (Life Technologies) has been inserted in pL4 (Syngenta Ltd) exploiting the Smal site between the pALCA promoter and the terminator. The pALCA promoter, the gateway cassette and the terminator have been excised from the plasmid through XbaI digestion and inserted in the pBIN19 (Clontech).

The ultimate purpose is to bring the blocking and the recovery construct on the same T-DNA in order to be inserted in linkage in the transgenic plants. This is necessary to avoid their segregation in the subsequent generations.

Therefore the final purpose was the construction of a final vector 35S::ALCR;pALCA::BnAGL23mut (mutated); PHERES::amiR-BnAGL23 and of a second vector 35S::ALCR;pALCA::BnAGL23 mut;LEC2::amiR-BnAGL23.

The strategy to obtain the final construct

The first passage has been the execution of a gateway LR recombination (for the description of the procedure see above) between the NOB10 and the NOB419 (figure 23). The final vector has been called NOB11 (figure 23) and therefore BnAGL23mutated has been produced (BnAGL23mut)
under the control of the pALCA inducible promoter (NOB11: pALCA::BnAGL23mut).

Subsequently PHERES::amiR-BnAGL23 (the reaction template was the NOB5 represented in figure 24) has been amplified (with Taq phusion, Finnzymes) using the primers Atp1552 and Ve50. These primers introduce a Xbal restriction site, then the PCR product has been digested with this restriction enzyme (Roche, the reaction has been done using the buffer H, composition of the stock 10X: 10 mM Tris-HCl, 0.1 M NaCl, 5 mM MgCl2, 1 mM 2-Mercaptoethanol, pH 8), purified (QIAquick PCR purification kit) and finally ligated with Promega T4 DNA ligase in the vector NOB11. Also NOB11 had been previously digested with XbaI and purified. The vector thus obtained has been named NOB12 (figure 25).

NOB12 has been digested at this point with the Roche restriction enzyme HINDIII (the digestion has been performed at 37°C with the buffer B, composition of the stock 10X: 0.5 M Tris-HCl, 1 M NaCl, 0.1 M MgCl2, 10 mM DTT, pH 7.5), digestion which allows to take off a fragment containing pALCA::BnAGL23mut, PHERES::amiR-BnAGL23. The digestion has been separated on an agarose gel and the fragment corresponding to the above-mentioned sequence has been extracted from the gel using the QIAquick Gel Extraction Kit. This fragment has been then inserted in the NOB 425 (containing the T-DNA with 35S::ALCR) also digested with HindIII (Roche). The ligation reaction has been incubated at 16°C for 12 hours. This is the first final construct which we have called NOB13 (figure 26).

Instead, for the production of the second final construct (35S::ALCR;pALCA::BnAGL23mut;LEC2::amiR-BnAGL23) we have operated
in the following way.

The LEC2::amiR-BnAGL23 fragment has been amplified using as template the NOB6 (figure 27) and the following primers have been used for the PCR reaction: Atp1554 and Ve50. These two primers introduce a restriction site recognized by the endonucleases XbaI, therefore after having purified the PCR product it has been digested and subsequently introduced in the NOB11 (see above) also digested with XbaI. The final construct (NOB11 + LEC2::amiR-BnAGL23) has been called NOB14 (figure 28).

As already reported for the construction of the NOB6, NOB 14 is digested with HindIII, the fragment containing pALCA::BnAGL23 mut;LEC2::amiR-BnAGL23 is eluted from the gel and ligated in the NOB 425 previously cut with HindIII. This final vector (35S::ALCR;pALCA::BnAGL23mut;LEC2::BnamiR-AGL23) has been named NOB16 (figure 29).

NOB14 and NOB16, like some of the previous vectors, have been introduced through electroporation in Agrobacterium tumefaciens. The Agrobacterium modified strains have been used for the transformation of B.napus. B. napus transformation is a long procedure which foresees some in vitro culturing steps, carried out according to what is reported by Bade Damm (1995). For the induction protocols with ethanol there have been followed the methods described by Rosian et al.(2001), Laufs et al. (2003) and the experience described in Battaglia et al. (2006).

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CLAIMS

1. A genic construct for the recoverable inhibition of the germination of transgenic seeds, comprising:

- a blocking genic element of the embryo development comprising a vegetal embryo promoter operably associated to a nucleotidic sequence capable of inactivating a gene involved in said embryo development;

- a recover genic element of the embryo development comprising an inducible promoter operably associated to a sequence of said gene involved in the embryo development mutated to result insensitive to the aforesaid sequence capable to inactivate a gene involved in the embryo development but active in the function of the embryo development.

2. Genic construct according to claim 1, further comprising one or more genes or transgenic nucleotidic sequences of interest operably associated to the construct in order to be contained within its closed right and left ends.

3. Genic construct according to claim 2, wherein said genes or sequences can be represented by genic elements which encode for a genic product having a desired function such as a protein, an enzyme including metabolites, hormones, antibiotics, toxins.

4. Genic construct according to any one of claims from 1 to 3, wherein said plant embryo promoter is chosen in the group consisting of the promoter of PHERES, of LEC2 and of napine.

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5. Genic construct according to any one of claims from 1 to 4, wherein said sequence capable of inactivating a gene involved in said embryo development is a sequence which encodes in turn for a nucleotidic sequence capable of binding itself to an active site of a gene involved in the embryo development to inactivate it.

6. Genic construct according to any one of claims from 1 to 5, wherein said sequence is a sequence which hybridizes with a target sequence to be inactivated and is able to provoke a breakage inside it.

7. Genic construct according to claim 6, wherein said sequence capable of inactivating said sequences or target genes is a sequence which encodes for the expression of an artificial micro RNA (amiRNA) or a small interference RNA (siRNA).

8. Genic construct according to claim 6 or 7, wherein said target sequence is a MADS-box sequence of plants.

9. Genic construct according to claim 8, wherein said MADS-box sequence has been selected from the genome of the plant *Arabidopsis thaliana*.

10. Genic construct according to claim 9, wherein said MADS-box sequence corresponds to the gene AGL23.

11. Genic construct according to any one of the claims from 7 to 10, wherein said nucleotidic sequence is capable of inactivating a gene involved in the embryo development comprises a sequence chosen among the sequence amiRNA-BnAFL23 and the sequences having an identity of at least 70%, preferably of at least 80%, with said sequence amiRNA-BnAFL23.

12. Genic construct according to any one of the claims from 1 to 11, wherein said genic recovery element of the embryo development comprises an
inducible promoter capable to react to an external stimulus by activating the
transcription of a nucleotidic sequence operably associated thereto.

13. Genic construct according to claim 12, wherein said inducible promoter is
chosen among the promoters of the inducible system mediated by
glucocorticoids, of the inducible system mediated by heat shock and of the
ethanol inducible system.

14. Genic construct according to any one of the claims from 1 to 13, further
comprising a costitutive promoter operably associated to the gene which
synthesizes for a protein which, when activated by the external stimulus, is
able to bind to the inducible promoter and to activate in turn its transcription.

15. Genic construct according to claim 14, in which said constitutive promoter
is in the group which constis of the 35S-CaMV promoter, ubiquitin promoter,
actin promoter and tubulin promoter.

16. Genic construct according to claim 14 or 15, wherein said constitutive
promoter is operably associated to a nucleotidic sequence which encodes for
an inactive protein activable through an external stimulus.

17. Genic construct according to claim 16, wherein said encoding sequence is
chosen among the sequences of the inducible system mediated by
glucocorticoids, the heat shock inducible system and the ethanol inducible
system.

18. Genic construct according to any one of claims from 1 to 17, wherein said
sequence of a gene involved in the embryo development mutated to result
insensitive to the inactivation sequence of said gene but active in its natural
function is represented by the mutated sequence BnAGL23.

19. Method for the preparation of a genic construct for the recoverable
inhibition of the germination of transgenic seeds according to any one of
claims from 1 to 18, it comprising the steps of:

1) isolation from the genome of interest of the nucleic sequence of
a plant embryo promoter;

2) amplification of said sequence;

3) digestion of the sequence with appropriate restriction enzymes
to allow the insertion into a suitable cloning plasmid;

4) digestion of a suitable cloning plasmid with the same restriction
enzymes used in the preceding step to consent the integration of said
sequence in said plasmid;

5) integration of said embryo promoter in said plasmid;

6) isolation from the genome of interest of a target sequence;

7) amplification of said sequence;

8) synthesis of a sequence capable of hybridizing and inactivating
such target sequence;

9) cloning said sequence capable of hybridizing and inactivating
the target sequence in appropriate plasmids;

10) recombination between the products of step 5) and of step 9) to
obtain a genic element for blocking the embryo development;

11) synthesis of a nucleic sequence of a gene involved in the
embryo development mutated so as to result insensitive to said
inactivation sequence;

12) recombination of the product of step 11) in an appropriate
vector;

13) isolation from the genome of interest of the nucleotidic
sequence of an inducible promoter;

14) amplification of said sequence;

15) digestion of the sequence with appropriate restriction enzymes to consent its insertion into a suitable cloning plasmid;

16) digestion of a suitable cloning plasmid with the same restriction enzymes used in the preceding step to consent the integration of said sequence in said plasmid;

17) integration of the product of step 15) in the plasmid of step 16);

18) ligation between the insert of the vector obtained in step 12) and the linearized plasmid of step 16) to obtain a plasmid containing the genic element for recovering the embryo development;

19) recombination between the products of step 12) and of step 18) to obtain the genic construct for the recoverable inhibition of the germination in transgenic seeds.

20. Method for the transformation of a vegetable organism through the genic construct according to any one of claims from 1 to 18, comprising the steps of:

c) making available a genic construct for the recoverable inhibition of the germination in transgenic seeds according to any one of the claims from 1 to 18;

d) inserting said construct in a host organism capable to transform a target organism or parts of this one

e) transforming said vegetable organism or parts of it with said host organism bearing said construct.
21. Method according to claim 21, wherein step a) comprises the cloning and the transferring of said genic construct in heterologous expression vectors, such as binary vectors.

22. Method according to claim 20 or 21, wherein step b) comprises the introduction of cloning plasmids containing the construct of interest by electroporation in a host organism to transfer transgenes in vegetable organisms.

23. Method according to claim 22, wherein said host is Agrobacterium tumefaciens.

24. Seeds, plants or parts of genetically modified plants comprising a genic construct according to any one of claims from 1 to 18.
FIG. 1

AtAGL23

ATGGTGA........ACTTTGCTGTAGAAGGTAGATAGTGAATGGAAATGCCTTCAACGTACATTTTC
CAAGGGAGCTGCTCTTTAAAGAGGCTAGTGAATTTGCGCAATTATGTGATAGTGGTTTT
CACCAGCTGAGAAAAAGTATTTTTGCTCATCATAAAATTTGATGTTCCTGTGACCACCTTCTGAGGCTGTGTTT
CACAAACAACGAAAACTTTGATGAAAAGCTACTCAAAAGCTTTTCAAAAAATCCCACACTGAGTAAATGT
CAATTGTGGCAATATCTAATATTGTTCCTCTCTTTTTGATTTGATATATATTATAAATTTTCTCACAAT
TCATGATATCTTTAAACAACTATTATTTCACTGAGATTATCCTGTAAGGTGAGGGCAGAGTGAGAAAGACACAA
AGCACTCGGGGCTCAAAAATGAAAGAACAGAAACACGCTGAGAAGGTGAGGTGAGTAAGCTTCCATTAGAACTCACT
AGTCAATCACAACCTGATAGTACGCTTCTTTAAAGATTTGGAAGAAGATATTGTGATGAAAGACAATTCAATTATCCATCA
AACCACACACGTTATGTTTGAGTCTACACTACACACACTGTTAGTGTGCTAAATATCTTCACAA
ACCCGGGCTCTTTGATCAAAAACGGAAATGACGATATCTCTACTCAACACTTCTGTGATTTGATATATGAAATAGC
ACACCAGAGGTTA
FIG. 2

Phores promoter
FIG. 3

LEC2 promoter

GCACAAAAATCTATCATCTACGTACATCTTTCTAAGACCTATATATTGTTATATATATTGTTATGCCGTTGGTTCTGATTCCAATAA
ATTTTAGCGCATAGTAATATTTCTAAATGCAAGAAGAATTGCTTCAAAAGTGTAAGAATGACAATTAATTGAGTTCTTCTACA
ATAAGAATATATTGACTCGATTTTCGACAAAAGCAGTTGATGCTAATATCAGATATTACCTTACTTATATTTATTTAAAAATTG
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
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ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
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ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
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ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
ATAGATTATATTTGATTTCTACGAAATGCAAAAAAGCAAAAAATAAAAATTTTACAGTATTCAGTTTTTTCAGATATACGGAA
FIG. 4

amiR-BnAGL23

CTGCAAGCGCATTAAGTTGGTACCCAGGGTTTTTCCCAATCGACAGCTGGATTTAAACGACGGCCAGTGAAATT
GTAATACGACTCACTATAAGAGGCGAATTTGCACTCCGGGCCTCCCTCGAGGCTGACGGTAATAGCTGGAT
ATCGAATTCTGCAGCCCCAACACAGCTCGGAGCCATATACACATGTGTCTACATTACATTAGGTGCTTT
TTGAATTTGAGTTTTACGAATATAATATAGTGAGGAGCACAGTAAGGACCACAATTACCAGTCTGATGATATGATT
CAATTTAGCTTCCGACTGATCCATCCAAATACCCAGATCGGCACAAAAATCAATAGACTCTGTTAAATGAAAT
GATGCCTGATAGCAAATTGGATGATTGATTCTCTCTGTATAGTTATGAGACTTACTGCGGCTCTCTCTCTTGTATT
CAATTTTCTCGTAttAATCTTTTCTCGCAAAAAACATGTCTTGTACCTCAACTAATGACTGATATAGCTGCTTCTGAT
TATATAGTTCTGGTTAAATACATTTTCGTTTTTCATTAAAAAAAAAGGATCTGACATGGGGGATCACTAGT
CTAGAGCGGCCAGCCACAGCGGCGGTGAGCTCCAGCTTTTTGGTCTCCCTTTAGTAGGCTAAATTCCGCAGCTTTGCC
TAATCATGGTCATAGCTTTTCTGGTAAATGTTATCCGC

FIG. 4a
5'-GGTGAGATAGTCTCCATTAG-3'

FIG. 4b
5'-TTAATGGAGACTTACTCGGCC-3'

FIG. 4c
5'-TGAACACGTATCATAACAGCTT-3'.
FIG. 5

BnAGL23

ATGGTGAAAAAACCTGGTCGTAAGGAGTAGATTTGAAATTGACTAAGGAATCAAAACCTCCAAGTCACATTTTC
CAAGAGAAAGGCTGGCTTTTTTAGAAGGCTAGTGAAATTTCACATTATGTGATGCAAAAATTTGGAGATAGTCTGTTTT
CACCAAGTGGAAAAAGTATTCTACCTGGTGTATCCAAATGGTGAATGTTCTGGACACATTTCGAGGTTCTGGTAGAGA
CACAAACACAAAACCTTTGATGAAGCTACACAAAGCTTTACTTGGAAAATGCTCACAACAAATCTACACTGAGGTGAAGGC
GGAGATGAAAGAAAAGACAGAAGAATAGCAGTCGCGGGCTCAAAATGAAAGAGAAAAACGCTGAGGAGTGGTGGAG
GTAAGTCTCCATTAGAACTCACTTTAAGTCAATCAACCTCTGTATAGTACTAGTTCTTTAAAGATTTTGAAGAGAGATAGTGTAT
GAAAAAGCAATTCAAATACCATCAACACAAAACCCACAAACCTCTATGGTGGAGTTCTAGCAATGCTGTCCACAGCAAC
TGTTAGTGTTGGTAATATCCCAAAACCAACGGGTTCTTTGGATCAAAAACGGGATGACGACTAATCCTACTCAAAACACTTC
TGTTGGATTGTATATGAGTCGACACCCAGGAGTTAA
6/30

FIG. 6

BnAGL23 mutated

ATGGTGAAAAAATCTTTGGTCTAGAAAAGTAGAATGAAAATGACTAAGGAAATCAACCCCTTCAAGTCACATTTTC
CAAGAGAAAGCTGGTCTTTTTAAAGAAAGGCTAGTGAAATTTGACATTTATGTAGATGCAAAAAATTTGAGATGACATGTTTT
CACCCAGCTGAAAAGTATTTTCTTTTGTCTAGCTCAGAACCGTTGAGGTTGTTAGGAGC
CACAACACAAAAACCTTTGAGAAAAGCTACACAAAGGCTTCATGGTTCAAATGCTCAACAAAAATCCCTACACTGAGGTGAGG
GGAAGTAAAGAAAAAGAAGAGAGATGGGCTCGCCGCTCAAAAAAGAAGAGAAAAAGGCTGAAGGAGGTGTTGAGG
GAAAAATCTCCATTAGAACATCAAATTTAGCAAATCGAGTATAGCCTTTTTAAAGATTTGAAAGAGATATAGTTGAT
GAAAAAGCAATTCAATTAACTCAAATCAACAACCAACCCCAAAACTTTCTATGTTGAAAGTTCTAGCAATGCTGCTCAGCAAC
TGTTAGTGTTGTAATCTCCTCACAACACACAGGGGTTCTTTGTACAAAACGGGATGACGACATATCTACTCAAAACACTTC
TGTTTGGATTTGATATTAGGTCACACCGAGGTTAAA
FIG. 8

LEC2::amiR-BnAGL23

GCACAAAATCTACTACATCGTACCATTTCATTAAAGACTATATATATTATAGATGTTATATTTTATGCGGTGGTTCTGTAGTCCAAATAA
ATTTTACGTCCATAGTTAAATTATTTCTAAAAAAGCAAATTTTTTCTCAAAAGTGTACTATGACAAATTAAATGAGGTTTCTCAAA
ATAAGAATAAATTTGACTGATTTTTCACAAAAATCTGAATGCTAAATATACATTACTATTAAATTTAACATGAAAATATGTTAC
TTTTCCATATTGGAATAAGAATAATTAAAACTACTAAGTATTTAAATATATATGGGAATTTGTTCTTTCTGCAATAGGTTTGA
ATACAAAAATAATCTCTATAAATAATTTATATTTAATAAAAATAACACGTTTTTTTCCAAATACACAGCAAAAAAATA
TTAATTACAAAAGAAAATTATGTGTATACAAAAAAATATAGAAATGCTTATAATGTGTTTTTACTCTAAATTATATATAGCAT
TTAGCTAAAATCTTCTAAAAAGTACAAGAGGCTTTTTACTGATTGAACTGCAATAGTGTAGTTTTGACCTT
AACAGAGTTTAAATATATAGGAGAAAGAGCAGCCAGCGATAATATCAAATAAAGAGCTCTCTCTCTCTCTCTGAGAAAA
AACACATATAGCCAATGACCTTCTCTGTTGCTCTGCTGTGCCATAAAAAGCCATTATATACATGCACAACTACACTGCGCCCA
CAGTATACACATGTACTAGTGTAAGTAGTAATAGGCTATTAACGTGATTTGCTATATCCTGTTCTGCGGTTGCTGAGGG
TATCCCCATAAGCTGCCAACATAGGACCATCTGCAACATTTTGGACTCGTTTTTTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTT
TCTCTCCTGCTCTCTCTCTAAAAATACCTCCACGACCTCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT
CTCTCTCTCTCTGCGGAAAAATAAGTAAAAATCTCTCCTGCCAAAGGCGATTAGTGGGTAGTAAACCGCCAGGGTTTT
TCCACAGTACGACAGTTGTAACAGACGCGCATGAAATTGTATACTAGCAGCACTGATAGGCGGATATGGGTCGCGGCCCC
CCTGAGGTCGACGATCGATACTGATATCTGCACGCAGCCAAAACACAGCGCTCGGACAGCATTTACAGATG
TTCAATAACCTTAAATACTCTGCTTTTGATGATTGTGGTTTTCGAATTATATTATGAGGACGATGAATGACTCATTACCC
CAGTGCTGATAGTCTTTACAAATATTGCTCTCCGACTATCTTCAATCCAAATACGAGTCCGCAAAAAATTACAAACTAGACTGCTTAA
ATGAAATGAGGCGGATGACAAATTATGGGAATTTGAGATTTGTTCTTTTTGTTAATATGGGACTTACTGCGGCTCTCTCCTTTGa
TTCCAAATTATTCTTTAATTCTCTGCACAAAAAAATCTGCACGCTGCTTCATAGATATCATCCTGTGTGTTATAT
TAGTTGTCGTTAAATTACATTTTGTTGTTATCTTTTATTATTAGGAGCATAGCCATAGGGGGAATACCATTTCTTAGAGCGGC
GCCACCAGCGCGGAGGAGCACCAGCAGTTTGTTCTCTGCTATGGTAAATTCGGAGGAATTATCCAGAGCTTGGCCTAAATTCGTCATAGCTG
TTCCCTGTGTAAGAATTGTTATACCGCG
PCR product PHERES promoter

AatII

Atp1553

XbaI

Atp1552

XbaI

9569 bp

pBGW,0

9569 bp

Rb

ClaI (5048)

SacII (1292)

PstI (1771)

SacI (1275)

EcoRV (1749)

SacI (1749)

PstI (1614)

Smal (1506)

Smal (9651)

XbaI (9560)

NcoI (799)

NcoI (24)

EcoRV (38)

AprR1

EcoRV (458)

CmR-ccdB

XbaI (1207)

PstI (1614)

AprR2

XbaI (9649)

LB

Sm.SpR

XbaI (9649)
FIG. 12

NOB 2

PCR product LEC2 promoter

XbaI → AatII

Atp1554

Atp1555

Smal (9601)

EcoRV (36)

atR1

NcoI (24)

AatII (77)

NcoI (769)

EcoRV (469)

atR2

NcoI (1207)

SmaI (1309)

PalI (1614)

PalI (1771)

SacI (1792)

HindII (1798)

pBGW.0

9669 bp

ClaI (5240)

SmSpR

LB
Ve49 (oligo A): 5' - CACCTGCAAGGCGATTAAGTTGGGTAAC-3'
Ve50 (oligo B): 5' - GCGGATAACAATTTCAACACAGGAAACAG-3'
Bnp1: 5'-GGGGACACTTTGTACAAGAAAGCTGGGTAAATGGTGAAAAACTCTTGG-3'
Bnp2: 5'-CTAATGGAGATTTACTCCACC-3'
Bnp3: 5'-GGTGGAGTAATCTCCATTAG-3'
Bnp4: 5'-GGGGACACTTTGTACAAGAAAGCTGGGTAAACTCTGGTGTCG-3'
Cloning procedure to obtain 35S::ALCR;pALCA::mutatedAGL23;PHERES::amiR_AGL23

FIG. 23

NOB10

LR recombination

+  

NOB419

NOB11

Xbal

mutatedAGL23

Xbal