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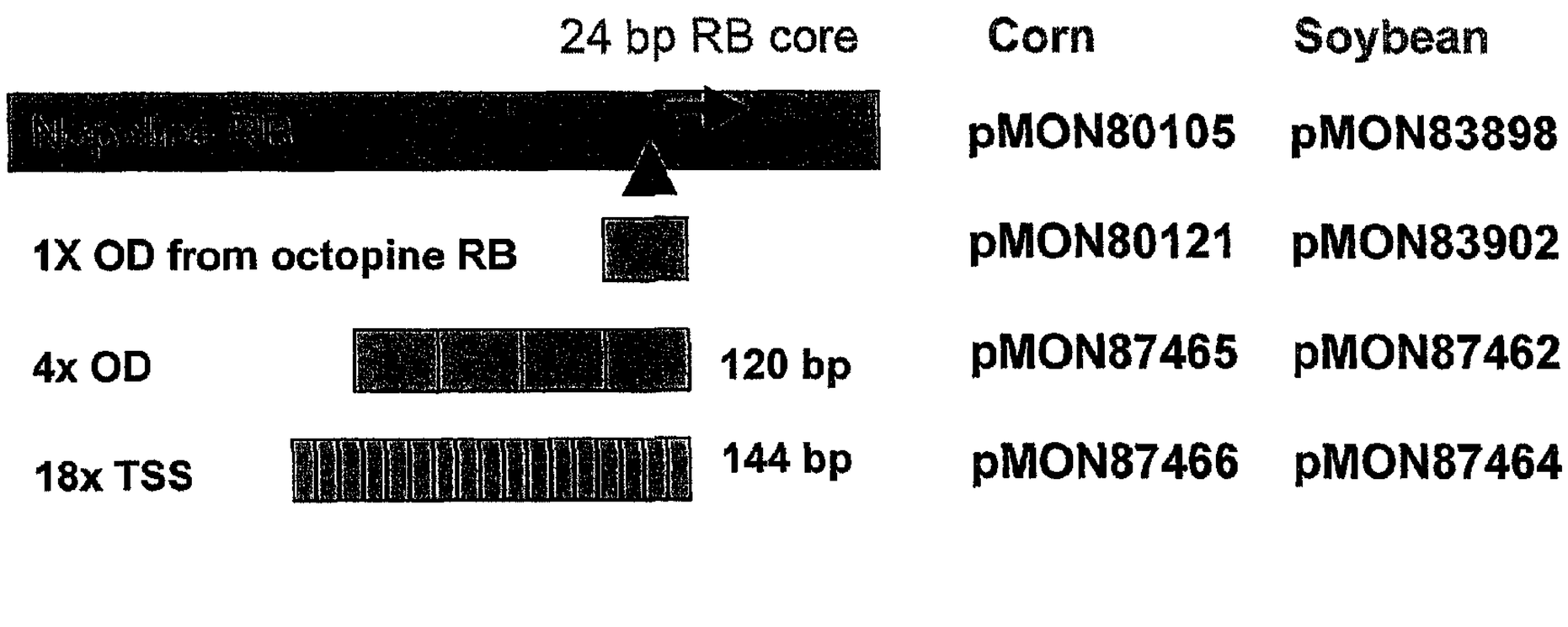
(54) Title: USE OF MULTIPLE TRANSFORMATION ENHANCER SEQUENCES TO IMPROVE PLANT TRANSFORMATION EFFICIENCY



(57) Abrégé/Abstract:
A method of increasing the efficiency of bacterially-mediated plant transformation, comprising the steps of: a) introducing at least one additional transformation enhancer sequence into a plant transformation vector comprising at least one T-DNA border region including a transformation enhancer sequence; and b) transforming a plant cell with said vector by bacterially-mediated transformation, wherein the bacterium is competent for the transformation of said plant cell, wherein the additional transformation enhancer sequence comprises a sequence selected from the group consisting of: SEQ ID NO:6, SEQ ID NO:11, and a sequence complementary to SEQ ID NO:6 or SEQ ID NO:11.

ABSTRACT

A method of increasing the efficiency of bacterially-mediated plant transformation, comprising the steps of: a) introducing at least one additional transformation enhancer sequence into a plant transformation vector comprising at least one T-DNA border region including a transformation enhancer sequence; and b) transforming a plant cell with said vector by bacterially-mediated transformation, wherein the bacterium is competent for the transformation of said plant cell, wherein the additional transformation enhancer sequence comprises a sequence selected from the group consisting of: SEQ ID NO:6, SEQ ID NO:11, and a sequence complementary to SEQ ID NO:6 or SEQ ID NO:11.

DESCRIPTION

USE OF MULTIPLE TRANSFORMATION ENHANCER SEQUENCES TO IMPROVE PLANT TRANSFORMATION EFFICIENCY

BACKGROUND OF THE INVENTION

This application is a division of application number **2,657,631**, filed in Canada on **July 18, 2007**.

1. Field of the Invention

The invention relates generally to plant biotechnology. More specifically, the invention relates to methods and compositions for improving the efficiency of bacterially–mediated plant transformation.

2. Description of Related Art

During natural *Agrobacterium*-mediated transformation of plant cells, a piece of DNA from the Ti plasmid of *A. tumefaciens* or Ri plasmid of *A. rhizogenes* is transferred into the plant cell (*e.g.* Gelvin, 2003). This transferred DNA (T-DNA) fragment is flanked by imperfect 24 bp direct repeats that are recognized by *Agrobacterium* endonuclease VirD2 to produce a single stranded T-strand by nicking at a specific site in one strand of each repeat. The repeat that initiates formation of single stranded T-strand has been termed the "right border" (RB), while the repeat terminating formation of single-stranded T-DNA has been termed the "left border" (LB). The VirD2 protein is attached to the 5' end of the strand after nicking, and guides the T-strand into plant cells where the T-strand is integrated into the plant genome with the help of other *Agrobacterium* and plant-encoded proteins. Sequences downstream (in a 5' to 3' direction) of the T-DNA region, including vector backbone sequence, may be transferred as well (*e.g.* Kononov *et al.*, 1997). This likely occurs by inefficient nicking of at least one of the borders in *Agrobacterium* prior to transfer to a plant cell.

Comparison of the RB and LB sequences from a variety of *Agrobacterium* strains indicated that both RB and LB share a consensus motif (Canaday *et al.*, 1992), which indicates that other elements may be involved in modulating the efficiency of

RB processing. Cis-acting sequences next to the RB are present in many *Agrobacterium* strains, including *A. tumefaciens* and *A. rhizogenes*. These sequences are necessary for wild type virulence (Veluthambi *et al.*, 1988; Shurvington and Ream, 1991; Toro *et al.*, 1989; Toro *et al.*, 1988; Hansen *et al.*, 1992). The sequence in *A. tumefaciens* was called an "overdrive" or "T-DNA transmission enhancer" by Peralta *et al.*, (1986). In *A. rhizogenes* the sequence has been termed the "T-DNA transfer stimulator sequence" (TSS) by Hansen *et al* (1992). The overdrive ("OD") sequence was initially defined as a particular 24 bp motif present immediately in front of the RB repeat of octopine Ti TL-DNA (Peralta *et al.*, 1986). A similar sequence is present in front of the RB repeat of octopine Ti TR-DNA and also in front of nopaline Ti RB and agropine Ri TL right border (Peralta *et al.*, 1986, Shaw *et al.*, 1984, Barker *et al.*, 1983, Slighton *et al.*, 1985). Further comparison of different *A. tumefaciens* strains revealed a 8 bp overdrive core sequence present in front of all right border sequences including nopaline strain pTiT37, octopine strain pTiA6 and *A. rhizogenes* pRiA4 (Peralta *et al.*, 1986).

The presence of octopine overdrive sequence enhanced single strand T-DNA formation in *Agrobacterium* cells and improved T-DNA transfer into plant cells, and was necessary for wild type virulence (Peralta *et al.*, 1986, Shurvinton and Ream 1991). The LB repeat from nopaline-producing Ti plasmid pTiT37 is capable of producing single-stranded T-DNA with high efficiency when the pTiT37 RB proximal cis-acting sequence was placed in front of it, indicating that an overdrive-like sequence indeed is also present on a nopaline Ti plasmid (Culianez-Macia and Hepburn 1988, Peralta *et al.*, 1986), just as it is in the other identified (octopine-producing) Ti plasmids. Integration of a heterologous octopine overdrive sequence in front of nopaline pTiT37 RB resulted in much greater virulence than the parental strain which contained only a synthetic pTiT37 RB repeat (Peralta *et al.*, 1986).

The VirC1 protein binds to overdrive and is thought to improve VirD2 nicking (Toro *et al.*, 1988, 1989), while mutation of *virC* results in attenuated virulence in plants (Close *et al.*, 1987) and reduced production of processed single stranded T-DNA sequence. Both *A. tumefaciens* octopine and nopaline Ti plasmids contain *virC* and can complement the *virC* mutation *in trans* to restore the attenuated virulence to wild type level (Close *et al.*, 1987).

The TSS found in *A. rhizogenes* strains 8196, A4 and 2659 plays a similar role as the overdrive sequence in *A. tumefaciens*. Each *A. rhizogenes* strain has a different but related sequence (Hansen *et al.*, 1992). The 8 bp TSS core sequence repeats 5 times in pRiA4, 6 times in pRi8196 and 17 times (rather than 16x as Hansen *et al.*, 1992) in pRi2659 (Genbank accession AJ271050). pRiA4 has a conserved 8 bp overdrive core sequence in addition to the repeats. Shorter core sequence repeats in pRiA4 and pRi8196 were not sufficient for wild type virulence (Hansen *et al.*, 1992).

Depicker *et al.* (U.S. Patent Publication 2003/0140376, and corresponding international publication WO01/44482) describe recombinant constructs with modified T-DNA borders in order to lessen or prevent transference of vector backbone sequences. Conner *et al.*, (WO 05/121346) describe creation and use of sequences from T-DNA border-like regions that comprise sequences derived from plants. Heim *et al.* (U.S. Publ. 2003/0188345) describe vectors for *Agrobacterium*-mediated transformation of plants with modified border regions. Lassner *et al.*, (U.S. Publ. 2006/0041956) describe modifications to T-DNA border regions to enable identification of transgenic events that do not comprise non T-DNA sequences.

While the foregoing studies have increased understanding in the art, what remains needed is a method to improve the efficiency of *Agrobacterium*-mediated plant transformation. Although the presence of overdrive or TSS sequences increases virulence of wild type *Agrobacterium* and improves T-DNA transfer into plant cells compared to plasmids lacking the sequences, it has remained unclear how to further improve transformation efficiency including through the use of overdrive or TSS sequences.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a method of increasing the efficiency of bacterially-mediated plant transformation, comprising the steps of: a) introducing at least one additional transformation enhancer sequence into a plant transformation vector comprising at least one T-DNA border region; and b) transforming a plant cell with the vector by bacterially-mediated transformation, wherein the bacterium is competent for the transformation of the plant cell. The method may optionally comprise regenerating a transgenic plant from the plant cell. In one embodiment, the additional transformation enhancer sequence comprises a consensus core sequence of

TGTWTGTK (SEQ ID NO:20). In other embodiments, the additional transformation enhancer sequence is selected from the group consisting of: SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:13, and a sequence complementary to any of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:13. In particular embodiments, the invention provides a recombinant DNA construct comprising SEQ ID NO:14, SEQ ID NO:15, or SEQ ID NO:16.

The transformation enhancer sequence used with the invention may be located proximal to a T-DNA border region or sequence, such as a right border (RB) sequence, *i.e.* between flanking sequence such as vector sequence and the border sequence. The transformation enhancer sequence may be from a Ti plasmid of *A. tumefaciens*, such as a nopaline or octopine plasmid, or may be from an Ri plasmid of *A. rhizogenes*. In certain embodiments, the bacterially-mediated transformation may utilize a technique selected from *Agrobacterium*-mediated transformation, *Rhizobium*-mediated transformation, and *Sinorhizobium*-, *Mesorhizobium*- or *Bradyrhizobium*-mediated transformation. In certain embodiments, the transformation enhancer sequence may comprise SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:17, or SEQ ID NO:18. In further embodiments, the T-DNA border region may comprise from 1 to about 18 copies of the transformation enhancer sequence, including from about 2 or about 4 to about 18 copies of the transformation enhancer sequence.

In accordance with one embodiment of the present invention, there is provided a method of increasing the efficiency of bacterially-mediated plant transformation, comprising the steps of: a) introducing at least one additional transformation enhancer sequence into a plant transformation vector comprising at least one T-DNA border region including a transformation enhancer sequence; and b) transforming a plant cell with the vector by bacterially-mediated transformation, wherein the bacterium is competent for the transformation of the plant cell, wherein the additional transformation enhancer sequence comprises a sequence selected from the group consisting of: SEQ ID NO:11, and a sequence complementary to SEQ ID NO:11, and wherein the plant cell is from a plant selected from corn and soybean.

A plant cell in accordance with the invention may be any plant cell. In certain embodiments, the plant cell is from a plant selected from the group consisting of soybean, corn, cotton, canola, rice, wheat, alfalfa, common bean, peanut, tobacco, sunflower, barley, beet, broccoli, cabbage, carrot, cauliflower, celery, Chinese cabbage, cucumber, eggplant, leek, lettuce, melon, oat, onion, pea, pepper, peanut, potato, pumpkin, radish, sorghum, spinach, squash, sugarbeet, tomato and watermelon. In particular embodiments, the plant cell is a corn cell or a soybean cell.

In another aspect, the invention provides a recombinant DNA construct comprising a T-DNA border sequence of a Ti or Ri plasmid, operably linked to a transformation enhancer sequence that comprises two or more copies of a sequence selected from the group consisting of: SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:13, a sequence complementary to any of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:13, and combinations

thereof. In particular embodiments, the invention provides a recombinant DNA construct comprising SEQ ID NO:14, SEQ ID NO:15, or SEQ ID NO:16.

In such a construct, the enhancer sequence may comprise at least about four copies of the sequence. The border sequence may be a right border (RB) or left border (LB) sequence. In certain embodiments, the construct may comprise SEQ ID NO:10 and/or SEQ ID NO:11. The RB sequence may be from a nopaline Ti plasmid, or an agropine, mannopine, succimanopine, cucumopine, or octopine Ti or Ri plasmid and may comprise SEQ ID NO:12.

In accordance with one embodiment, the present invention provides a recombinant DNA construct comprising a T-DNA border sequence, operably linked to a transformation enhancer sequence of SEQ ID NO:11.

In another aspect, the invention provides a cell transformed with a construct provided herein. The cell may be a plant or bacterial cell, including an *Agrobacterium* cell and *Rhizobium* cell. In one embodiment, the plant cell is from a plant selected from the group consisting of soybean, corn, cotton, canola, rice, wheat, alfalfa, common bean, peanut, tobacco and sunflower. The invention also provides transgenic plants transformed a construct of the invention. In particular embodiments, the transgenic plant may be selected from the group consisting of soybean, corn, cotton, canola, rice, wheat, alfalfa, common bean, peanut, tobacco and sunflower.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings are part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to the drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1: Outline of various transformation enhancer sequences used for improving transformation efficiency.

FIG. 2: Schematic map of pMON87464.

FIG. 3: Schematic map of pMON87465.

FIG. 4: Engineered RB sequences; overdrive sequence is in bold and the 24 bp RB core sequence underlined. (A) sequence of the Nopaline RB+1x overdrive (SEQ ID NO:14); (B) Nopaline RB+4x overdrive (SEQ ID NO:15); (C) Nopaline RB+18x TSS (SEQ ID NO:16).

DESCRIPTION OF SEQUENCE LISTING

SEQ ID NO:1 Forward primer Xd463 for 2X OD sequence preparation.

SEQ ID NO:2 Reverse primer Xd464 for 2X OD sequence preparation.

SEQ ID NO:3 Forward primer Xd465 for 6X TSS sequence preparation.

SEQ ID NO:4 Reverse primer Xd466 for 6X TSS sequence preparation.

SEQ ID NO:5 24 bp core OD of pTiA6.

SEQ ID NO:6 8 bp 1x TSS sequence.

SEQ ID NO:7 30 bp 1x OD of pTiA6; reverse complement of SEQ ID NO:17.

SEQ ID NO:8 1X OD sequence from pTiAB3.

SEQ ID NO:9 1X OD from pTi15955.

SEQ ID NO:10 4X stacked OD.

SEQ ID NO:11 18X stacked TSS.

SEQ ID NO:12 Border region with 1X OD sequence.

SEQ ID NO:13 Partial OD sequence.

SEQ ID NO:14 Nopaline RB region with 1X OD.

SEQ ID NO:15 Nopaline RB region with 4X OD.

SEQ ID NO:16 Nopaline RB region with 18X TSS.

SEQ ID NO:17 1X OD; reverse complement of SEQ ID NO:7.

SEQ ID NO:18 4X stacked OD; reverse complement of SEQ ID NO:10.

SEQ ID NO:19 Consensus OD sequence (Toro *et al.*, 1988).

SEQ ID NO:20 Consensus 8 bp core OD sequence.

DETAILED DESCRIPTION OF THE INVENTION

The following is a detailed description of the invention provided to aid those skilled in the art in practicing the present invention. Those of ordinary skill in the art may make modifications and variations in the embodiments described herein without departing from the spirit or scope of the present invention.

The invention provides methods and compositions for improving the efficiency of *Agrobacterium*-mediated transformation of plant cells. Sequencing of the 20 kb T-DNA region of *A. rhizogenes* K599, a soybean super virulent strain, led to the recognition that the pRi plasmid in *A. rhizogenes* K599 is identical to the *A. rhizogenes* NCPBB 2659 strain. The super-virulence of the K599 strain may thus be related to the number of TSS sequences present near the RB. Therefore, stacking of multiple overdrive and TSS repeats was tested in binary vectors with a nopaline RB (*e.g.* from pTiT37) to improve transformation efficiency. The octopine Ti plasmid's 30 bp overdrive (Shurvinton and Ream 1991) from pTiA6, present in 4 copies, and the *A. rhizogenes* NCPBB2659 TSS 8 bp core sequence, present in 18 copies, was used to enhance T-DNA transmission efficiency.

Transformation studies comparing the use of constructs containing varying numbers of overdrive or TSS sequences demonstrated that the presence of additional "stacked" copies of these sequences improved transformation efficiency by improving transformation frequency as well as the quality of the resulting transgenic events. For example, the proportion of events with single copy insertions, and also lacking vector backbone sequences (*e.g. oriV*), was increased. Increased transformation frequency and quality events improve the overall efficiency of the transformation process by reducing the amount of resources required to select event for further commercial development.

The invention therefore provides improved methods for obtaining fertile transgenic plants and for the transformation of plant cells or tissues and regeneration of the transformed cells or tissues into fertile transgenic plants. To initiate a transformation process in accordance with the invention, the genetic components desired to be inserted into the plant cells or tissues will first be selected. Genetic components may include any nucleic acid that is to be introduced into a plant cell or tissue using the method according to the invention. Genetic components can include non-plant DNA, plant DNA, or synthetic DNA.

In certain embodiments of the invention, genetic components are incorporated into a DNA composition such as a recombinant, double-stranded plasmid or vector molecule comprising genetic components such as: (a) a promoter that functions in plant cells to cause the production of an RNA sequence, (b) a structural DNA sequence that causes the production of an RNA sequence that encodes a desired

protein or polypeptide, and (c) a 3' non-translated DNA sequence that functions in plant cells to cause the polyadenylation of the 3' end of the RNA sequence. The vector may also contain genetic components that facilitate transformation and regulate expression of the desired gene(s).

The genetic components are typically oriented so as to express an mRNA, which in one embodiment can be translated into a protein. The expression of a plant structural coding sequence (a gene, cDNA, synthetic DNA, or other DNA) that exists in double-stranded form involves transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase and subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region that includes polyadenylation of the 3' ends of the mRNA.

General methods for preparing plasmids or vectors that contain desired genetic components and can be used to transform plants and methods of making those vectors are known in the art. Vectors typically consist of a number of genetic components, including, but not limited to, regulatory elements such as promoters, leaders, introns, and terminator sequences. Regulatory elements are also referred to as cis- or trans-regulatory elements, depending on the proximity of the element to the sequences or gene(s) they control. The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA and to initiate the transcription into mRNA using one of the DNA strands as a template to make a corresponding complementary strand of RNA.

The constructs may also contain the plasmid backbone DNA segments that provide replication function and antibiotic selection in bacterial cells, for example, an *Escherichia coli* origin of replication such as *ori*322, a broad host range origin of replication such as *ori*V or *oriRi*, and a coding region for a selectable marker such as Spec/Strp that encodes for Tn7 aminoglycoside adenyltransferase (*aadA*) conferring resistance to spectinomycin or streptomycin, or a gentamicin (Gm, Gent) selectable marker gene. For plant transformation, the host bacterial strain is often *Agrobacterium tumefaciens* ABI, C58, LBA4404, EHA101, or EHA105 carrying a plasmid having a transfer function for the expression unit. Other bacterial strains known to those skilled in the art of plant transformation can function in the present invention, including *A. rhizogenes, Sinorhizobium* sp., *Mesorhizobium* sp., *Bradyrhizobium* sp., and *Rhizobium* sp. strains.

A number of promoters that are active in plant cells have been described in the literature. Such promoters include, but are not limited to, the nopaline synthase (NOS) and octopine synthase (OCS) promoters, which are carried on tumor-inducing plasmids of *A. tumefaciens*; the caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S promoters and the figwort mosaic virus (FMV) 35S promoter; the enhanced CaMV35S promoter (e35S); and the light-inducible promoter from the small subunit of ribulose bisphosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide). All of these promoters have been used to create various types of DNA constructs that have been expressed in plants. Promoter hybrids can also be constructed to enhance transcriptional activity or to combine desired transcriptional activity, inducibility, and tissue or developmental specificity.

Thus, promoters that function in plants may be inducible, viral, synthetic, constitutive as described, temporally regulated, spatially regulated, and/or spatio-temporally regulated. Other promoters that are tissue-enhanced, tissue-specific, or developmentally regulated are also known in the art and envisioned to have utility in the practice of this invention. Useful promoters may be obtained from a variety of sources such as plants and plant DNA viruses. It is preferred that the particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of the gene product of interest.

The promoters used in the DNA constructs (*i.e.*, chimeric/recombinant plant genes) of the present invention may be modified, if desired, to affect their control characteristics. Promoters can be derived by means of ligation with operator regions, random or controlled mutagenesis, etc. Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression.

The mRNA produced by a DNA construct of the present invention may also contain a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. Such "enhancer" sequences may be desirable to increase or alter the translational efficiency of the resultant mRNA and are usually known as translational enhancers. Other genetic components that serve to enhance expression or affect transcription or translation of a gene are also envisioned as genetic components. The 3' non-translated

region of the chimeric constructs preferably contains a transcriptional terminator, or an element having equivalent function, and a polyadenylation signal, which functions in plants to polyadenylate the 3' end of the RNA. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylation signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (*nos*) gene, and (2) plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene. An example of a preferred 3' region is that from the ssRUBISCO E9 gene from pea.

Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. These DNA sequences are referred to herein as transcription-termination regions. The regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA) and are known as 3' non-translated regions. RNA polymerase transcribes a coding DNA sequence through a site where polyadenylation occurs.

In many transformation systems, it is preferable that the transformation vector contains a selectable, screenable, or scoreable marker gene. These genetic components are also referred to herein as functional genetic components, as they produce a product that serves a function in the identification of a transformed plant, or a product of desired utility.

The DNA that serves as a selection device may function in a regenerable plant tissue to produce a compound that confers upon the plant tissue resistance to an otherwise toxic compound. Genes of interest for use as a selectable, screenable, or scoreable marker would include, but are not limited to, β-glucuronidase (*gus*), green fluorescent protein (*gfp*), luciferase (*lux*), antibiotics like kanamycin (Dekeyser *et al.*, 1989), genes allowing tolerance to herbicides like glyphosate (Della-Cioppa *et al.*, 1987), such as CP4 EPSPS (U.S. Patent 5,627,061; U.S. Patent 5,633,435; U.S. Patent 6,040,497; U.S. Patent 5,094,945; WO04/074443; WO04/009761); glufosinate (US Patent 5,646,024, US Patent 5,561,236, US Patent 5,276,268; US Patent 5,637, 489; US Patent 5,273, 894); 2,4-D (WO05/107437) and dicamba, such as DMO (U.S. Patent 7,022,896). Other selection methods can also be implemented, including, but not limited to, tolerance to phosphinothricin, bialaphos, and positive selection mechanisms (Joersbo *et al.*, 1998) and would still fall within the scope of the present

invention. Examples of various selectable/screenable/scoreable markers and genes encoding them are disclosed in Miki and McHugh (2004).

The present invention can be used with any suitable plant transformation plasmid or vector containing a selectable or screenable marker and associated regulatory elements as described, along with one or more nucleic acids (a structural gene of interest) expressed in a manner sufficient to confer a particular desirable trait. Examples of suitable structural genes of interest envisioned by the present invention include, but are not limited to, genes for insect or pest tolerance, genes for herbicide tolerance, genes for quality improvements such as yield, nutritional enhancements, environmental or stress tolerances, or genes for any desirable changes in plant physiology, growth, development, morphology, or plant product(s).

Alternatively, the DNA coding sequences can affect these phenotypes by encoding a non-translatable RNA molecule that causes the targeted inhibition of expression of an endogenous gene, for example via double-stranded RNA mediated mechanisms, including antisense- and cosuppression-mediated mechanisms (see, for example, Bird *et al.*, 1991). The RNA could also be a catalytic RNA molecule (*i.e.*, a ribozyme) engineered to cleave a desired endogenous mRNA product (see for example, Gibson and Shillitoe, 1997). More particularly, for a description of antisense regulation of gene expression in plant cells see U.S. Pat. No. 5,107,065, and for a description of gene suppression in plants by transcription of a dsRNA see U.S. Pat. No. 6,506,559, U.S. Patent Application Publication No. 2002/0168707 A1, and U.S. patent application Ser. Nos. 09/423,143 (see WO 98/53083), 09/127,735 (see WO 99/53050) and 09/084,942 (see WO 99/61631).

Use of sequences that result in silencing of other endogenous genes (*e.g.* RNAi technologies including miRNA) to result in a phenotype is also envisioned. For instance RNAi may be used to silence one or more genes resulting in a scoreable phenotype. One embodiment is to assemble a DNA cassette that will transcribe an inverted repeat of sequences, to produce a double-stranded RNA (dsRNA), typically at least about 19-21 bp in length and corresponding to a portion of one or more genes targeted for silencing. Thus, any gene that produces a protein or mRNA that expresses a phenotype or morphology change of interest is useful for the practice of the present invention.

Exemplary nucleic acids that may be introduced by the methods encompassed by the present invention include, for example, heterologous DNA sequences- that is, sequences or genes from another species, or even genes or sequences that originate with or are present in the same species but are incorporated into recipient cells by genetic engineering methods rather than classical reproduction or breeding techniques. The term heterologous, however, is also intended to refer to genes that are not normally present in the cell being transformed or to genes that are not present in the form, structure, etc., as found in the transforming DNA segment or to genes that are normally present but a different expression is desirable. Thus, the term "heterologous" gene or DNA is intended to refer to any gene or DNA segment that is introduced into a recipient cell, regardless of whether a similar gene may already be present in such a cell. The type of DNA included in the heterologous DNA can include DNA that is already present in the plant cell, DNA from another plant, DNA from a different organism, or a DNA generated externally, such as a DNA sequence containing an antisense message of a gene, or a DNA sequence encoding a synthetic or modified version of a gene or sequence.

In light of this disclosure, numerous other possible selectable or screenable marker genes, regulatory elements, and other sequences of interest will be apparent to those of skill in the art. Therefore, the foregoing discussion is intended to be exemplary rather than exhaustive.

After the construction of the plant transformation vector or construct, the nucleic acid molecule, prepared as a DNA composition *in vitro*, is generally introduced into a suitable host such as *Escherichia coli* and mated into another suitable host such as *Agrobacterium* or *Rhizobium*, or directly transformed into competent *Agrobacterium* or *Rhizobium*. These techniques are well-known to those of skill in the art and have been described for a number of plant systems including soybean, cotton, and wheat (see, for example, U.S. Pat. Nos. 5,569,834 and 5,159,135 and WO 97/48814). Those of skill in the art would recognize the utility of *Agrobacterium*-mediated transformation methods. Strains may include, but are not limited to, disarmed derivatives of *A. tumefaciens* strain C58, a nopaline strain that is used to mediate the transfer of DNA into a plant cell; octopine strains, such as LBA4404; or agropine strains, e.g., EHA101, EHA105, or *R. leguminosarum*

USDA2370 with a Ti or Ri plasmid. The use of these strains for plant transformation has been reported, and the methods are familiar to those of skill in the art.

Plant tissue to be transformed is typically inoculated and co-cultured with *Agrobacterium* or *Rhizobium* containing a recombinant construct comprising at least one heterologous overdrive or TSS sequence, a sequence of interest to be transferred, and at least one RB sequence that serves to define the DNA to be transferred, and is selected under appropriate conditions. In certain embodiments, at least one LB sequence is also present on the recombinant construct. In certain other embodiments, a border sequence can be a "plant derived border-like sequence(s)." Methods of identifying and using such sequences are described in Rommens *et al.*, 2005; Rommens 2004a; Rommens *et al.*, 2004b

The present invention can be used with any transformable cell or tissue. Those of skill in the art recognize that transformable plant tissue generally refers to tissue that can have exogenous DNA inserted in its genome and under appropriate culture conditions can form into a differentiated plant. Such tissue can include, but is not limited to, cell suspensions, callus tissue, hypocotyl tissue, cotyledons, embryos, meristematic tissue, roots, and leaves. For example, transformable tissues can include calli or embryoids from anthers, microspores, inflorescences, and leaf tissues. Other tissues are also envisioned to have utility in the practice of the present invention, and the desirability of a particular explant for a particular plant species is either known in the art or may be determined by routine screening and testing experiments whereby various explants are used in the transformation process and those that are more successful in producing transgenic plants are identified.

Methods for transforming dicots by use of *Agrobacterium* or *Rhizobium* and obtaining transgenic plants have been published for a number of crops including cotton, soybean, *Brassica*, and peanut. Successful transformation of monocotyledonous plants by *Agrobacterium*- or *Rhizobium*-based methods has also been reported. Transformation and plant regeneration have been achieved and reported at least in asparagus, barley, maize, oat, rice, sugarcane, tall fescue, and wheat. Techniques that may be particularly useful in the context of cotton transformation are disclosed in U.S. Patent Nos. 5,846.797, 5,159,135, 5,004,863, and 6,624,344. Techniques for transforming *Brassica* plants in particular are disclosed, for example, in U.S. Patent 5,750,871. Techniques for transforming soybean are

disclosed in for example in Zhang *et al.*, (1999) and US Patent 6,384,301; and techniques for transforming corn are disclosed in for example in U.S. Patent 5,981,840, U.S. Patent 7,060,876, U.S. Patent 5,591,616, WO95/06722, and U.S. Patent Pub. 2004/244075.

In one embodiment, after incubation on medium containing antibiotics to inhibit *Agrobacterium* or *Rhizobium* growth without selective agents (delay medium), the explants are cultured on selective growth medium including, but not limited to, a callus-inducing medium containing a plant cell selective agent. Typical selective agents have been described and include, but are not limited to, antibiotics such as G418, paromomycin, kanamycin, or other chemicals such as glyphosate, dicamba, and glufosinate. The plant tissue cultures surviving the selection medium are subsequently transferred to a regeneration medium suitable for the production of transformed plantlets. Regeneration can be carried out over several steps. Those of skill in the art are aware of the numerous types of media and transfer requirements that can be implemented and optimized for each plant system for plant transformation and regeneration.

The transformants produced are subsequently analyzed to determine the presence or absence of a particular nucleic acid of interest contained on the transformation vector. Molecular analyses can include, but are not limited to, Southern blots or PCR (polymerase chain reaction) analyses. These and other well known methods can be performed to confirm the stability of the transformed plants produced by the methods disclosed, as well as the copy number of insertions, and the presence of vector backbone sequences flanking the T-DNA. These methods are well known to those of skill in the art and have been reported (see, for example, Sambrook *et al.*, 1989).

The previous discussion is merely a broad outline of standard transformation and regeneration protocols. One of skill in the art knows that specific crops and specific protocols can vary somewhat from the broad outline. A variety of media can be used in each system as well. Those of skill in the art are familiar with the variety of tissue culture media that, when supplemented appropriately, support plant tissue growth and development. These tissue culture media can either be purchased as a commercial preparation or custom prepared and modified by those of skill in the art. Examples of such media include, but are not limited to those described by Murashige

and Skoog (1962); Chu *et al.* (1975); Linsmaier and Skoog (1965); Uchimiya and Murashige (1962); Gamborg *et al.* (1968); Duncan *et al.* (1985); McCown and Lloyd (1981); Nitsch and Nitsch (1969); and Schenk and Hildebrandt (1972), or derivations of these media supplemented accordingly. Those of skill in the art are aware that media and media supplements such as nutrients and growth regulators for use in transformation and regeneration are usually optimized for the particular target crop or variety of interest. Reagents are commercially available and can be purchased from a number of suppliers (see, for example Sigma Chemical Co., St. Louis, Mo.).

"Overdrive" sequences have been identified in numerous Ti plasmids, including pTiA6, pTiAB3, and pTi15955. Other sequences with high similarity to overdrive or TSS can be identified, for example, using the "BestFit," "Gap," or "FASTA" programs of the Sequence Analysis Software Package, Genetics Computer Group, Inc., University of Wisconsin Biotechnology Center, Madison, Wis. 53711, or using the "BLAST" program (Altschul *et al.*, 1990), or another available DNA sequence analysis package. Such sequences when present in multicopy near an RB sequence may be assayed for transformation enhancement activity, similarly to the sequences whose enhancer activity is described below.

"Frequency of transformation" or "transformation frequency," as used herein, refers to the percentage of transgenic events produced per explant or the percentage of transgenic plants produced per explant.

"Border sequence," *e.g.* right border (RB) or left border (LB), refers to a directly repeated nucleic acid sequence defining an end of the transferred DNA (T-DNA) region, typically about 24 bp in length. Border sequences may be from a Ti or Ri plasmid of *Agrobacterium* sp., or may be plant derived sequences that function similarly.

"T-DNA Border region" refers to the RB or LB sequence and associated flanking sequence, typically about 100 bp in length, and, as found in nature, may include a transformation enhancer sequence.

"Transformation efficiency" as used herein, refers to any improvement, such as increase in transformation frequency and quality events that impact overall efficiency of the transformation process by reducing the amount of resources required to select event for further commercial development.

"Transformation enhancer" as used herein refers to overdrive and TSS sequences.

A first nucleic acid sequence is "operably linked" with a second nucleic acid sequence when the sequences are so arranged that the first nucleic acid sequence affects the function of the second nucleic-acid sequence. Preferably the two sequences are part of a single contiguous nucleic acid molecule. The overdrive or TSS enhancer sequence may be placed immediately adjacent to the border sequence, such as the RB sequence. Alternatively, in certain embodiments the overdrive or TSS sequence is located about 1, 10, 25, 50, 100, 250, 500, 1000 or more nucleotides from the end of the border sequence, including all intermediate ranges. The overdrive sequence may be placed in either orientation relative to the border.

EXAMPLES

Those of skill in the art will appreciate the many advantages of the methods and compositions provided by the present invention. The following examples are included to demonstrate the preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result.

EXAMPLE 1

Synthesis of transformation enhancer sequences

1) Synthesis of 4x overdrive sequence

To synthesize a 4x 30 bp overdrive (OD) sequence (5' caaacaaacatacacagcgacttattcacacaaacaaacatacacagcgacttattcacacaaacaaacatacacagcgac ttattcacacaaacaaacatacacagcgacttattcaca 3'; SEQ ID NO:18), 2x 30 bp overdrive primer pair 5' caaacaaacatacacagcgacttattcacacaaacaaacatacacagcgacttattcaca 3'

(Xd463; SEQ ID NO:1) and 5' tgtgaataagtcgctgtgtatgtttgtttgtgtgaataagtcgctgtgtatgtttgtttg 3' (Xd464; SEQ ID NO:2) were synthesized, mixed and PCR amplified for 20 cycles in the presence of high fidelity PfuTurbo® polymerase from Stratagene (La Jolla, CA). The PCR product was fractionated on a 1% Agarose gel, and the portion of the gel corresponding to the size ranging between 100-300 bp was excised, purified and ligated into TOPO Zero blunt PCR vector from Invitrogen (Carlsbad, CA). The repeat stacking was confirmed by sequencing. Up to 6x overdrive sequence was observed following PCR, although only 4x 30 bp overdrive insert was utilized in subsequent cloning of a multicopy overdrive construct.

2) Synthesis of 18x TSS sequence

6x 8 bp TSS repeat primer pairs:

5' ctgacgaactgacgaactgacgaactgacgaactgacgaactgacgaa 3'(Xd465; SEQ ID NO:3), and 5' ttcgtcagttcgtcagttcgtcagttcgtcagttcgtcagttcgtcag 3' (Xd466; SEQ ID NO:4) were synthesized and equally mixed and amplified for 5 cycles in the presence of Pfu Turbo® polymerase from Stratagene (La Jolla, CA).. The 100-300 bp size gel slice was cut, purified and ligated into TOPO Zero blunt PCR vector from Invitrogen. Up to 35x TSS repeat was confirmed by sequencing, but only 18x TSS repeat was kept for further cloning. The size of overdrive and TSS was dependent on the PCR cycles and the excised gel position.

EXAMPLE 2

Construction of vectors having RB with overdrive, additional overdrive, or 18x TSS

To place the overdrive or TSS in front of a 24 bp RB, an *Eco*RI site was introduced into a nopaline RB, 11 bp away from the upstream of the RB (of pMON83900). The 4x overdrive or 18x TSS was excised from the corresponding TOPO cloning vector digested by *Eco*RI and inserted into pMON83900 opened by *Eco*RI, resulting in pMON83903 and pMON83909, respectively.

The modified RB containing either 4x overdrive or the 18x TSS from pMON83903 or pMON83909 were digested with *Hin*dIII/*Spe*I and used to replace the 1x overdrive RB of pMON83902 with the *Hin*dIII/*Spe*I fragment comprising the 4x overdrive or 18x TSS enhancer sequences, resulting in pMON87462 and

pMON83864, respectively, for soy transformation. Alternatively, the RB of pMON80105 was modified so as to comprise the 4x overdrive or 18x TSS by inserting the *SpeI/SalI* fragment from pMON83903 or pMON83909 to yield pMON87465 and pMON87466, respectively, for corn transformation. The modified RB with 1x, 4x and 18x transformation enhancer sequences are shown in FIGs. 1 and 4 and SEQ ID NOs:14-16.

A construct containing 1x overdrive sequence (SEQ ID NO:17) was synthesized by first assembling the oligonucleotide containing the 30 bp overdrive sequence according to standard protocol and then cloning it into pBlueScript®II (Stratagene Inc., La Jolla, CA), resulting in pMON80088. Then the *Spe*I and *Not*I (filled-in with polymerase) fragment from pMON80088 was inserted into pMON80105 digested with *Spe*I and *Sma*I, resulting into pMON80121 for corn transformation. For soybean transformation, 1x overdrive RB construct, pMON83902, was made by replacing the RB in pMON83898 with the 1x overdrive RB fragment from pMON80121 using *Pme*I/*Nde*I restriction enzyme sites.

EXAMPLE 3

Transformation of Corn with Overdrive or TSS-enhanced RB sequences

Corn (*Zea mays*) cells were transformed with *oriV* containing vectors pMON80105, pMON80121, pMON87465, or pMON87466 essentially as described in U.S. Patent Application Publn. 2004/244075 in order to assess the ability of stacking of additional overdrive and TSS enhancer sequences to improve transformation frequency and the proportion of events comprising low copy number T-DNA insertion and lacking vector backbone sequence (*e.g.* *E. coli*-derived *ori*V). The control treatment consisted of transformation with pMON80105, lacking an overdrive or TSS sequence. As shown in Table 1, use of constructs comprising stacked enhancer sequences resulted in a statistically significant increase in transformation frequency. With these constructs, a higher percentage of quality TF was also obtained. Quality TF combines TF and events with one or two copies. Also, the percentage of events having one or two copies increased.

Table 1: Effect of transformation enhancer sequences on transformation frequency and event quality in corn.

Overdrive in RB	Construct (pMON)	% Transformation Frequency (TF)[a]	% Quality TF	% of one or two copies events regardless of backbone
4X OD	87465	25.3	12.7%	50.1
1X OD	80121	24.1	10.4%	43.2
18X TSS	87466	22.8	10.2%	44.7
Control	80105	17.7	6.4%	39.0

[a] denotes statistical significance

EXAMPLE 4

Transformation of Soybean with Overdrive or TSS-enhanced RB sequences

Soybean (*Glycine max*) cells were transformed with *oriV* containing vectors pMON83898, pMON83902, pMON87462, or pMON87464 essentially as described in U.S. Patent 6,384,301 in order to assess the ability of stacking of additional overdrive and TSS enhancer sequences to improve transformation frequency and the proportion of events comprising low copy number T-DNA insertion and lacking vector backbone sequence (*e.g. E. coli*-derived *ori*V). The sequences of the stacked 4x overdrive and 18x TSS enhancers are found in SEQ ID NO:10 and SEQ ID NO:11, respectively. The control treatment consisted of transformation with pMON83898, lacking an overdrive or TSS sequence. As shown in Tables 2-3, use of constructs comprising stacked transformation enhancer sequences resulted in an increase in transformation frequency. The proportion of single copy and backbone free events also increased (Table 3; column 5). Also, the percentage of events having one or two copies increased.

Table 2: Effect of transformation enhancer sequences on transformation frequency in soybean.

Enhancer sequence	pMON plasmid	Transformation frequency (%)
Control	83898	2.79
1X overdrive	83902	3.06
4X overdrive	87462	4.22*
18X TSS	87464	4.10*

* statistically significant increase

Table 3: Effect of transformation enhancer sequences on event quality.

pMON plasmid	Total events	*oriV* positive / total GOI positive	*oriV* negative / total GOI positive	1 copy / *oriV* negative	1 or 2 copy regardless of *oriV* presence or absence
83898 (control)	31	6/24 (25%)	18/24 (75%)	1 (4%)	19 (79%)
83902 (1X OD)	61	16/45 (35.5%)	29/45 (64.5%)	6 (13.3%)	30 (66.7%)
87462 (4X OD)	43	12/33 (36.4%)	21/33 (63.6%)	4 (12.1%)	23 (69.7%)
87464 (18X TSS)	71	10/50 (20%)	40/50 (80%)	17 (34%)	37 (74%)

EXAMPLE 5

Additional Transformation Enhancer sequences

In addition to the overdrive sequence of pTiA6 used above (SEQ ID NO:17), other overdrive sequences (including the reverse complementary sequences) are known in the art (*e.g.* Shurvinton and Ream, 1991), and may be used similarly. These sequences may include but are not limited to those from pTiAB3 (GenBank M63056) (TGTGAATAAATCGCTGTGTATGTTTGTTTG; SEQ ID NO:8), and pTi15955 (GenBank AF242881) (TTGTCTAAATTTCTGTATTTGTTTGTTTG; SEQ ID NO:9), and the consensus sequence AAACAAACATACACAGCGACTTATTCACA (SEQ ID NO:13), and TAARTYNCTGTRTNTGTTTGTTTG; (SEQ ID NO:19, Toro *et al.,* 1988) among others. Primers may be synthesized accordingly and PCR carried out as described in Example 1 to create DNA segments comprising these sequences for use in construction of recombinant plasmids analogous to pMON83902, pMON80121, pMON87462, and pMON87465, among others. Crop plants can be transformed with constructs comprising one or more of these transformation enhancer sequences and can be assessed for their ability to improve transformation frequency and the proportion of events comprising low copy number T-DNA insertion and lacking vector backbone sequence.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of the foregoing illustrative embodiments, it will be apparent to those of skill in the art

that variations, changes, modifications, and alterations may be applied to the composition, methods, and in the steps or in the sequence of steps of the methods described herein. More specifically, it will be apparent that certain agents that are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. The scope of the claims should not be limited by the preferred embodiments set forth herein, but should be given the broadest interpretation consistent with the description as a whole.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are referred to herein.

U.S. Patent 5,004,863, U.S. Patent 5,094,945; U.S. Patent 5,107,065; U.S. Patent 5,159,135; US Patent 5,273,894; US Patent 5,276,268; US Patent 5,561,236; U.S. Patent 5,569,834; U.S. Patent 5,591,616; U.S. Patent 5,627,061; U.S. Patent 5,633,435; US Patent 5,637,489; US Patent 5,646,024; U.S. Patent 5,750,871; U.S. Patent 5,846.797, U.S. Patent 5,981,840; U.S. Patent 6,040,497; U.S. Patent 6,506,559; U.S. Patent 6,624,344; US Patent 6,384,301; U.S. Patent 7,022,896; U.S. Patent 7,060,876.

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The embodiments of the present invention for which an exclusive property or privilege is claimed are defined as follows:

1. A method of increasing the efficiency of *Rhizobia*-mediated plant transformation, comprising the steps of:

a) introducing at least one additional transformation enhancer sequence into a plant transformation vector comprising at least one T-DNA border region including a transformation enhancer sequence; and

b) transforming a plant cell with said vector by *Rhizobia*-mediated transformation, wherein the bacterium is competent for the transformation of said plant cell,

wherein the additional transformation enhancer sequence comprises a sequence selected from the group consisting of: SEQ ID NO:6, and a sequence complementary to SEQ ID NO:6, wherein the vector comprises two or more copies of SEQ ID NO:6 or a sequence complementary to SEQ ID NO:6, and

wherein said plant cell is from a plant selected from the group consisting of: corn and soybean.

2. The method of claim 1, wherein the additional transformation enhancer sequence is located proximal to a T-DNA right border (RB) sequence.

3. The method of claim 2, wherein the additional transformation enhancer sequence is from an Ri plasmid of *A. rhizogenes*.

4. The method of claim 1, wherein the *Rhizobia*-mediated transformation is *Agrobacterium*-mediated transformation.

5. The method of claim 1, wherein the Rhizobia-mediated transformation is *Agrobacterium-*, *Rhizobium-*, *Sinorhizobium-*, *Mesorhizobium-*, or *Bradyrhizobium*-mediated transformation.

6. The method of claim 1, further comprising the step of:

c) regenerating a transgenic plant from said plant cell.

7. A recombinant DNA construct comprising a T-DNA border sequence, operably linked to a transformation enhancer sequence that comprises two or more copies of SEQ ID NO:6.

8. The construct of claim 7, wherein the border sequence is a right border (RB) sequence.

9. The construct of claim 7, wherein the border sequence is a left border (LB) sequence.

10. The construct of claim 8, wherein the RB sequence is from a nopaline Ti plasmid.

11. The construct of claim 8, wherein the RB sequence is from an octopine Ti plasmid.

12. A transgenic cell transformed with and comprising the construct of claim 7.

13. The cell of claim 12, defined as a plant or bacterial cell.

14. The cell of claim 13, wherein the cell is an *Agrobacterium* cell.

15. The cell of claim 13, wherein the cell is a *Rhizobium* cell.

16. The cell of claim 13, wherein the plant cell is from a soybean plant or a corn plant.

17. A method of producing a recombinant construct comprising:

introducing at least one additional transformation enhancer sequence into a plant transformation vector, said vector comprising at least one T-DNA border region, and said T-DNA border region including a first transformation enhancer sequence, thereby producing a recombinant construct comprising at least two transformation enhancer sequences, wherein the transformation enhancer sequences are operably linked to the T-DNA border region and comprise one or more copies of SEQ ID NO:6 and one or more copies of a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:13.

18. A recombinant DNA construct comprising a T-DNA border sequence operably linked to a transformation enhancer sequence that comprises a combination of one or more copies of SEQ ID NO:6, and one or more copies of a sequence selected from the group consisting of SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:13.

19. The construct of claim 18, wherein the border sequence is a right border (RB) sequence.

20. The construct of claim 18, wherein the border sequence is a left border (LB) sequence.

21. The construct of claim 19, wherein the RB sequence is from a nopaline Ti plasmid.

22. The construct of claim 19, wherein the RB sequence is from an octopine Ti plasmid.

23. A transgenic cell transformed with the construct of claim 18.

24. The cell of claim 23, defined as a plant or bacterial cell.

25. The cell of claim 24, wherein the cell is an *Agrobacterium* cell.

26. The cell of claim 24, wherein the cell is a *Rhizobium* cell.

27. The cell of claim 24, wherein the plant cell is from a plant selected from the group consisting of soybean, corn, cotton, canola, rice, wheat, alfalfa, common bean, peanut, tobacco, sunflower, barley, beet, broccoli, cabbage, carrot, cauliflower, celery, Chinese cabbage, cucumber, eggplant, leek, lettuce, melon, oat, onion, pea, pepper, peanut, potato, pumpkin, radish, sorghum, spinach, squash, sugarbeet, tomato, and watermelon.

28. The method of claim 17, wherein the border region is a right border (RB) sequence.

29. The method of claim 17, wherein the border region is a left border (LB) sequence.

30. The method of claim 17, further comprising transforming a plant cell with said recombinant construct by Rhizobia-mediated transformation, wherein the Rhizobia bacterium is competent for the transformation of said plant cell.

31. The method of claim 30, wherein the Rhizobia-mediated transformation is *Agrobacterium*-mediated transformation.

32. The method of claim 30, wherein the Rhizobia-mediated transformation is *Agrobacterium*-, *Rhizobium*-, *Sinorhizobium*-, *Mesorhizobium*-, or *Bradyrhizobium*-mediated transformation.

33. The method of claim 30, wherein the plant cell is from a plant selected from the group consisting of soybean, corn, cotton, canola, rice, wheat, alfalfa, common bean, peanut, tobacco,

sunflower, barley, beet, broccoli, cabbage, carrot, cauliflower, celery, Chinese cabbage, cucumber, eggplant, leek, lettuce, melon, oat, onion, pea, pepper, peanut, potato, pumpkin, radish, sorghum, spinach, squash, sugarbeet, tomato and watermelon.

34. The method of claim 33 wherein the plant cell is a corn or soybean cell.

35. The method of claim 17, wherein the T-DNA border region comprises from 2 to about 18 copies of said transformation enhancer sequence.

36. The method of claim 30, further comprising regenerating a transgenic plant from said plant cell.

37. The method of claim 1, wherein the additional transformation enhancer sequence comprises a sequence selected from the group consisting of: SEQ ID NO:11, and a sequence complementary to SEQ ID NO:11, wherein the vector comprises two or more copies of SEQ ID NO:11 or a sequence complementary to SEQ ID NO:11.

38. The recombinant DNA construct of claim 7, comprising a T-DNA border sequence, operably linked to a transformation enhancer sequence of SEQ ID NO:11.

39. The method of claim 1, wherein the plant transformation vector further comprises a sequence selected from the group consisting of: SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9, and SEQ ID NO:13.

40. A recombinant DNA construct comprising a T-DNA border sequence operably linked to a transformation enhancer sequence that comprises a combination of one or more copies of SEQ ID NO:6, and one or more copies of SEQ ID NO:20.

41. The construct of claim 40, wherein the border sequence is a right border (RB) sequence.

42. The construct of claim 40, wherein the border sequence is a left border (LB) sequence.

43. The construct of claim 41, wherein the RB sequence is from a nopaline Ti plasmid.

44. The construct of claim 41, wherein the RB sequence is from an octopine Ti plasmid.

45. A method of increasing the efficiency of *Rhizobia*-mediated plant transformation, comprising the step of: transforming a plant cell with the recombinant DNA construct of claim 40 by *Rhizobia*-mediated transformation, wherein the bacterium is competent for the transformation of said plant cell, and wherein said plant cell is from a plant selected from the group consisting of: corn and soybean.

46. A transgenic cell transformed with the construct of claim 40.

47. The cell of claim 46, defined as a plant or bacterial cell.

48. The cell of claim 47, wherein the cell is an *Agrobacterium* cell.

49. The cell of claim 47, wherein the cell is a *Rhizobium* cell.

50. The cell of claim 47, wherein the plant cell is from a plant selected from the group consisting of soybean, corn, cotton, canola, rice, wheat, alfalfa, common bean, peanut, tobacco, sunflower, barley, beet, broccoli, cabbage, carrot, cauliflower, celery, Chinese cabbage, cucumber, eggplant, leek, lettuce, melon, oat, onion, pea, pepper, peanut, potato, pumpkin, radish, sorghum, spinach, squash, sugarbeet, tomato, and watermelon.

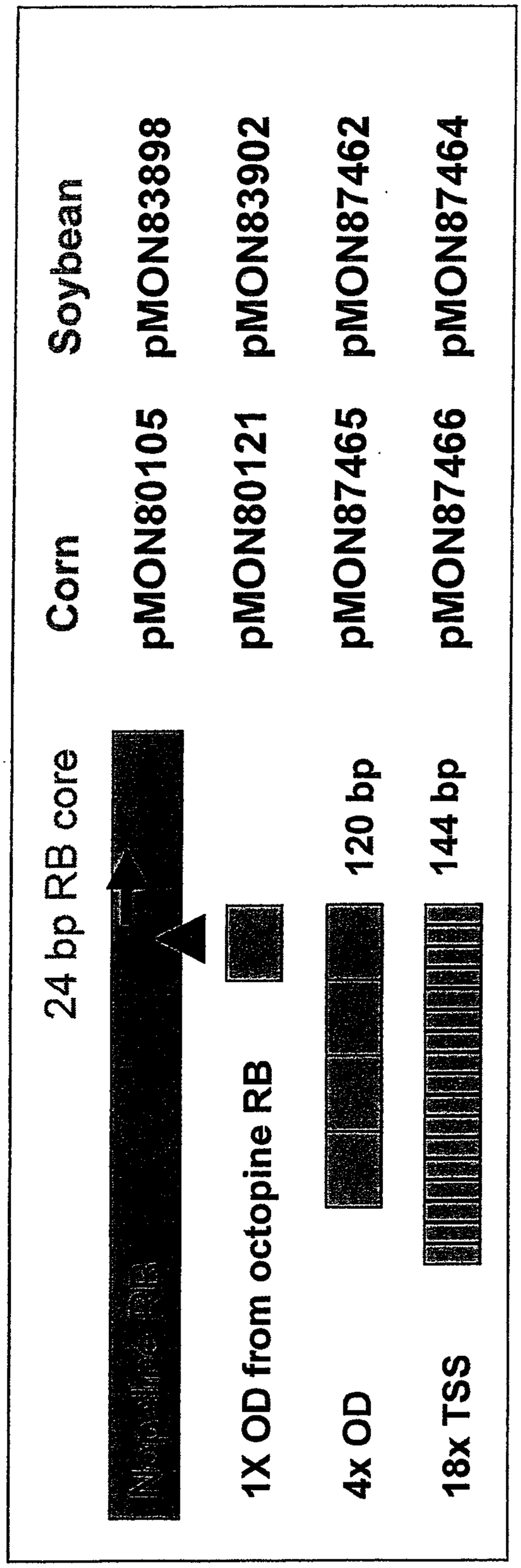

Soybean
pMON83898
pMON83902
pMON87462
pMON87464
Corn
pMON80105
pMON80121
pMON87465
pMON87466
24 bp RB core
120 bp
144 bp
1X OD from octopine RB
4x OD
18x TSS

FIG. 1

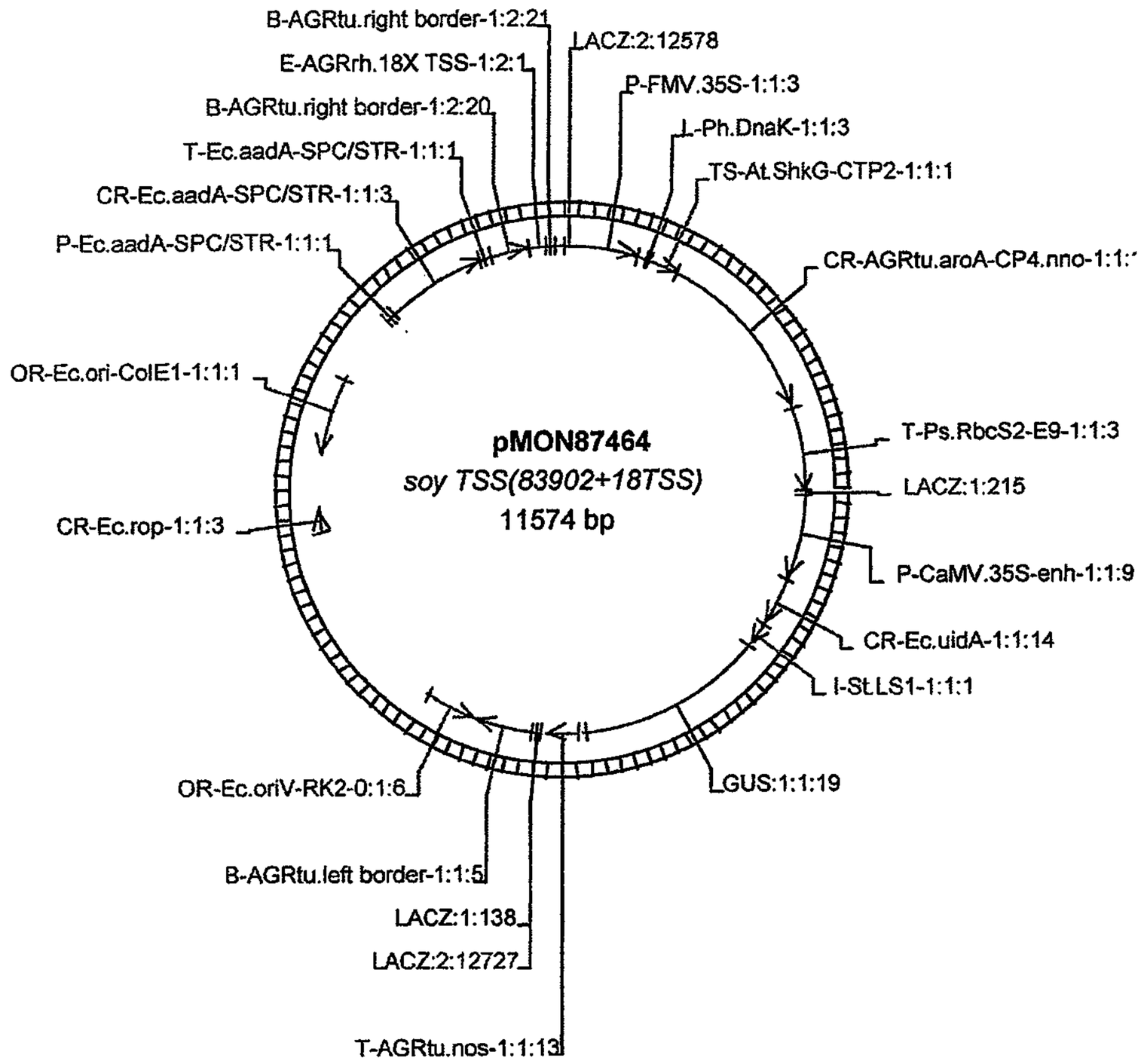

B-AGRtu.right border-1:2:21
E-AGRrh.18X TSS-1:2:1
B-AGRtu.right border-1:2:20
T-Ec.aadA-SPC/STR-1:1:1
CR-Ec.aadA-SPC/STR-1:1:3
P-Ec.aadA-SPC/STR-1:1:1
OR-Ec.ori-ColE1-1:1:1
CR-Ec.rop-1:1:3
OR-Ec.oriV-RK2-0:1:6
B-AGRtu.left border-1:1:5
LACZ:1:138
LACZ:2:12727
T-AGRtu.nos-1:1:13
LACZ:2:12578
P-FMV.35S-1:1:3
L-Ph.DnaK-1:1:3
TS-At.ShkG-CTP2-1:1:1
CR-AGRtu.aroA-CP4.nno-1:1:
T-Ps.RbcS2-E9-1:1:3
LACZ:1:215
P-CaMV.35S-enh-1:1:9
CR-Ec.uidA-1:1:14
I-St.LS1-1:1:1
GUS:1:1:19
pMON87464
soy TSS(83902+18TSS)
11574 bp

FIG. 2

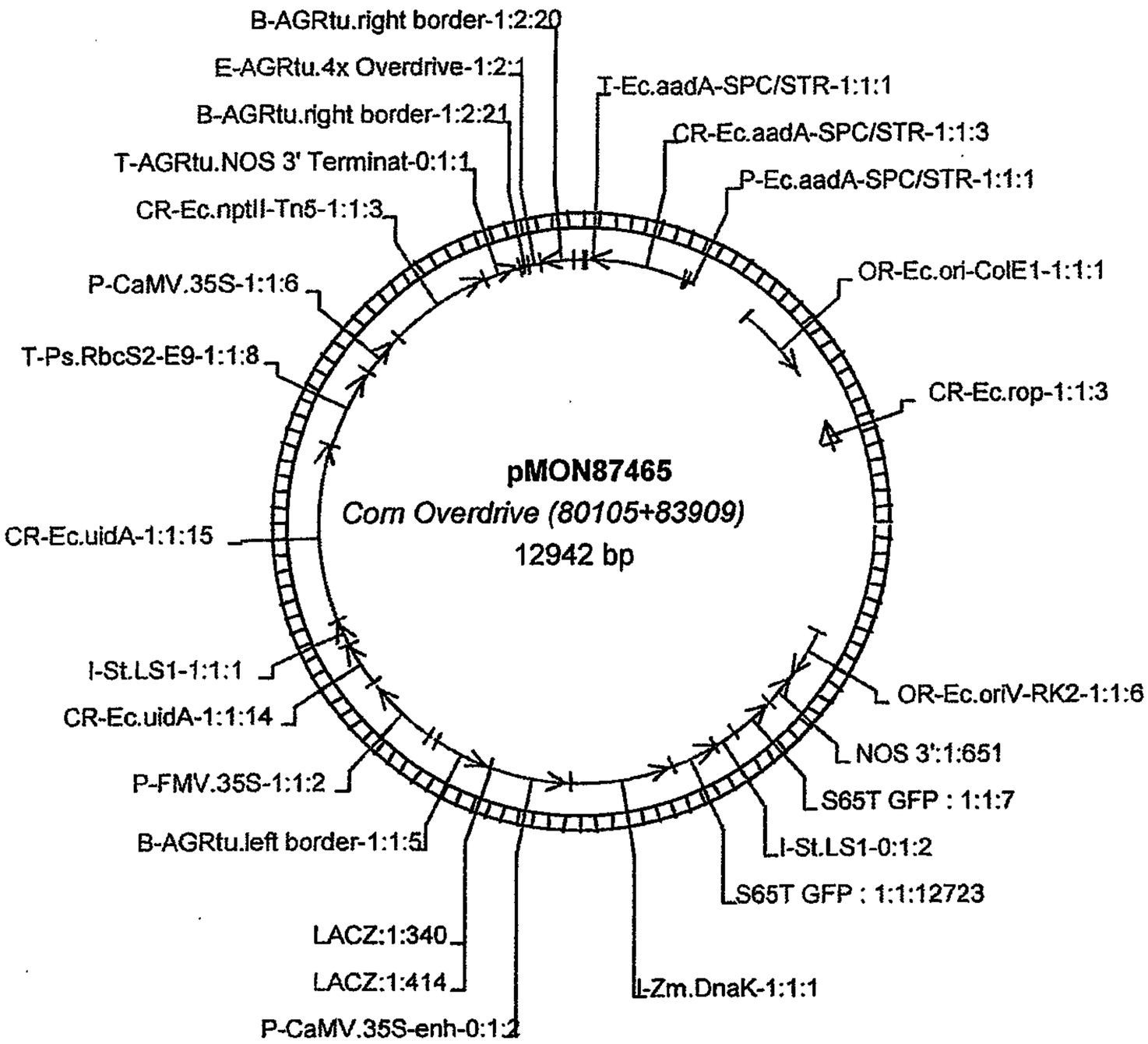

B-AGRtu.right border-1:2:20
E-AGRtu.4x Overdrive-1:2:1
B-AGRtu.right border-1:2:21
T-AGRtu.NOS 3' Terminat-0:1:1
CR-Ec.nptII-Tn5-1:1:3
P-CaMV.35S-1:1:6
T-Ps.RbcS2-E9-1:1:8
I-Ec.aadA-SPC/STR-1:1:1
CR-Ec.aadA-SPC/STR-1:1:3
P-Ec.aadA-SPC/STR-1:1:1
OR-Ec.ori-ColE1-1:1:1
CR-Ec.rop-1:1:3
pMON87465
Corn Overdrive (80105+83909)
12942 bp
CR-Ec.uidA-1:1:15
I-St.LS1-1:1:1
CR-Ec.uidA-1:1:14
P-FMV.35S-1:1:2
B-AGRtu.left border-1:1:5
LACZ:1:340
LACZ:1:414
P-CaMV.35S-enh-0:1:2
I-Zm.DnaK-1:1:1
S65T GFP : 1:1:12723
I-St.LS1-0:1:2
S65T GFP : 1:1:7
NOS 3':1:651
OR-Ec.oriV-RK2-1:1:6

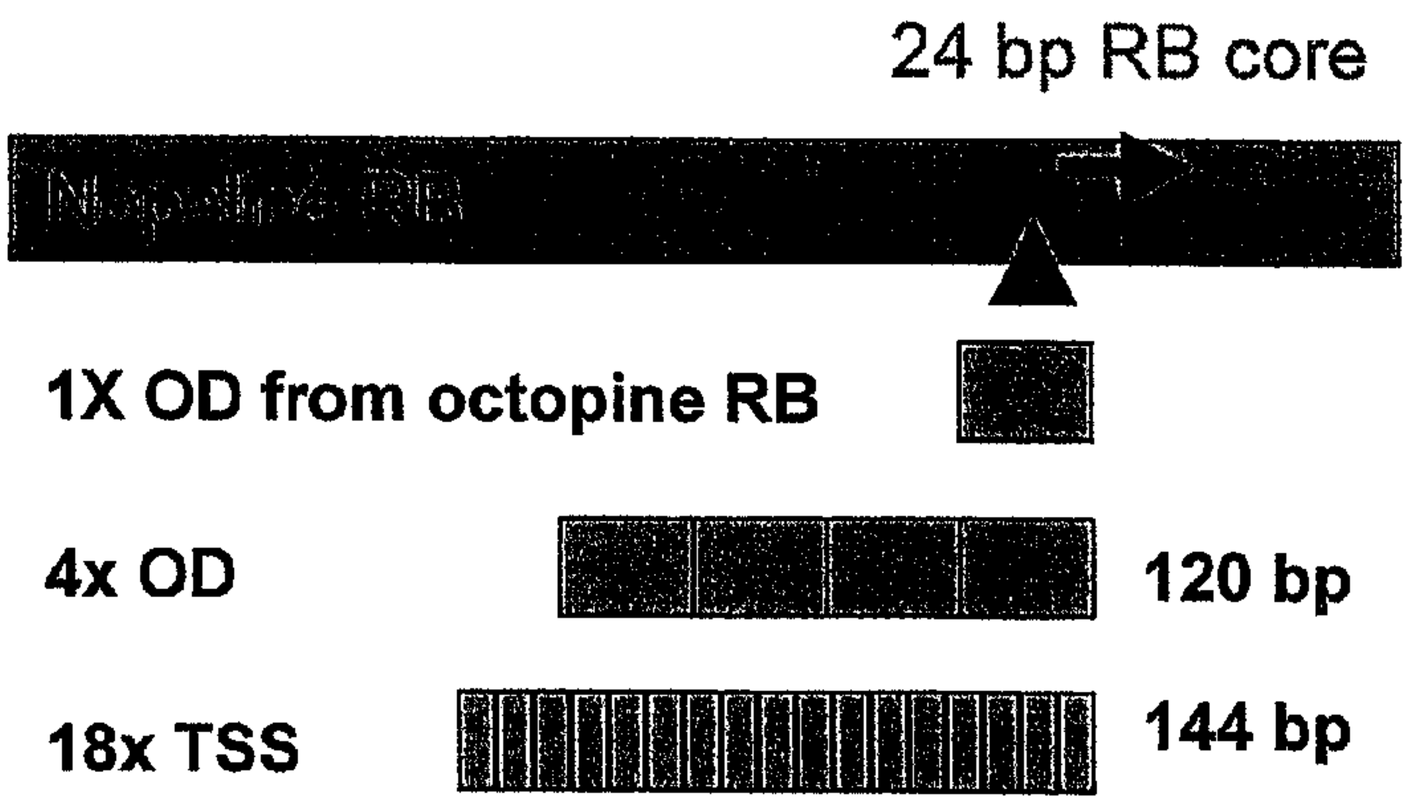
FIG. 3

(A)
Nopaline RB+1x overdrive
AGGATTTTTCGGCGCTGCGCTACGTCCGCGACCGCGTTGAGGGATCAAGC
CACAGCAGCCCACTCGACCTTCTAGCCGACCCAGACGAGCCAAGGGATCTTTTTG
GAATGCTGCTCCGTCGTCAGGCTTTCCGACGTTTGGGTGGTTGAACAGAAGTCAT
TATCGCACGGAATGCCAAGCACTCCCGAGGGGAACCCTGTGGTTGGCATGCACA
TACAAATGGACGAACGGATAAACCTTTTCACGCCCTTTTAAATATCCGATTATTC
TAATAAACGCTCTTT**caaacaaacatacacagcgacttattcaca**TCTCTTAGGTTTACCCGCCA
ATATATCCTGTCAAACACTGATAGTTTAAACTGAAGGCGGGAAACGACAATCT

(B)
Nopaline RB+4x overdrive
AGGATTTTTCGGCGCTGCGCTACGTCCGCgACCGCGTTGAGGGATCAAGCC
ACAGCAGCCCACTCGACCtTCTAGCCGACCCAGACGAGCCAAGGGATCTTTTTGG
AATGCTGCTCCGTCGTCAGGCTTTCCGACGTTTGGGTGGTTGAACAGAAGTCATT
ATCGcACGGAATGCCAAGCACTCCCGAGGGGAACCCTGTGGTTGGCATGCACATA
CAAATGGACGAACGGATAAACCTTTTCACGCCCTTTTAAATATCCGaTTATTCTAA
TAAACGCTCTTTGAATTCGCCCTT**caaacaaacatacacagcgacttattcacacaaacaaacatacaca**
gcgacttattcacacaaacaaacatacacagcgacttattcacacaaacaaacatacacagcgacttattcacaAAGGG
CGAATTCTCTTAGGTTTACCCGCCAATATATCCTGTCAAACACTGATAGTTTAAA
CTGAAGGCGGGAAACGACAATCT

(C)
Nopaline RB+18x TSS
AGGATTTTTCGGCGGCGCTACGTCCGCgACCGCGTTGAGGGATCAAGCCA
CAGCAGCCCACTCGACCtTCTAGCCGACCCAGACGAGCCAAGGGATCTTTTTGGA
ATGCTGCTCCGTCGTCAGGCTTTCCGACGTTTGGGTGGTTGAACAGAAGTCATTA
TCGcACGGAATGCCAAGCACTCCCGAGGGGAACCCTGTGGTTGGCATGCACATAC
AAATGGACGAACGGATAAACCTTTTCACGCCCTTTTAAATATCCGaTTATTCTAAT
AAACGCTCTTTGAATTCGCCCTT**ctgacgaactgacgaactgacgaactgacgaactgacgaactgacga**
actgacgaactgacgaactgacgaactgacgaactgacgaactgacgaactgacgaactgacgaactgacga
actgacgaactgacgaaGGGCGAATTCTCTTAGGTTTACCCGCCAATATATCCTGTCAAAC
ACTGATAGTTTAAACTGAAGGCGGGAAACGACAATCT

FIG. 4



	Corn	Soybean
	pMON80105	pMON83898
	pMON80121	pMON83902
	pMON87465	pMON87462
	pMON87466	pMON87464