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Mirkov et al.

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(54) **COMPOSITIONS, ORGANISMS, SYSTEMS,
AND METHODS FOR EXPRESSING A GENE
PRODUCT IN PLANTS USING SCBV
EXPRESSION CONTROL SEQUENCES
OPERABLE IN MONOCOTS AND DICOTS**

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10, 2010, provisional application No. 61/400,976,
filed on Aug. 5, 2010.

(51) **Int. Cl.**

C07H 21/04 (2006.01)
C12N 1/20 (2006.01)
C12N 15/11 (2006.01)
C12N 15/63 (2006.01)
C12N 15/79 (2006.01)
C12N 15/82 (2006.01)
A01H 4/00 (2006.01)
A01H 5/00 (2006.01)

(52) **U.S. Cl.**

USPC **536/24.1**; 536/23.5; 536/23.6; 536/23.7;
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435/410; 435/411; 435/414; 435/417; 435/419;
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800/278; 800/293; 800/294; 800/295; 800/298;
800/302; 800/316; 800/317; 800/320

(58) **Field of Classification Search**

None
See application file for complete search history.

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(57) **ABSTRACT**

The present disclosure relates, in some embodiments, to compositions, organisms, systems, and methods for expressing a gene product in a plant using a expression control sequence (ECS) operable in monocots and/or dicots. For example, (i) an isolated nucleic acid may comprise an ECS (e.g., a sugarcane bacilliform virus promoter) and, optionally, an exogenous nucleic acid (ExNA) operably linked to the ECS; (ii) an expression vector may comprise an ECS; an ExNA; and, optionally, a 3' termination sequence, wherein the ECS has promoter activity sufficient to express the ExNA in at least one monocot and at least one dicot; (iii) a microorganism, plant cell, or plant may comprise an isolated nucleic acid; (iv) a method for constitutively expressing an ExNA in a plant (e.g., a monocot and/or a dicot) may comprise, contacting an expression vector with the cytosol of a cell of the plant, wherein the expression vector comprises the ExNA and an ECS operable to drive expression of the ExNA; and/or (v) a method of directing constitutive expression of a nucleic acid in a plant (e.g., a monocot and/or a dicot) may comprise transforming the plant with an expression nucleic acid comprising an ECS, an ExNA, and a 3' termination sequence.

52 Claims, 17 Drawing Sheets
(6 of 17 Drawing Sheet(s) Filed in Color)

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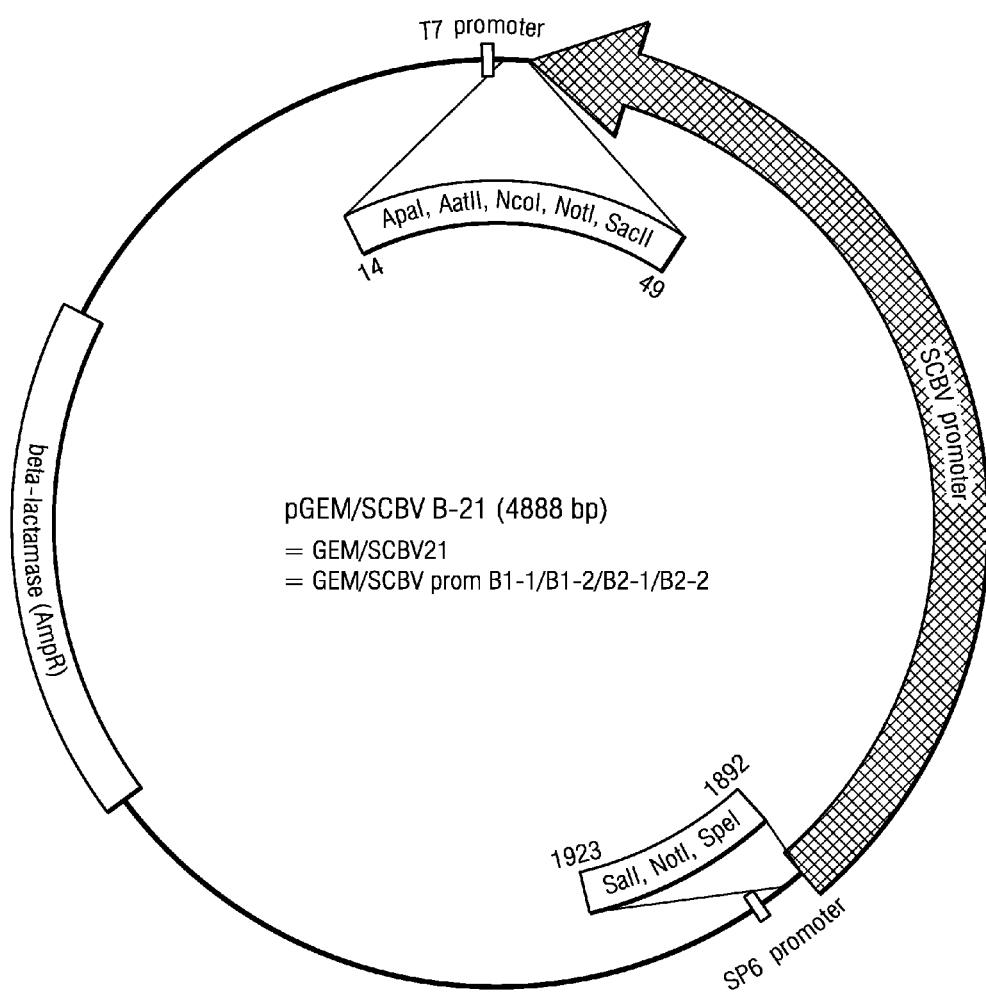


FIG. 1

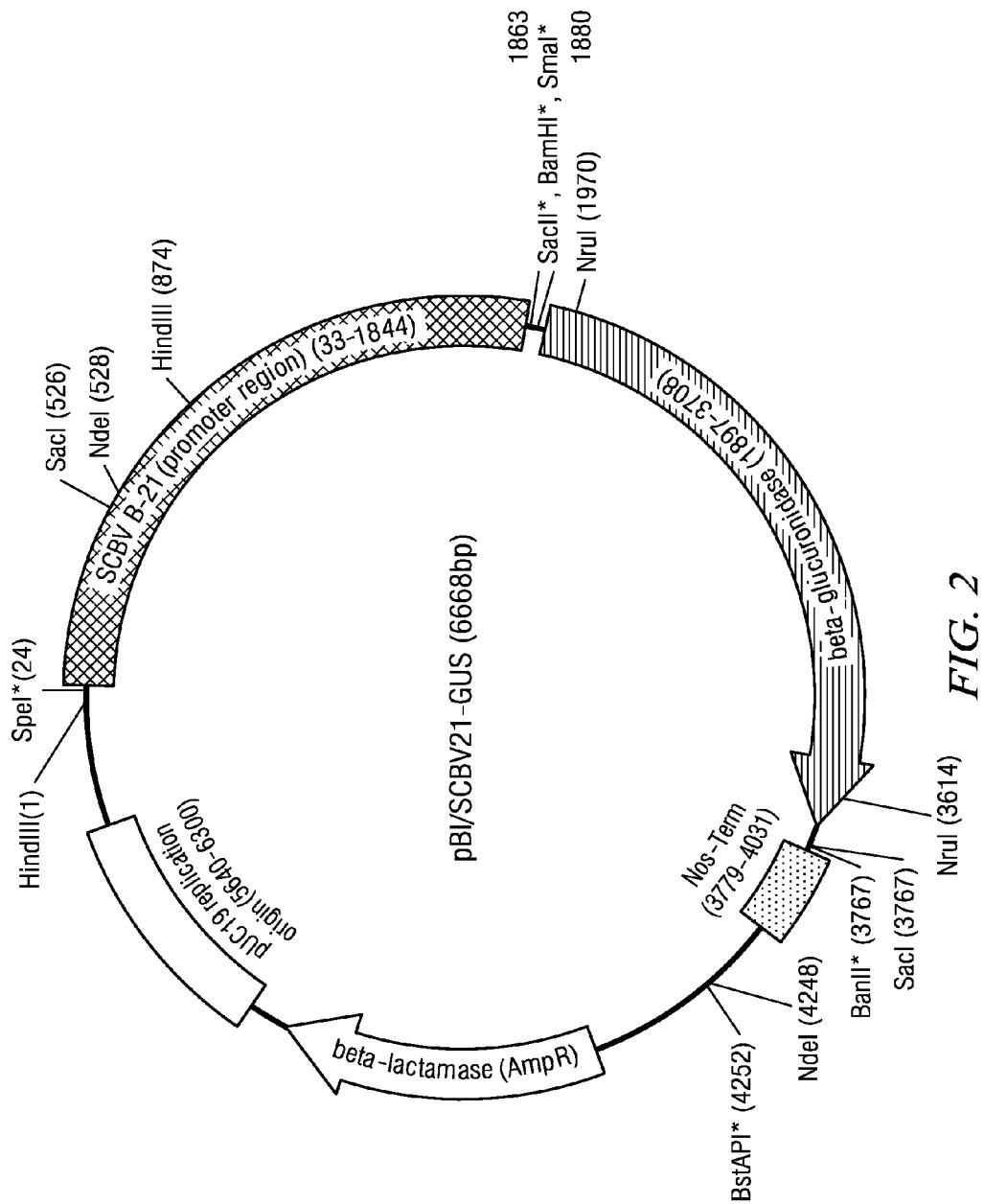


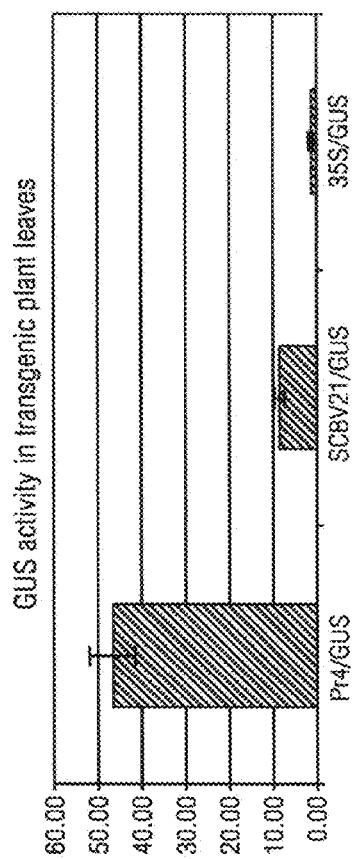
FIG. 2

GUS driven by 35S promoter



(A) (B)

FIG. 3



Construct

FIG. 4

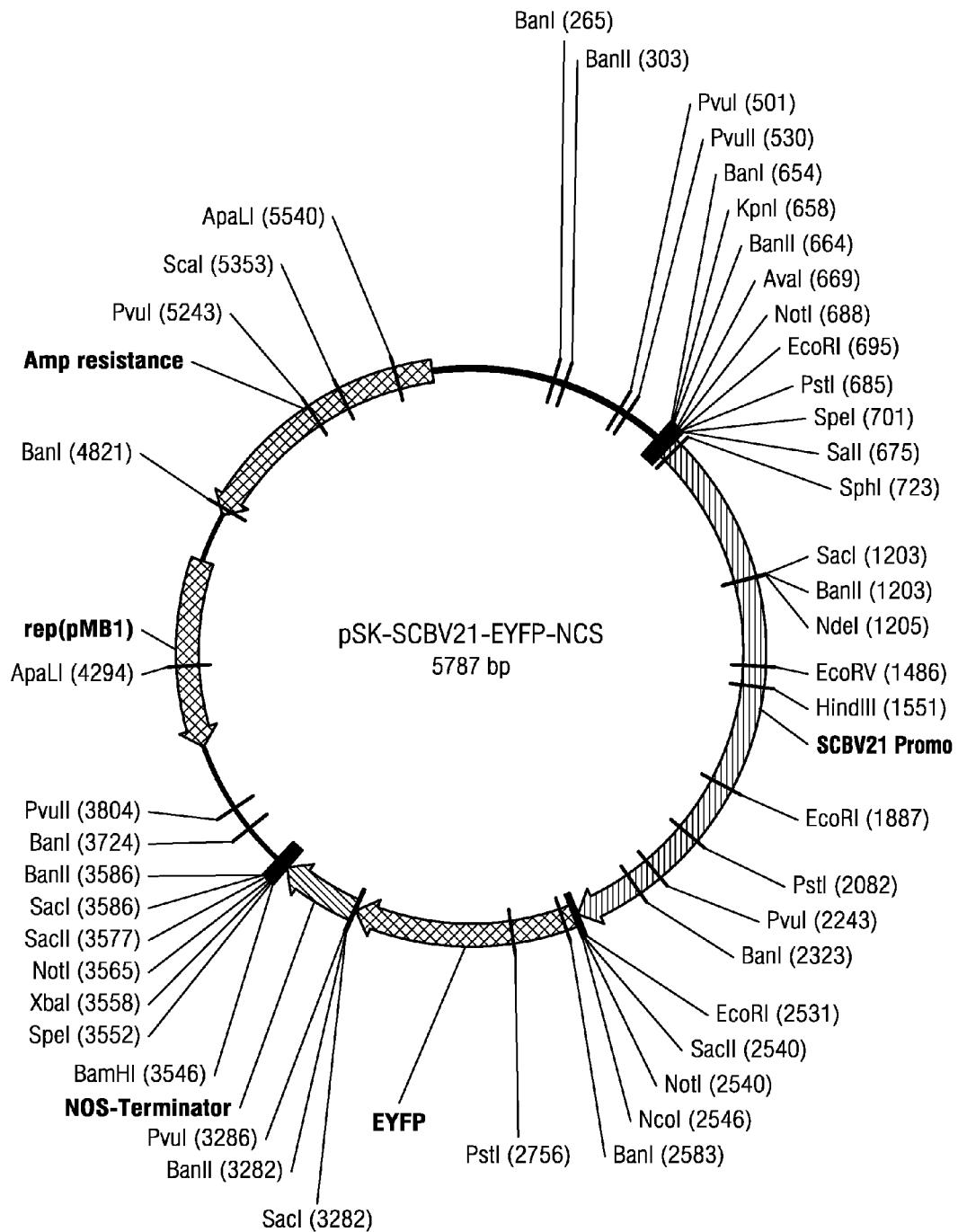


FIG. 5

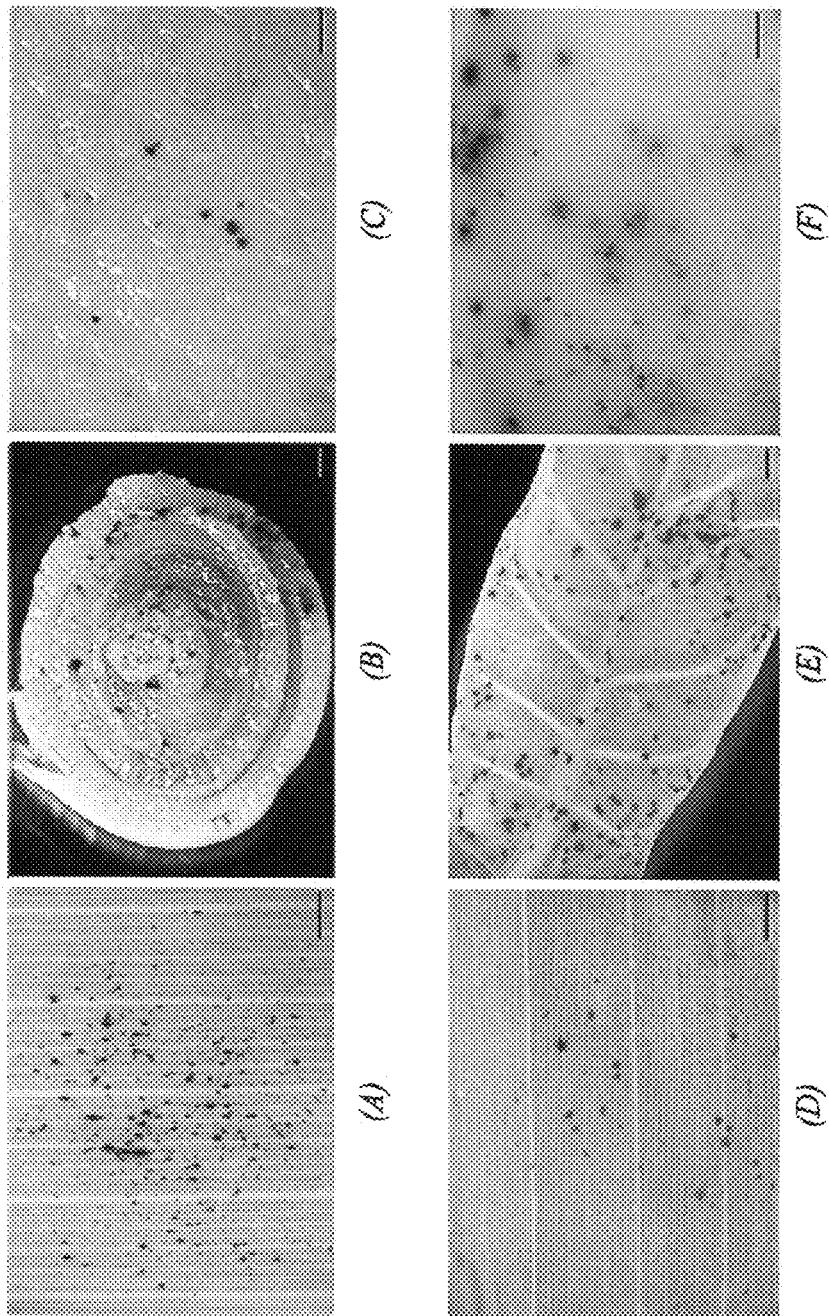


FIG. 6

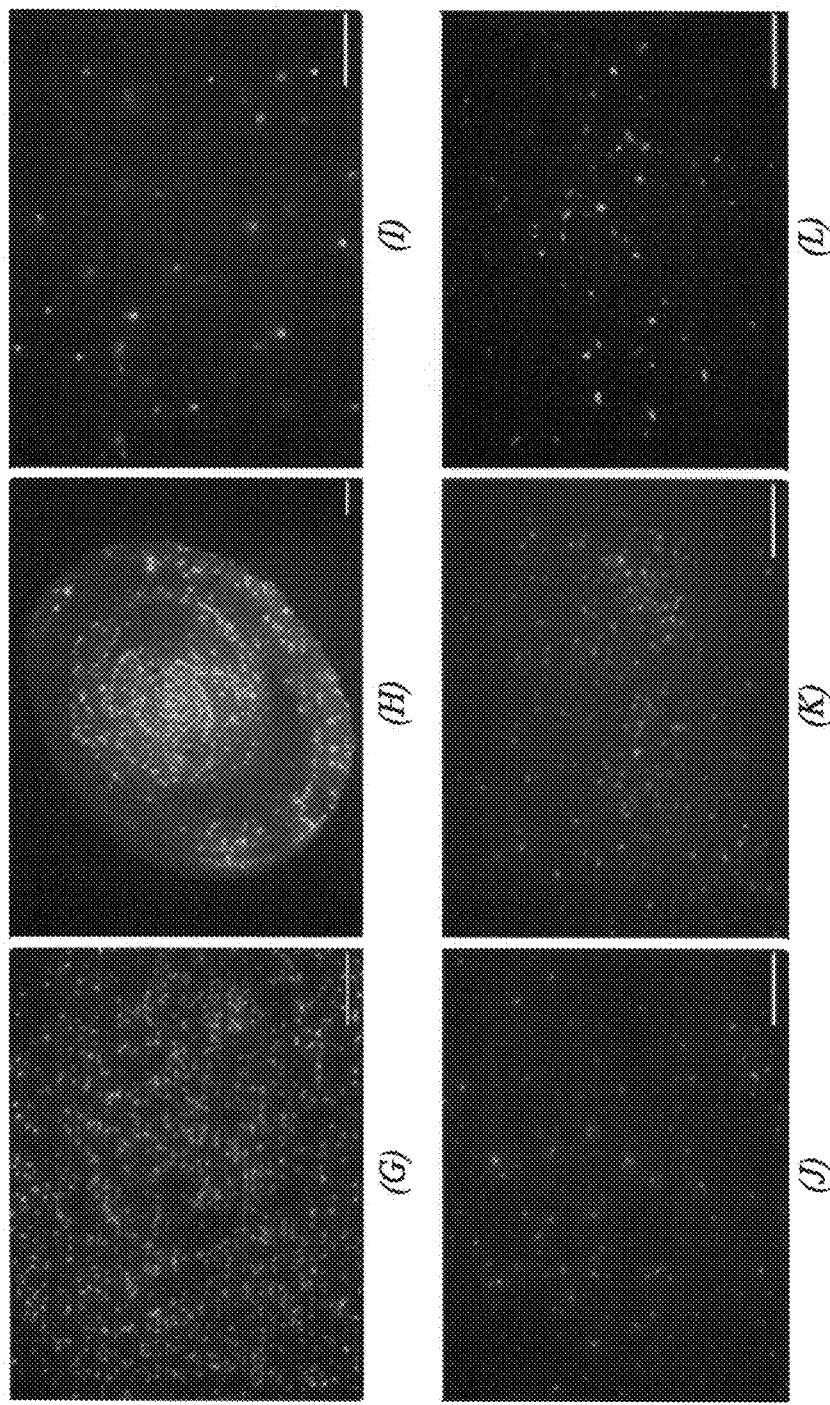


FIG. 6

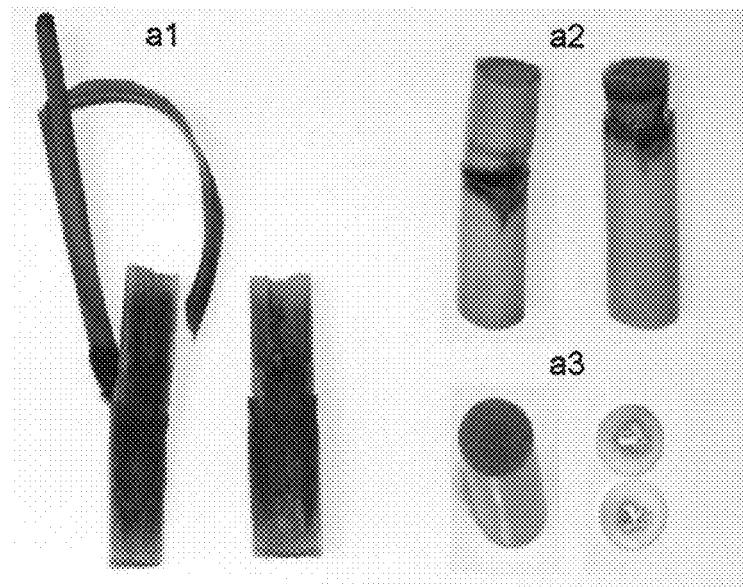


FIG. 7A

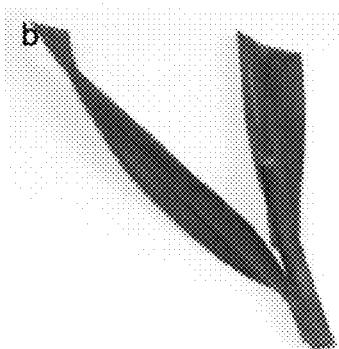


FIG. 7B

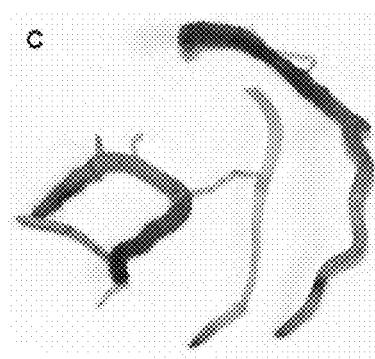


FIG. 7C

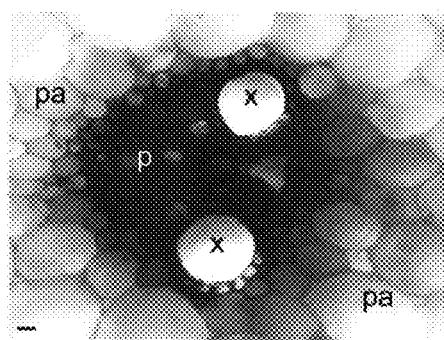


FIG.8A

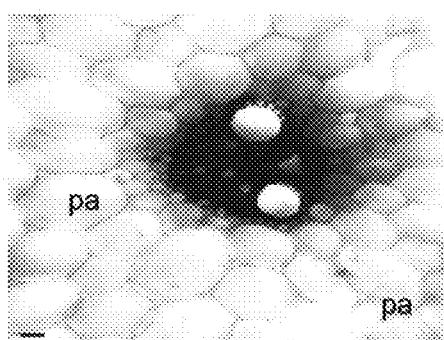


FIG.8B

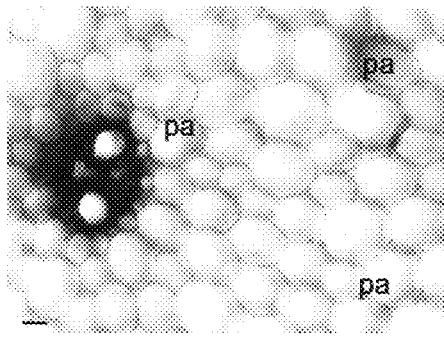


FIG.8C

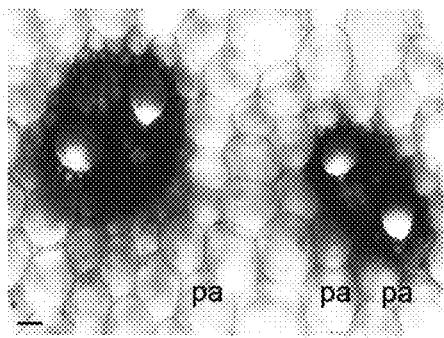


FIG.8D

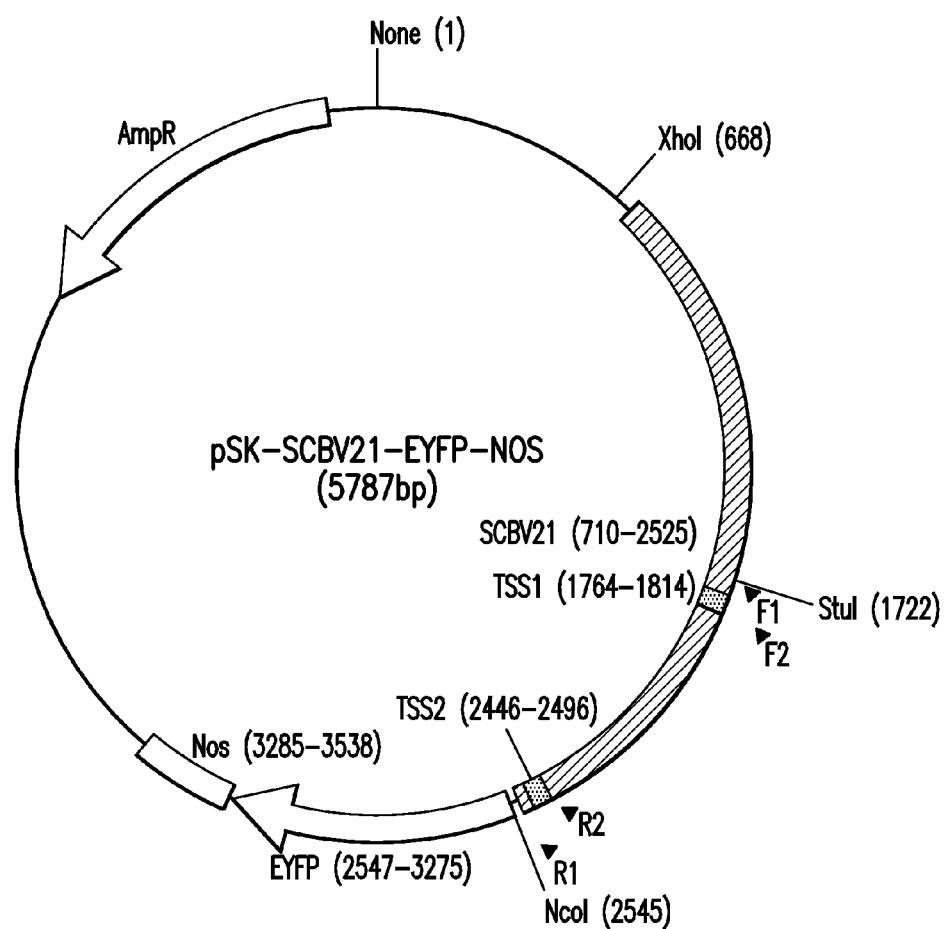


FIG. 9A

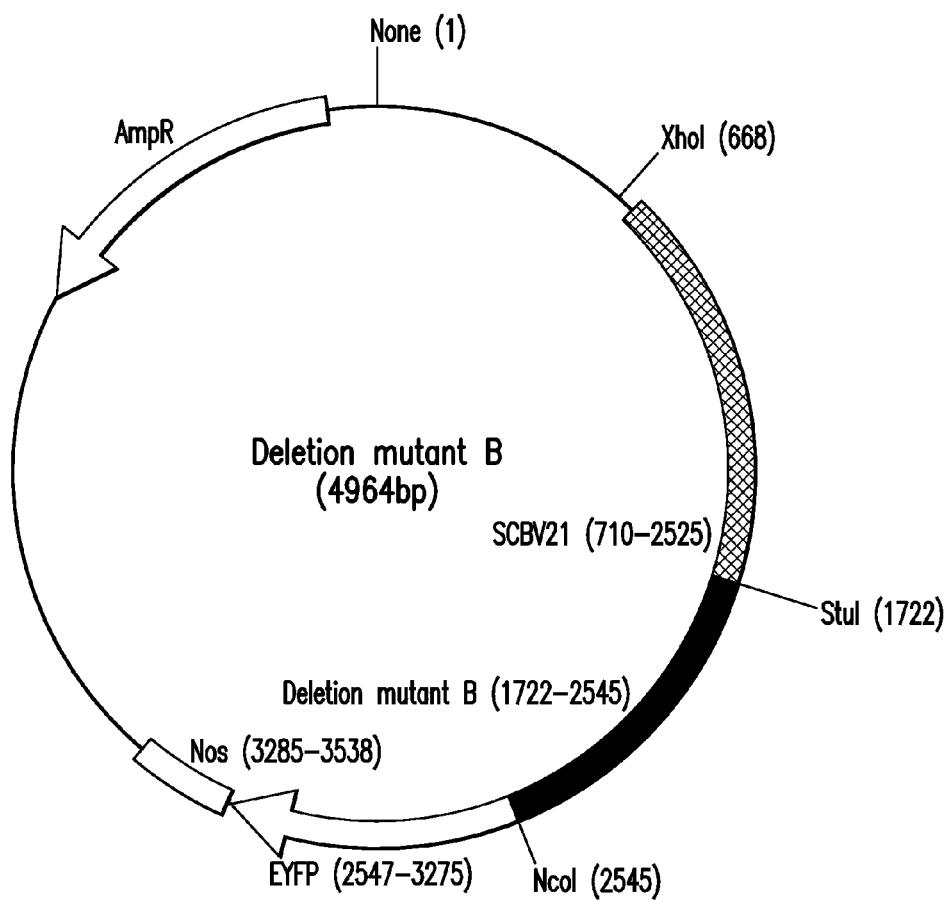


FIG.9B

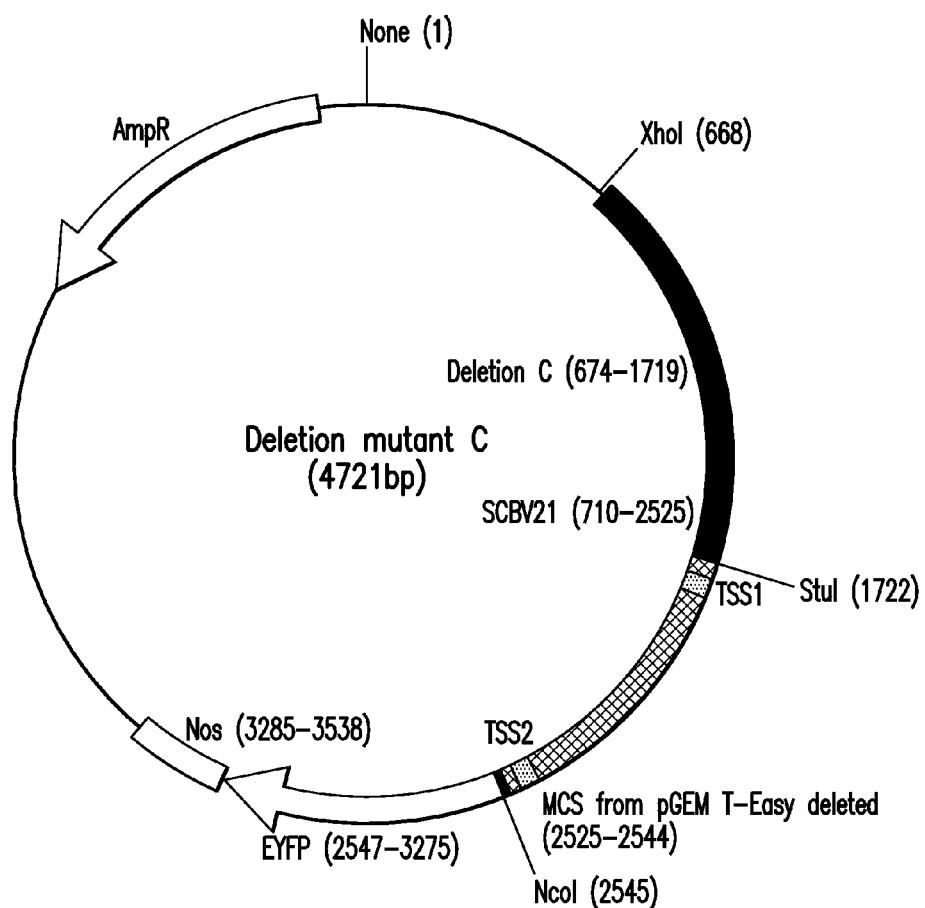


FIG.9C

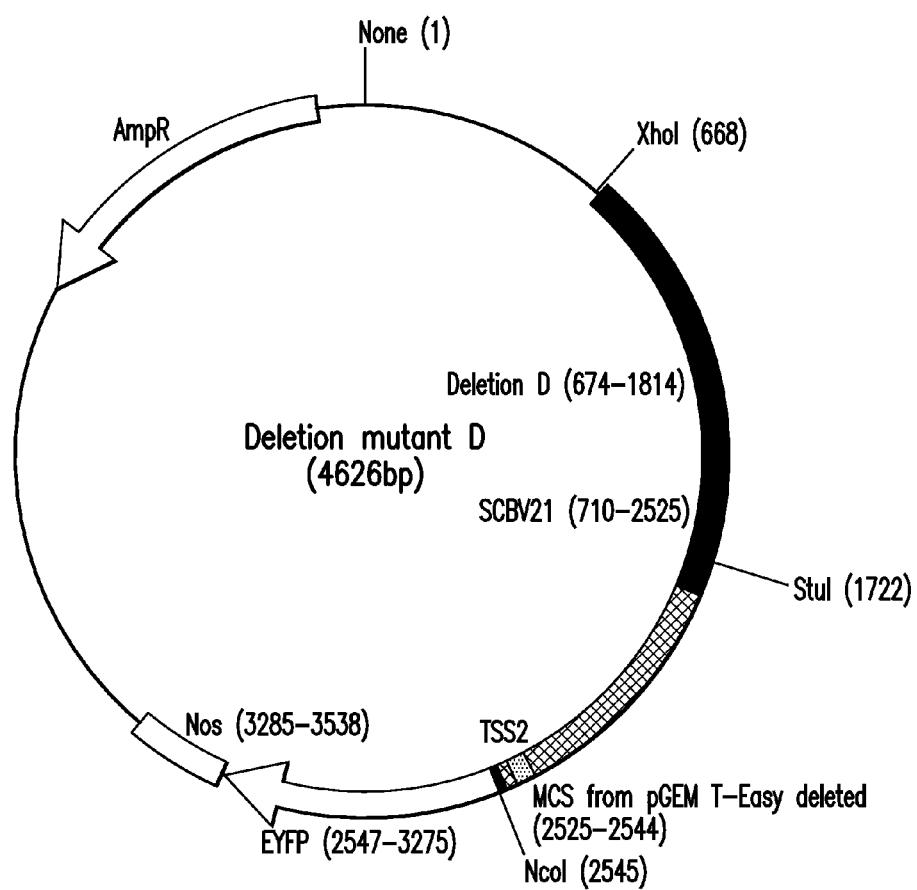


FIG. 9D

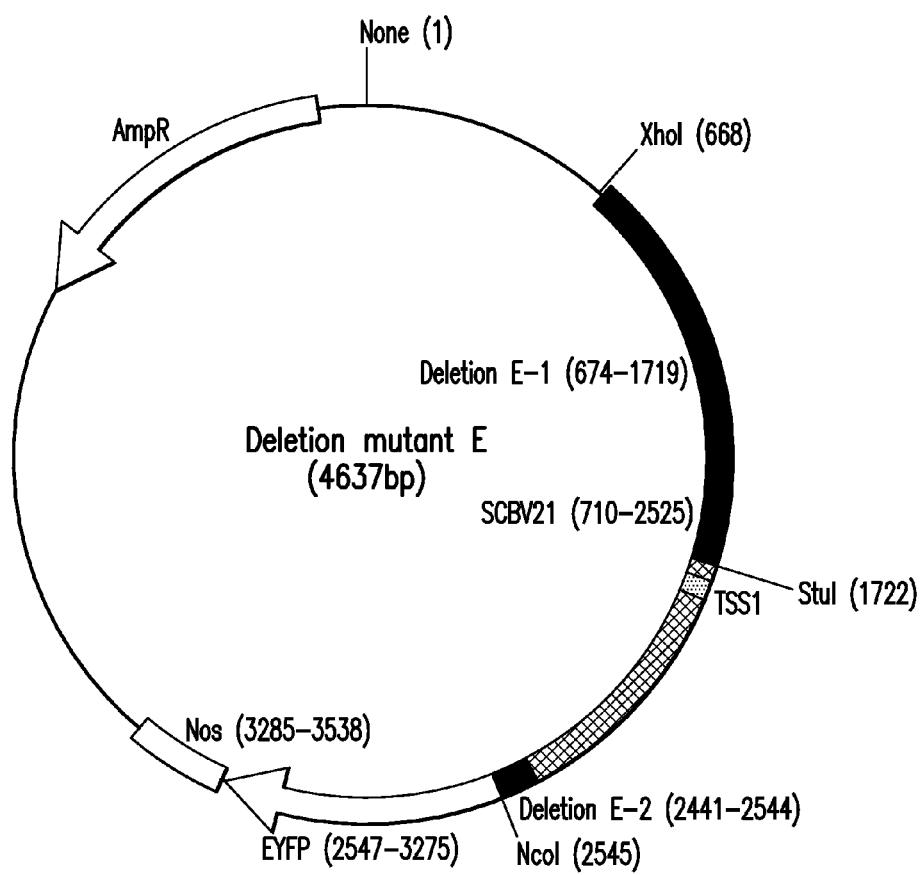
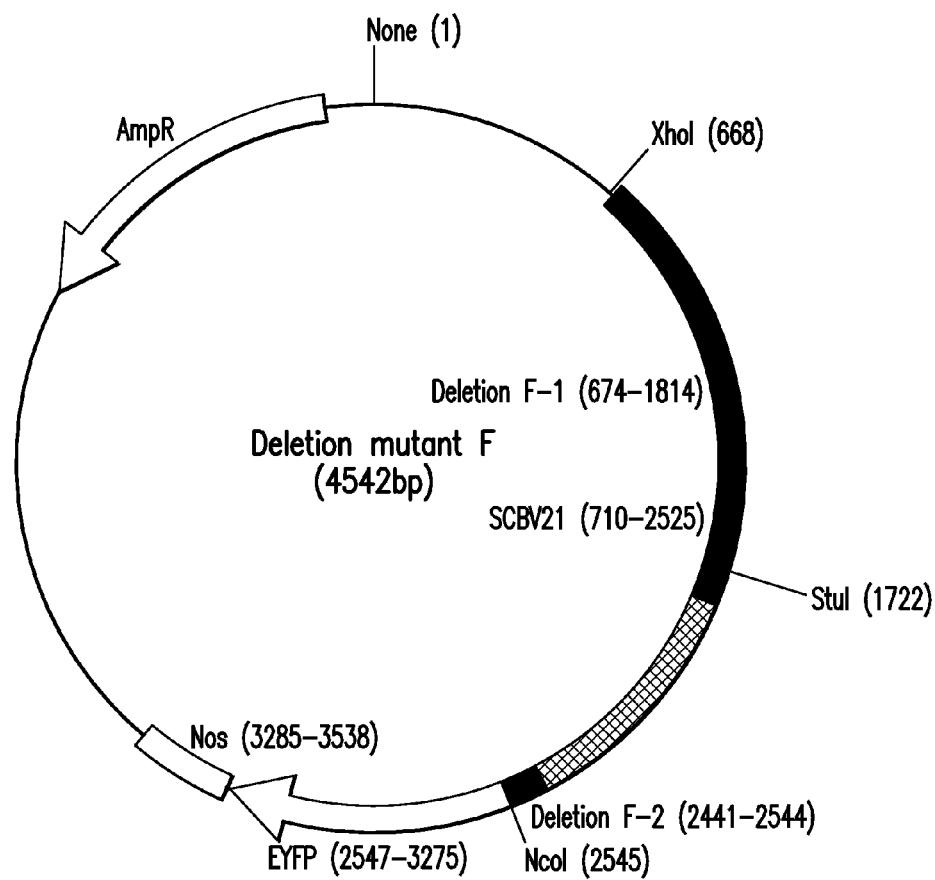
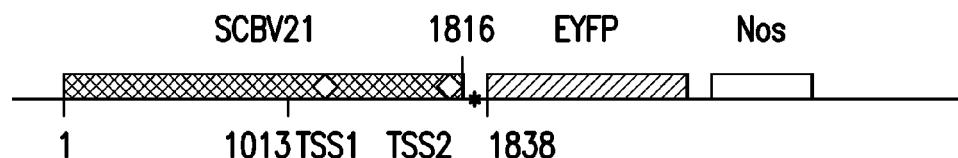
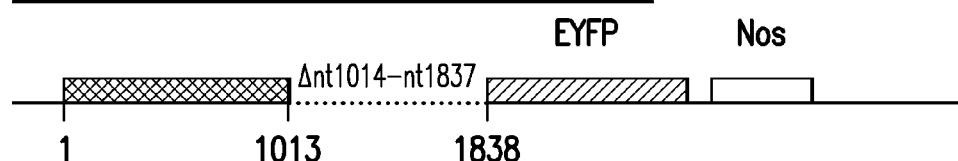
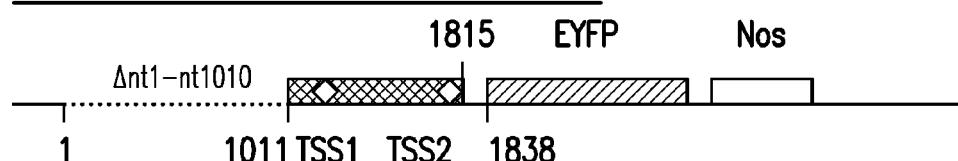
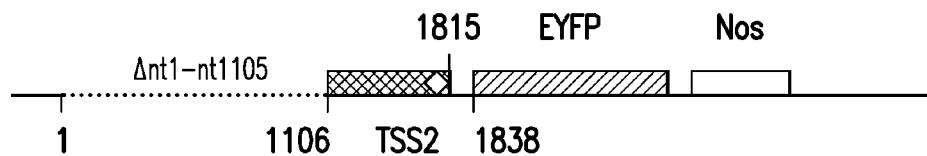
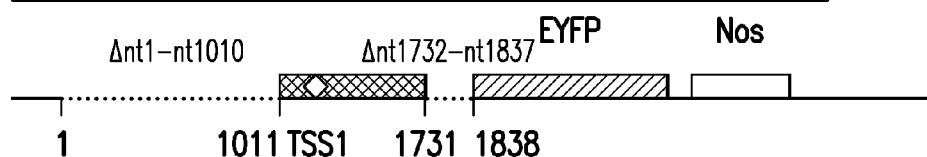
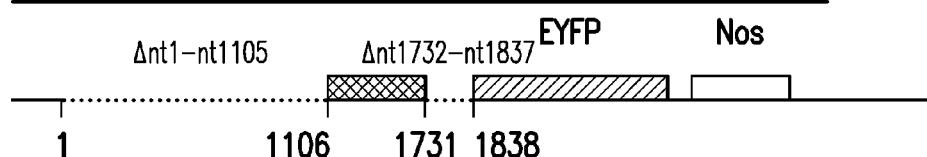
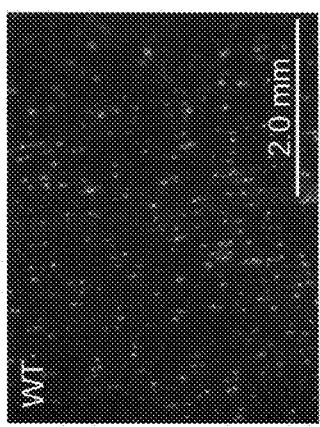


FIG. 9E

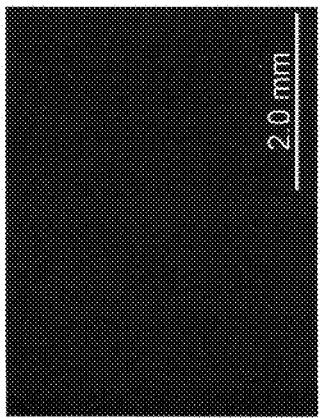
**FIG. 9F**

A. SCBV21-EYFP-Nos (Wild type)**FIG. 10A****Mutant B. SCBV21 Δ nt1014-nt1837-EYFP-Nos****FIG. 10B****Mutant C. SCBV21 Δ nt1-nt1010-EYFP-Nos****FIG. 10C**

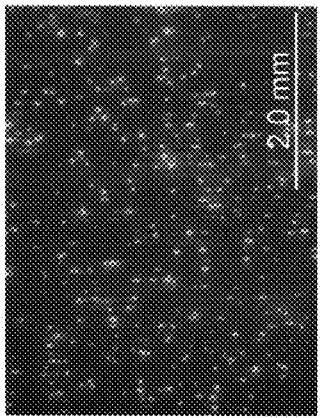
Mutant D. SCBV21 Δ nt1-nt1105-EYFP-Nos**FIG. 10D****Mutant E. SCBV21 Δ nt1-nt1010_Δnt1732-nt1837-EYFP-Nos****FIG. 10E****Mutant F. SCBV21 Δ nt1-nt1105_Δnt1732-nt1837-EYFP-Nos****FIG. 10F**



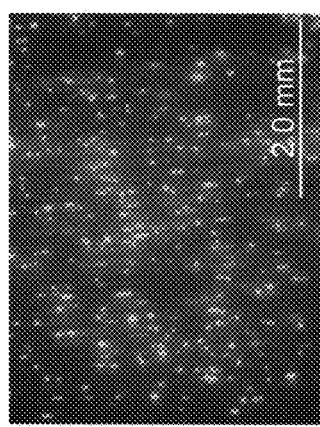
SCBV21 Δnt1-nt1104-
EYFP-Nos
FIG. 11A



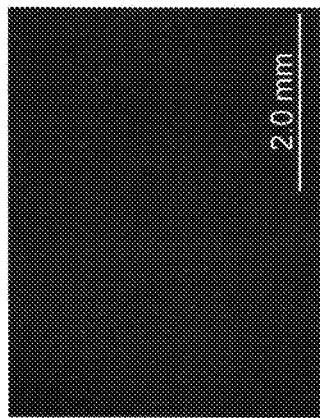
SCBV21 Δnt1-nt11837-
EYFP-Nos
FIG. 11B



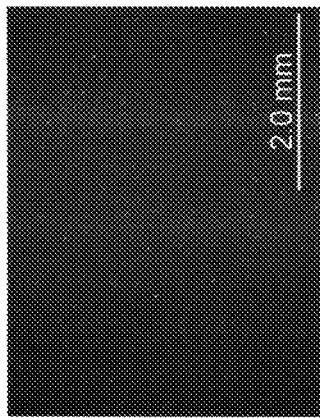
SCBV21 Δnt1-nt1010-
EYFP-Nos
FIG. 11C



SCBV21 Δnt1-nt1104-
EYFP-Nos
FIG. 11D



SCBV21 Δnt1-nt1010-
Δnt1732-nt11837-EYFP-Nos
FIG. 11E



SCBV21 Δnt1-nt1010-
EYFP-Nos
FIG. 11F

**COMPOSITIONS, ORGANISMS, SYSTEMS,
AND METHODS FOR EXPRESSING A GENE
PRODUCT IN PLANTS USING SCBV
EXPRESSION CONTROL SEQUENCES
OPERABLE IN MONOCOTS AND DICOTS**

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/395,197 filed May 10, 2010 and U.S. Provisional Application No. 61/400,976 filed Aug. 5, 2010, the entire contents of which are hereby incorporated in their entirety by this reference.

FIELD OF THE DISCLOSURE

The present disclosure relates, in some embodiments, to compositions, organisms, systems, and methods for expressing a gene product in a plant using a promoter operable in monocots, dicots, or both monocots and dicots.

BACKGROUND OF THE DISCLOSURE

Biotechnology promises to revolutionize everything from agriculture to modern medicine. For example, methods of genetically engineering plants are being explored to increase productivity through greater drought and insect resistance as well as increased yields. In addition, plants are being examined as potential biofactories for the production of proteins (e.g., antibodies) and other compounds for use in human and veterinary medicine. However, a limited number of promoters exist for driving expression of a gene product of interest in plants. Some of these are effective at driving expression in only some plants. Others are effective at driving expression in some tissues and/or cells, but not others.

SUMMARY

Accordingly, a need has arisen for promoters operable in plants including promoters that are operable monocots, dicots, or both monocots and dicots and/or promoters that are operable in multiple tissues and/or cells.

The present disclosure relates, in some embodiments, to compositions, organisms, systems, and methods for expressing a gene product in a plant using a promoter operable in monocots, dicots, or both monocots and dicots. For example, a composition may comprise a nucleic acid (e.g., an isolated and/or purified nucleic acid) comprising an expression control sequence (e.g., a promoter). In some embodiments, an expression control sequence may comprise a nucleic acid sequence at least about 85% identical to a sequence selected from (a) nucleotides 1-1786 of SEQ ID NO: 1, (b) nucleotides 1450-1786 of SEQ ID NO: 1, (c) SEQ ID NO: 1, (d) SEQ ID NO: 17, (e) SEQ ID NO: 18, (f) SEQ ID NO: 26, (g) SEQ ID NO: 27, (h) SEQ ID NO: 32, and/or (i) SEQ ID NO: 33; wherein the expression control sequence has promoter activity in at least one monocot and at least one dicot. An expression control sequence may have promoter activity in at least two monocots and at least two dicots according to some embodiments. A nucleic acid may comprise, in some embodiments, a expression control sequence (e.g., SEQ ID NO: 1) and an exogenous nucleic acid, wherein the expression control sequence is operable to drive transcription of the exogenous nucleic acid in at least one monocot and at least one dicot. According to some embodiments, an isolated nucleic acid according to claim 3, wherein the exogenous nucleic acid comprises a transgene. An isolated nucleic acid may comprise

an exogenous nucleic acid that (a) alters carbon metabolism in the plant cell when expressed or transcribed and/or (b) encodes an insecticide effective against at least one stem-boring insect, in some embodiments.

According to some embodiments, the present disclosure relates to an expression vector. For example, an expression vector may comprise, in a 5' to 3' direction, a sugarcane bacilliform virus (SCBV) promoter (e.g., (a) nucleotides 1-1786 of SEQ ID NO: 1, (b) nucleotides 1450-1786 of SEQ ID NO: 1, (c) SEQ ID NO: 1, (d) SEQ ID NO: 17, (e) SEQ ID NO: 18, (f) SEQ ID NO: 26, (g) SEQ ID NO: 27, (h) SEQ ID NO: 32, and/or (i) SEQ ID NO: 33); an exogenous nucleic acid (e.g., a transgene); and a 3' termination sequence, wherein the SCBV promoter has promoter activity sufficient to express the exogenous nucleic acid in at least one monocot and at least one dicot. An expression vector may be located in a cell (e.g., a bacterial cell, a yeast cell, a plant cell, an insect cell, a mammalian cell) according to some embodiments. An expression vector may comprise, according to some embodiments, the nucleotide sequence of AAAATGG at positions -3 to +4 (e.g., at nucleotides 1819-1825 of SEQ ID NO: 18). In some embodiments, an expression vector may comprise a linker (e.g., between the expression control sequence and the exogenous nucleic acid) having a length of from about 1 to about 200 nucleotides.

The present disclosure further relates, in some embodiments, to a cell (e.g., a bacterial cell, a yeast cell, a plant cell, an insect cell, a mammalian cell) comprising an expression vector comprising an expression control sequence (e.g., an SCBV promoter). For example, a cell may comprise an expression vector having an SCBV promoter (e.g., having a nucleotide sequence at least about 85% identical to SEQ ID NO: 1); an exogenous nucleic acid; and a 3' termination sequence, wherein the SCBV promoter has promoter activity sufficient to express the exogenous nucleic acid in at least one monocot and at least one dicot. An exogenous nucleic acid may comprise a transgene in some embodiments. For example, an exogenous nucleic acid may (a) alter carbon metabolism in the plant cell when expressed or transcribed and/or (b) encode an insecticide effective against at least one stem-boring insect. A cell, in some embodiments, may be a plant cell (e.g., located in a plant). A cell may comprise a plant cell (or plant) selected from a monocot cell and a dicot cell. A monocot may be selected from sugarcane, miscanthus, a miscanthus x sugarcane hybrid, switch grass, oats, wheat, barley, maize, rice, banana, yucca, onion, asparagus, page sorghum and hybrids thereof. A dicot may be selected from coffee, tomato, pepper, tobacco, lima bean, *Arabidopsis*, rubber, orange, grapefruit, potato, squash, peas, and sugar beet. A plant, in some embodiments, may comprise an expression vector having a promoter (e.g., having a nucleotide sequence at least about 85% identical to SEQ ID NO: 1), an exogenous nucleic acid operably linked to the promoter, and a 3' termination sequence, wherein the promoter has promoter activity sufficient to express the exogenous nucleic acid in at least one monocot and at least one dicot.

The present disclosure relates, in some embodiments, to methods for constitutively expressing an exogenous nucleic acid in a plant. For example, a method may comprise contacting an expression cassette or expression vector with the cytosol of a cell of the plant, wherein the expression cassette or expression vector comprises (i) the exogenous nucleic acid, (ii) an expression control sequence (e.g., a SCBV promoter having a nucleotide sequence at least about 85% identical to SEQ ID NO: 1) operable to drive expression of the exogenous nucleic acid, and (iii) a 3' termination sequence operably linked to the exogenous nucleic acid, and wherein the plant is

selected from the group consisting of a monocot and a dicot. Contacting, according to some embodiments, may comprise biolistically bombarding the cell with a particle comprising the expression cassette and/or co-cultivating the cell with a *Agrobacterium* cell comprising the expression cassette.

In some embodiments, the present disclosure relates to methods directing constitutive expression of a nucleic acid in a plant. For example, a method may comprise transforming a plant (e.g., a plant, a plant cell, a leaf disc, an embryonic callus) with an expression nucleic acid, the expression nucleic acid (e.g., an expression vector) comprising an expression control sequence (e.g., a promoter having a nucleotide sequence at least about 85% identical to SEQ ID NO: 1), an exogenous nucleic acid and a 3' termination sequence, wherein the plant is selected from the group consisting of a monocot and a dicot. Transforming may comprise, according to some embodiments, biolistically bombarding the plant with a particle comprising the expression cassette and/or co-cultivating the plant with a *Agrobacterium* cell comprising the expression cassette. A method may comprise, in some embodiments, regenerating a plant from a transformed cell (e.g., embryonic callus) to form one or more progeny of the transformed cell. A method may comprise cultivating and/or breeding progeny of a transformed cell according to some embodiments.

The present disclosure relates, according to some embodiments, to an isolated nucleic acid (e.g., an isolated and/or purified nucleic acid) comprising an expression control sequence. An expression control sequence may comprise, for example, the sequence of nucleotides 632-716 of SEQ ID NO: 33; wherein the expression control sequence has promoter activity in at least one monocot and at least one dicot. In some embodiments, an expression control sequence may have sufficient length (e.g., more than about 0.3 kb, more than about 0.4 kb, more than about 0.5 kb, more than about 0.6 kb, more than about 0.7 kb, and/or more than about 0.8 kb,) to be operable as an expression control sequence in at least one monocot and at least one dicot. For example, an expression control sequence may be at least about 0.75 kb. In some embodiments, a nucleic acid (e.g., an isolated and/or purified nucleic acid) may comprise an expression control sequence having a sequence selected from the group consisting of SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33 and combinations thereof; wherein the expression control sequence has promoter activity in at least one monocot and at least one dicot. For example, a nucleic acid may comprise (e.g., in a 5' to 3' direction) an expression control sequence comprising the sequence of SEQ ID NO: 30 and the sequence of SEQ ID NO: 31 separated by a spacer of about 630 nucleotides, a linker of from about 1 to about 75 nucleotides in length, and a start codon, wherein the expression control sequence has promoter activity in at least one monocot and at least one dicot. A linker may have, in some embodiments, at least about 85%, at least about 90%, at least about 95%, at least about 98%, and/or at least about 99% identity to the sequence of nucleotides 778-805 of SEQ ID NO: 32. According to some embodiments, a spacer may have at least about 85%, at least about 90%, at least about 95%, at least about 98%, and/or at least about 99% identity to sequence of nucleotides 96-726 of SEQ ID NO: 32. An expression control sequence may comprise at its 5' end a sequence having at least about 85%, at least about 90%, at least about 95%, at least about 98%, and/or at least about 99% identity to the sequence of nucleotides 1-44 of SEQ ID NO: 32.

The present disclosure further relates, in some embodiments, to expression systems for expressing desirable amounts of a gene product (e.g., protein) of interest. An

expression system may comprise, for example, nucleic acids, expression cassettes, cells, and/or plants comprising two or more expression control sequences, each operably linked to a coding sequence (e.g., transgene) encoding a gene product (e.g., protein) of interest. An expression system may comprise, according to some embodiments, a plant having two or more expression cassettes, each comprising an expression control sequence (e.g., promoter), a coding sequence encoding the gene product (e.g., protein) of interest, and/or one or more terminators, wherein each expression control sequence (e.g., promoter) is different from the other(s) and/or each copy of the coding sequence encoding the gene product (e.g., protein) of interest is substantially identical and/or identical to the other(s). An expression control sequence (e.g., promoter) may comprise any promoter operative in a plant including, for example, a maize ubiquitin 1 promoter (with or without a heat shock element), a sugarcane proline-rich protein promoter, a sugarcane elongation factor 1 α promoter, a sugarcane jasmonate-inducible promoter, a sugarcane bacilliform virus promoter, a sugarcane O-methyltransferase promoter, and/or combinations thereof. An expression system may comprise a plant having 2, 3, 4, 5, or more promoters, each independently selected from the group consisting of a maize ubiquitin 1 promoter (with or without a heat shock element), a sugarcane proline-rich protein promoter, a sugarcane elongation factor 1 α promoter, a sugarcane jasmonate-inducible promoter, a sugarcane bacilliform virus promoter, a sugarcane O-methyltransferase promoter, and/or combinations thereof, and each operably linked (e.g., in trans) to a coding sequence (e.g., transgene) encoding a gene product (e.g., protein) of interest, wherein each copy of the coding sequence encoding the gene product (e.g., protein) of interest is substantially identical and/or identical to the other(s). A coding sequence of interest may include, in some embodiments, any of the coding sequences cited in the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

40 The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Some embodiments of the disclosure may be understood 45 by referring, in part, to the present disclosure and the accompanying drawings, wherein:

FIG. 1 illustrates a vector for a promoter according to a specific example embodiment of the disclosure;

FIG. 2 illustrates a vector with a promoter according to a 50 specific example embodiment of the disclosure;

FIG. 3 illustrates GUS expression under the control of a promoter according to a specific example embodiment of the disclosure compared to a 35S promoter;

FIG. 4 illustrates a bar graph showing GUS activity in 55 transgenic plant leaves according to a specific example embodiment of the disclosure;

FIG. 5 illustrates a vector with a promoter according to a specific example embodiment of the disclosure;

FIG. 6A illustrates GUS expression in sugarcane young 60 leaf under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6B illustrates GUS expression in sugarcane young leaf (roll) under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6C illustrates GUS expression in sugarcane stem under the control of a promoter according to a specific 65 example embodiment of the disclosure;

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FIG. 6D illustrates GUS expression in sweet sorghum young leaf under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6E illustrates GUS expression in tobacco leaf under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6F illustrates GUS expression in lima bean seed under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6G illustrates GFP expression in sugarcane young leaf under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6H illustrates GFP expression in sugarcane young leaf (roll) under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6I illustrates GFP expression in sugarcane stem under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6J illustrates GFP expression in sweet sorghum young leaf under the control of a promoter according to a specific example embodiment of the disclosure;

FIG. 6K illustrates GFP expression in tobacco leaf under the control of a promoter according to a specific example embodiment of the disclosure; and

FIG. 6L illustrates GFP expression in lima bean seed under the control of a promoter according to a specific example embodiment of the disclosure.

FIG. 7A illustrates expression of GUS in stalks of sugarcane transformed with an expression cassette according to an example embodiment of the disclosure;

FIG. 7B illustrates expression of GUS in leaves of sugarcane transformed with an expression cassette according to an example embodiment of the disclosure;

FIG. 7C illustrates expression of GUS in roots of sugarcane transformed with an expression cassette according to an example embodiment of the disclosure;

FIG. 8A illustrates expression of GUS in stalks of sugarcane transformed with an expression cassette according to an example embodiment of the disclosure;

FIG. 8B illustrates expression of GUS in stalks of sugarcane transformed with an expression cassette according to an example embodiment of the disclosure;

FIG. 8C illustrates expression of GUS in stalks of sugarcane transformed with an expression cassette according to an example embodiment of the disclosure;

FIG. 8D illustrates expression of GUS in stalks of sugarcane transformed with an expression cassette according to an example embodiment of the disclosure;

FIG. 9A illustrates a vector (SEQ ID NO:19) with a promoter according to a specific example embodiment of the disclosure;

FIG. 9B illustrates a vector (SEQ ID NO:20) with a promoter (deletion mutant B) according to a specific example embodiment of the disclosure;

FIG. 9C illustrates a vector (SEQ ID NO:21) with a promoter (deletion mutant C) according to a specific example embodiment of the disclosure;

FIG. 9D illustrates a vector (SEQ ID NO:22) with a promoter (deletion mutant D) according to a specific example embodiment of the disclosure;

FIG. 9E illustrates a vector (SEQ ID NO:23) with a promoter (deletion mutant E) according to a specific example embodiment of the disclosure;

FIG. 9F illustrates a vector (SEQ ID NO:24) with a promoter (deletion mutant F) according to a specific example embodiment of the disclosure;

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FIG. 10A illustrates a promoter according to a specific example embodiment of the disclosure;

FIG. 10B illustrates a promoter (deletion mutant B) according to a specific example embodiment of the disclosure;

FIG. 10C illustrates a promoter (deletion mutant C) according to a specific example embodiment of the disclosure;

FIG. 10D illustrates a promoter (deletion mutant D) according to a specific example embodiment of the disclosure;

FIG. 10E illustrates a promoter (deletion mutant E) according to a specific example embodiment of the disclosure;

FIG. 10F illustrates a promoter (deletion mutant F) according to a specific example embodiment of the disclosure;

FIG. 11A illustrates expression of EYFP in leaves of sugarcane transformed with an expression cassette according to an example embodiment of the disclosure;

FIG. 11B illustrates expression of EYFP in leaves of sugarcane transformed with an expression cassette (comprising deletion B) according to an example embodiment of the disclosure;

FIG. 11C illustrates expression of EYFP in leaves of sugarcane transformed with an expression cassette (comprising deletion C) according to an example embodiment of the disclosure;

FIG. 11D illustrates expression of EYFP in leaves of sugarcane transformed with an expression cassette (comprising deletion D) according to an example embodiment of the disclosure;

FIG. 11E illustrates expression of EYFP in leaves of sugarcane transformed with an expression cassette (comprising deletion E) according to an example embodiment of the disclosure; and

FIG. 11F illustrates expression of EYFP in leaves of sugarcane transformed with an expression cassette (comprising deletion F) according to an example embodiment of the disclosure.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

Some embodiments of the disclosure may be understood by referring, in part, to the present disclosure and the accompanying sequence listing, wherein:

SEQ ID NO: 1 illustrates a sugarcane bacilliform virus promoter according to a specific example embodiment of the disclosure;

SEQ ID NO: 2 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane bacilliform virus promoter, a GUS coding sequence, and a NOS terminator;

SEQ ID NO: 3 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane bacilliform virus promoter, an enhanced YFP coding sequence, and a NOS terminator;

SEQ ID NO: 4 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a 35S promoter, a GUS coding sequence, and a NOS terminator;

SEQ ID NO: 5 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a 35S promoter, an enhanced YFP coding sequence, and a NOS terminator;

SEQ ID NO: 6 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising an E35S promoter, an enhanced YFP coding sequence, and a NOS terminator;

SEQ ID NO: 7 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a maize ubiquitin promoter (mUbil no hse), a GUS coding sequence, and a NOS terminator;

SEQ ID NO: 8 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a maize ubiquitin promoter (mUbil no hse), an enhanced YFP coding sequence, and a NOS terminator;

SEQ ID NO: 9 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a maize ubiquitin promoter, a GUS coding sequence, and a NOS terminator;

SEQ ID NO: 10 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a ubiquitin promoter, an enhanced YFP coding sequence, and a NOS terminator;

SEQ ID NO: 11 illustrates a P-2 PCR primer according to a specific example embodiment of the disclosure;

SEQ ID NO: 12 illustrates a P-W3F PCR primer according to a specific example embodiment of the disclosure;

SEQ ID NO: 13 illustrates a P-W4F PCR primer according to a specific example embodiment of the disclosure;

SEQ ID NO: 14 illustrates a P-W1R PCR primer according to a specific example embodiment of the disclosure;

SEQ ID NO: 15 illustrates a SCBV/Prom/F PCR primer according to a specific example embodiment of the disclosure;

SEQ ID NO: 16 illustrates a SCBV/Prom/R PCR primer according to a specific example embodiment of the disclosure;

SEQ ID NO: 17 illustrates a sugarcane bacilliform virus promoter according to a specific example embodiment of the disclosure;

SEQ ID NO: 18 illustrates a sugarcane bacilliform virus promoter according to a specific example embodiment of the disclosure;

SEQ ID NO: 19 illustrates a vector (wild type pSK-SCBV21-EYFP-Nos) with a promoter according to a specific example embodiment of the disclosure;

SEQ ID NO: 20 illustrates a vector with a promoter (deletion mutant B) according to a specific example embodiment of the disclosure;

SEQ ID NO: 21 illustrates a vector with a promoter (deletion mutant C) according to a specific example embodiment of the disclosure;

SEQ ID NO: 22 illustrates a vector with a promoter (deletion mutant D) according to a specific example embodiment of the disclosure;

SEQ ID NO: 23 illustrates a vector with a promoter (deletion mutant E) according to a specific example embodiment of the disclosure;

SEQ ID NO: 24 illustrates a vector with a promoter (deletion mutant F) according to a specific example embodiment of the disclosure;

SEQ ID NO: 25 illustrates a sugarcane bacilliform virus promoter (deletion B) according to a specific example embodiment of the disclosure;

SEQ ID NO: 26 illustrates a sugarcane bacilliform virus promoter (deletion C) according to a specific example embodiment of the disclosure;

SEQ ID NO: 27 illustrates a sugarcane bacilliform virus promoter (deletion D) according to a specific example embodiment of the disclosure;

SEQ ID NO: 28 illustrates a sugarcane bacilliform virus promoter (deletion E) according to a specific example embodiment of the disclosure;

SEQ ID NO: 29 illustrates a sugarcane bacilliform virus promoter (deletion F) according to a specific example embodiment of the disclosure;

SEQ ID NO: 30 illustrates a transcription start site (TSS1) according to a specific example embodiment of the disclosure;

10 SEQ ID NO: 31 illustrates a transcription start site (TSS2) according to a specific example embodiment of the disclosure;

15 SEQ ID NO: 32 illustrates a sugarcane bacilliform virus promoter with TSS1 and TSS2 according to a specific example embodiment of the disclosure;

SEQ ID NO: 33 illustrates a sugarcane bacilliform virus promoter with TSS2 according to a specific example embodiment of the disclosure;

20 SEQ ID NO: 34 illustrates a Forward PCR primer F1 according to a specific example embodiment of the disclosure;

SEQ ID NO: 35 illustrates a Forward PCR primer F2 according to a specific example embodiment of the disclosure;

25 SEQ ID NO: 36 illustrates a Reverse PCR primer R1 according to a specific example embodiment of the disclosure;

SEQ ID NO: 37 illustrates a Reverse PCR primer R2 according to a specific example embodiment of the disclosure;

30 SEQ ID NO: 38 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane bacilliform virus promoter, a bovine lysozyme coding sequence, a 35S terminator, and a NOS terminator;

35 SEQ ID NO: 39 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a maize ubiquitin promoter (with no heat shock element), a bovine lysozyme coding sequence, and a 35S terminator;

40 SEQ ID NO: 40 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a maize ubiquitin promoter (with no heat shock element), a 5' untranslated region of *Sorghum* mosaic virus protein, and a 35S terminator;

45 SEQ ID NO: 41 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a maize ubiquitin promoter (with no heat shock element), a 5' untranslated region of *Sorghum* mosaic virus protein, a bovine lysozyme coding sequence, a 3' untranslated region of *Sorghum* mosaic virus protein, and a 35S terminator;

50 SEQ ID NO: 42 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a maize ubiquitin promoter (with no heat shock element), a bovine lysozyme coding sequence, a 35S terminator, and a NOS terminator;

55 SEQ ID NO: 43 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane proline rich promoter (with no 5' UTR), a bovine lysozyme coding sequence, a 3' untranslated region of *Sorghum* mosaic virus protein, and a 35S terminator;

60 SEQ ID NO: 44 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane proline rich promoter (with no 5' UTR), a 5' untranslated region of *Sorghum* mosaic virus protein, a

bovine lysozyme coding sequence, a 3' untranslated region of *Sorghum* mosaic virus protein, and a 35S terminator;

SEQ ID NO: 45 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane proline rich promoter (with no 5' UTR), a bovine lysozyme coding sequence, a 35S terminator, and a NOS terminator;

SEQ ID NO: 46 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane elongation factor 1 α promoter, a bovine lysozyme coding sequence, a 3' untranslated region of *Sorghum* mosaic virus protein, and a 35S terminator;

SEQ ID NO: 47 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane elongation factor 1 α promoter, a bovine lysozyme coding sequence, a 35S terminator, and a NOS terminator;

SEQ ID NO: 48 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a jasmonate responsive promoter, a bovine lysozyme coding sequence, and a 35S terminator;

SEQ ID NO: 49 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a jasmonate responsive promoter, a bovine lysozyme coding sequence, a 3' untranslated region of *Sorghum* mosaic virus protein, and a 35S terminator; and

SEQ ID NO: 50 illustrates an expression cassette according to a specific example embodiment of the disclosure comprising a sugarcane bacilliform virus promoter, a bovine lysozyme coding sequence, a 35S terminator, and a NOS terminator.

DETAILED DESCRIPTION

The present disclosure relates, in some embodiments, to compositions, organisms, systems, and methods for expressing a gene product in a plant using a promoter operable in monocots, dicots, or both monocots and dicots. For example, the present disclosure relates to expression control sequences (e.g., promoters), expression cassettes, expression vectors, microorganisms, and/or plants comprising a sugarcane bacilliform virus (SCBV) promoter. An expression control sequence, according to some embodiments, may be constitutively active or conditionally active in (a) an organ selected from root, leaf, stem, flower, seed, fruit, and/or tuber and/or (b) active in a tissue selected from epidermis, periderm, parenchyma, collenchyma, sclerenchyma, xylem, phloem, and/or secretory structures.

In some embodiments, an expression control sequence may be included in methods, compositions, systems, and/or organisms to alter carbon metabolism (e.g., in a sucrose accumulating tissue) and/or to express a protein (e.g., an insecticidal protein) in a plant (e.g., in sugarcane). An expression control sequence may be included, according to some embodiments, in methods, compositions, systems, and/or organisms to improve pest and/or disease tolerance and/or disease resistance (e.g., rice plants).

Sugarcane bacilliform virus (SCBV) belongs to the genus *Badnavirus* in the family Caulimoviridae. The virions of those species that belong to the genus *Badnavirus* have non-enveloped bacilliform particles. SCBV is serologically related to Banana streak virus (BSV). The genome of SCBV consists of a single double-stranded DNA of ~7600 bp in size encoding three open reading frames whose transcription is directed by a single promoter residing in between the 3' portion of ORF3 and near the 5' end of ORF1.

The promoter of SCBV Mor isolate may be active both in monocots and dicots. The promoters from other badnaviruses such as Rice tungro bacilliform virus, Commelina yellow mosaic virus, Banana streak virus and Taro bacilliform virus, have also been tested for foreign gene expression. In some embodiments, promoters from these viruses may be useful for transgene expression in monocots since the aforementioned badnaviruses infect monocots. While it seems that the promoters from RTBV, CoYMV and TaBV are typically active in vascular tissues, the promoters from SCBV and BSV direct constitutive expression of foreign genes.

SCBV is closely related to BSV and may display considerable sequence variation among different SCBV isolates. Similar sequence variations may be present in SCBV promoter regions cloned from SCBV-infected *Saccharum officinarum* species. While the PCR-derived promoter sequence cloned from *S. officinarum* Ireng Maleng showed only ~53% sequence homology with the promoter sequence of another SCBV Ireng Maleng isolate (SCBVM-12), this PCR-derived promoter showed ~74% sequence homology with BSV promoter regions.

Preliminary screening for SCBV incidence in sugarcane fields located in the Mid Rio Grande Valley, Tex. confirmed that SCBV is prevalent in the sugarcane fields in this region. A SCBV promoter from the SCBV-positive commercial sugarcane hybrid CP72-1210 has been isolated, purified, and cloned. Its promoter activity has been confirmed in various monocot and dicot plants and in transgenic sugarcane plants. Expression Control Sequences

In some embodiments, an expression control sequence may comprise one or more promoters, one or more operators, one or more enhancers, one or more ribosome binding sites, and/or combinations thereof. An expression control sequence may comprise, for example, a nucleic acid having (a) a promoter activity in a monocot, a dicot, or both a monocot and a dicot and (b) a nucleotide sequence more than about 70% identical to SEQ ID NO: 1, more than about 75% identical to SEQ ID NO: 1, more than about 80% identical to SEQ ID NO: 1, more than about 81% identical to SEQ ID NO: 1, more than about 82% identical to SEQ ID NO: 1, more than about 83% identical to SEQ ID NO: 1, more than about 84% identical to SEQ ID NO: 1, more than about 85% identical to SEQ ID NO: 1, more than about 86% identical to SEQ ID NO: 1, more than about 87% identical to SEQ ID NO: 1, more than about 88% identical to SEQ ID NO: 1, more than about 89% identical to SEQ ID NO: 1, more than about 90% identical to SEQ ID NO: 1, more than about 92% identical to SEQ ID NO: 1, more than about 94% identical to SEQ ID NO: 1, more than about 96% identical to SEQ ID NO: 1, more than about 98% identical to SEQ ID NO: 1, and/or more than about 99% identical to SEQ ID NO: 1. According to some embodiments, sequences that are not 100% identical over the full length of SEQ ID NO: 1 may have points and/or regions of variation that are dispersed (e.g., uniformly, haphazardly) over the length of the subject nucleic acid. For example, an expression control sequence may comprise one or more regions of sequence that are 100% identical to SEQ ID NO: 1 (e.g., in or near a TATA-box, a CCAAT-box, and/or a TSS-motif) and one or more regions that are less than 100% identical length and/or sequence. An expression control sequence may comprise, for example, a region that is about 95% identical to nucleotides 1-1450 of SEQ ID NO: 1 (in length and/or sequence) and a region that is 100% identical to nucleotides 1450-1786 of SEQ ID NO: 1. According to some embodiments, an expression control sequence may share similarity (e.g., from more than about 70% to 100% identity as disclosed above) to (a) nucleotides 1-1786 of SEQ ID NO: 1, (b) SEQ ID NO: 17, (c) SEQ ID

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NO: 18, (d) SEQ ID NO: 26, (e) SEQ ID NO: 27, (f) SEQ ID NO: 32, and/or (g) SEQ ID NO: 33). For example, an expression control sequence may be more than about 85% identical to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, and/or SEQ ID NO: 33; more than about 95% identical to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, and/or SEQ ID NO: 33; and/or more than about 98% identical to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, and/or SEQ ID NO: 33.

An expression control sequence, in some embodiments, may comprise TSS1 (SEQ ID NO: 30), TSS2 (SEQ ID NO: 31), or both TSS1 and TSS2. For example, an expression control sequence may be at least about 0.76 kb in length with the 5' end of TSS1, if present, within 100 nucleotides of the -760 position and the 5' end of TSS2, if present, within 100 nucleotides of the -80 position. An expression control sequence, for example, may be at least about 0.7 kb in length with the 5' end of TSS1, if present, within 100 nucleotides of the 5' end of the expression control sequence and the 5' end of TSS2, if present, within 100 nucleotides of the -80 position. In some embodiments, TSS2, if present, may be positioned such that it does not extend beyond the start codon. In some embodiments, TSS1 may be 5' of TSS2. An expression control sequence may comprise, in some embodiments, TSS1 and TSS2 separated by a spacer (e.g., more than about 500 nucleotides, more than about 550 nucleotides, more than about 600 nucleotides, and/or more than about 650 nucleotides), a linker of from about 1 to about 75 nucleotides in length, and/or a start codon. A spacer may have, for example, more than about 85% identity, more than about 90% identity, more than about 95% identity, and/or more than about 98% identity to the sequence of nucleotides 96-726 of SEQ ID NO: 32. A linker may have, for example, more than about 85% identity, more than about 90% identity, more than about 95% identity, and/or more than about 98% identity to the sequence of nucleotides 778-805 of SEQ ID NO: 32. An expression control sequence may further comprise (e.g., 5' of TSS1) a sequence having more than about 85% identity to the sequence of nucleotides 1-44 of SEQ ID NO: 32.

An expression control sequence may comprise a fragment of SEQ ID NO: 1 according to some embodiments. For example, an expression control sequence may comprise the portion of SEQ ID NO: 1 that is upstream of a transcription start site (e.g., upstream of nucleotide 1787). In some embodiments, an expression control sequence may comprise a nucleic acid having at least 70% identity to nucleotides 1-1786 of SEQ ID NO: 1.

According to some embodiments, an expression control sequence may comprise a sequence of a nucleic acid found in virus (e.g., a plant virus). For example, an expression control sequence may comprise, according to some embodiments, an SCBV promoter, a Rice tungro bacilliform virus promoter, a Commelina yellow mosaic virus promoter, a Banana streak virus promoter, a Taro bacilliform virus promoter, and/or combinations thereof. In some embodiments, an expression control sequence may comprise a nucleic acid having the nucleotide sequence of SEQ ID NO: 1.

An expression control sequence, according to some embodiments, may be operable to drive higher expression of a nucleic acid sequence (e.g., a coding sequence) in a cell compared to the 35S promoter (e.g., from about 5% higher to about 50% higher, from about 50% higher to about 500% higher). Metrics for expression may include, for example, rate of appearance and/or accumulation of a gene product (e.g., RNA and/or protein) and/or total accumulation of a gene product as of one or more time points (e.g., elapsed time after a starting point and/or a stage of development). Comparative assays for gene products may be qualitative, semi-

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quantitative, and/or quantitative in some embodiments. Comparative assays may indirectly and/or directly assess the presence and/or amount of gene product. In some embodiments, an expression control sequence may be sensitive to one or more stimuli (e.g., one or more small molecules, one or more plant defense-inducing agents, mechanical damage, temperature, pressure). For example, activity of an expression control sequence may be enhanced or suppressed upon infection with a virus (e.g., a bacilliform virus). An expression control sequence may comprise, in some embodiments, a light responsive element, a copper responsive element, a salicylic acid responsive element, an auxin responsive element, a sulfur responsive element, and/or a dehydration responsive element. Identification of motifs may be informed by available motif prediction software (e.g., PLACE database of the National Institute of Agrobiological Sciences, Japan) and/or experimental data.

The present disclosure relates, according to some embodiments, to one or more expression control sequences like a nucleotide sequence of nucleotides -1816 to -1 of SEQ ID NO: 1 and/or operable to direct expression in at least one monocot and/or at least one dicot. For example, an expression control sequence may include a nucleic acid sequence that differs from SEQ ID NO: 1 at one or more positions. Examples of expression control sequences that differ from SEQ ID NO: 1 may include, in some embodiments, a promoter from one or more bacilliform virus isolates. An expression control sequence, according to some embodiments, may hybridize to a nucleic acid having the nucleotide sequence of SEQ ID NO: 1 under stringent conditions. Stringent conditions may include, for example, (a) 4×SSC at 65° C. followed by 0.1×SSC at 65° for 60 minutes and/or (b) 50% formamide, 4×SSC at 65° C. An expression control sequence may comprise a deletion fragment of a nucleic acid having a sequence of SEQ ID NO: 1 and having the capacity to direct expression in at least one monocot and/or at least one dicot, in some embodiments. One of ordinary skill in the art having the benefit of the present disclosure may prepare one or more deletion fragments of a nucleic acid having a sequence of SEQ ID NO: 1.

An expression control sequence having a sequence like SEQ ID NO: 1 may be identified by database searches using the promoter or elements thereof as the query sequence using the Gapped BLAST algorithm (Altschul et al., 1997 *Nucl. Acids Res.* 25:3389-3402) with the BLOSUM62 Matrix, a gap cost of 11 and persistence cost of 1 per residue and an E value of 10. Sequence identity may be assessed by any available method according to some embodiments. For example, two sequences may be compared with either ALIGN (Global alignment) or LALIGN (Local homology alignment) in the FASTA suite of applications (Pearson and Lipman, 1988 *Proc. Nat. Acad. Sci.* 85:2444-24448; Pearson, 1990 *Methods in Enzymology* 183:63-98) with the BLOSUM50 matrix and gap penalties of -16, -4. Sequence similarity may be assessed according to ClustalW (Larkin et al., 2007, *Bioinformatics* 23(21): 2947-2948), BLAST, FASTA or similar algorithm. Expression Cassettes and Vectors

The disclosure relates, in some embodiments, to expression vectors and/or expression cassettes for expressing a nucleic acid sequence (e.g., a coding sequence) in a cell and comprising an expression control sequence and the nucleic acid sequence operably linked to the expression control sequence. A cassette, in some embodiments, may include a nucleotide sequence capable of expressing a particular coding sequence inserted so as to be operably linked to one or more expression control sequences present in the nucleotide sequence. Thus, for example, an expression cassette may

include a heterologous coding sequence which is desired to be expressed in a plant seed according to some embodiments.

The disclosure relates, in some embodiments, to an expression vector which may comprise, for example, a nucleic acid having an expression control sequence and a coding sequence operably linked to the expression control sequence. An expression vector may be contacted with a cell (e.g., a plant cell) under conditions that permit expression (e.g., transcription) of the coding sequence. An expression control sequence may be contacted with a plant cell (e.g., an embryonic cell, a stem cell, a callous cell) under conditions that permit expression of the coding sequence in the cell and/or cells derived from the plant cell according to some embodiments. An expression vector may be contacted with a cell (e.g., a plant cell), in some embodiments, under conditions that permit inheritance of at least a portion of the expression vector in the cell's progeny. Examples of expression vectors may include, without limitation the vectors shown in FIG. 1, FIG. 2, FIG. 5, and FIG. 9. According to some embodiments, an expression vector may include one or more selectable markers. For example, an expression vector may include a marker for selection when the vector is in a bacterial host, a yeast host, and/or a plant host.

According to some embodiments, the disclosure relates to an expression cassette which may comprise, for example, a nucleic acid having an expression control sequence and a coding sequence operably linked to the expression control sequence. An expression cassette may be comprised in an expression vector. A coding sequence, in some embodiments, may comprise any coding sequence expressible in at least one plant cell. For example, a coding sequence may comprise a human sequence (e.g., an antibody sequence), a non-human animal sequence, a plant sequence, a yeast sequence, a bacterial sequence, a viral sequence (e.g., plant virus, animal virus, and/or vaccine sequence), an artificial sequence, an antisense sequence thereof, a fragment thereof, a variant thereof, and/or combinations thereof. According to some embodiments, a coding sequence may comprise, a sugar transport gene and/or a sugar accumulation gene. Examples of sugar transport genes may include, without limitation, a disaccharide transporter (e.g., a sucrose transporter) and/or a monosaccharide transporter. A coding sequence may comprise, in some embodiments, a sequence encoding one or more gene products with insecticidal, antimicrobial, and/or antiviral activity. Examples of gene products that may have insecticidal activity, antimicrobial activity, and/or antiviral activity may include, without limitation, avidin, vegetative insecticidal proteins (e.g., Vip3A), insecticidal crystal proteins from *Bacillus thuringiensis* (e.g., Cry1, Cry1Ab, Cry2, Cry9), pea albumin (e.g., PALb), hirsutellin A, lectins (e.g., smow drop lily lectin, garlic lectin, onion lectin), amylase inhibitors (e.g., alpha amylase inhibitor), arcelins (e.g., arcelins from beans), proteinase inhibitors, lysozymes (e.g., bovine lysozyme, human lysozyme, mollusk lysozyme), defensin, chitinase, β -1,3-glucanase, variants thereof, and/or combinations thereof. A coding sequence may comprise an enzyme for forming and/or modifying a polymer according to some embodiments. Examples of enzymes for forming and/or modifying a polymer may include, without limitation, a polyhydroxyalkanoate synthases, 4-hydroxybutyryl-CoA transferases, variants thereof, and/or combinations thereof. In some embodiments, a coding sequence may comprise a sequence encoding one or more enzymes that hydrolyzes cellulose. Examples of enzymes that hydrolyze cellulose include, without limitation, cellulase, endoglucanases (e.g., endo β -1,4 glucanases), glucosidases (e.g., β glucosidase), hydrolases (e.g., β -1,4-glucan cellobiohydrolase), exocellu-

lases), variants thereof, and/or combinations thereof. In some embodiments, a coding sequence may comprise a sequence encoding one or more enzymes that form and/or modify a sugar (e.g., sucrose, trehalose, sorbitol, fructan, fructose, tagatose, sucralose). Examples of enzymes that form and/or modify a sugar may include, without limitation, trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). According to some embodiments, a coding sequence may comprise a sequence encoding an enzyme for forming or modifying glycine betaine, a polyamine, proline, threhalose, a variant thereof, and/or combinations thereof. A coding sequence may comprise, in some embodiments, a start codon, an intron, and/or a translation termination sequence. According to some embodiments, a coding sequence may comprise one or more natural or artificial coding sequences (e.g., encoding a single protein or a chimera). According to some embodiments, an expression cassette may optionally comprise a termination sequence.

An expression control sequence may be used, in some embodiments, to construct an expression cassette comprising, in the 5' to 3' direction, (a) the expression control sequence (e.g., a SCBV promoter), (b) a heterologous gene or a coding sequence, or sequence complementary to a native plant gene under control of the expression control sequence, and/or (c) a 3' termination sequence (e.g., a termination sequence comprising a polyadenylation site). Examples of expression cassettes may include, in some embodiments, SEQ ID NO: 2, SEQ ID NO:3, nucleotides 710-3538 of SEQ ID NO:19, nucleotides 674-2472 of SEQ ID NO:21, nucleotides 674-2377 of SEQ ID NO:22, SEQ ID NO:38, SEQ ID NO:50, and/or sequences having at least about 98% and/or at least about 99% identity thereto. An expression cassette may be incorporated into a variety of autonomously replicating vectors in order to construct an expression vector. An expression cassette may be constructed, for example, by ligating an expression control sequence to a sequence to be expressed (e.g., a coding sequence).

Some techniques for construction of expression cassettes are well known to those of ordinary skill in the art. For example, a variety of strategies are available for ligating fragments of DNA, the choice of which depends on the nature of the termini of the DNA fragments. Restriction and/or deletion fragments that contain a subject promoter TATA box may be ligated in a forward orientation to a promoterless heterologous gene or coding sequence such as the coding sequence of GUS. An expression control sequence and/or portions thereof may be provided by other means, for example chemical or enzymatic synthesis as artisan of ordinary skill having the benefit of the present disclosure may appreciate.

A nucleic acid may comprise, in a 5' to 3' direction, an expression control sequence, a linker (optional), and a coding sequence according to some embodiments. A linker may be, in some embodiments, from about 1 nucleotide to about 200 nucleotides in length and/or may comprise one or more restriction sites. Expression level of a nucleic acid sequence (e.g., a coding sequence) operably linked to an expression control sequence may be influenced by the length and/or sequence of a linker and/or the 5' sequence of the coding sequence. For example, expression level may be influenced by the sequence from about the -4 position to about the +4 position. In some embodiments, a nucleic acid may comprise, in a 5' to 3' direction, an expression control sequence, a linker, and a coding sequence, wherein the sequence of positions -4 to +4 comprises a sequence selected from the sequence shown in Table 1. A nucleic acid may comprise, in a 5' to 3' direction, an expression control sequence and a coding sequence, wherein the sequence of positions -4 to +4 comprises a

sequence selected from the sequence shown in Table 1 according to some embodiments. In some embodiments, a -3 to -1 sequence of AAA may be associated with higher (e.g., the highest) expression levels than other -3 to -1 sequences. A +1 to +4 sequence of ATGG may be associated with higher (e.g., the highest) expression levels than other +1 to +4 sequences (e.g., ATGC, ATGA, ATGT).

TABLE 1

Optional Junction Sequences								
-4	-3	-2	-1	+1	+2	+3	+4	
1	N	N	N	N	A	T	G	G/T
2	N	A/C	A/C	A/C	A	T	G	G
3	A/C	A/C	A/C	A/C	A	T	G	G
4	N	A	A	A	A	T	G	G
5	N	A	A	C	A	T	G	G
6	N	A	C	A	A	T	G	G
7	N	A	C	C	A	T	G	G
8	N	C	A	A	A	T	G	G
9	N	C	A	C	A	T	G	G
10	N	C	C	A	A	T	G	G
11	N	C	C	C	A	T	G	G
12	N	A	A	T	A	T	G	G
13	N	A	T	A	A	T	G	G
14	N	A	T	T	A	T	G	G
15	N	T	A	A	A	T	G	G
16	N	T	A	T	A	T	G	G
17	N	T	T	A	A	T	G	G
18	N	T	T	T	A	T	G	G
19	N	C	T	T	A	T	G	G
20	N	T	C	T	A	T	G	G
21	N	T	T	C	A	T	G	G
22	C	A	C	C	A	T	G	G
23	N	N	C	C	A	T	G	G
24	C	G	C	C	A	T	G	G
25	N	A/C	A/C	A/C	A	T	G	G
26	A/C	A/C	A/C	A/C	A	T	G	G
27	N	A	A	A	A	T	G	G
28	N	A	A	C	A	T	G	G
29	N	A	C	A	A	T	G	G
30	N	A	C	C	A	T	G	G
31	N	C	A	A	A	T	G	G
32	N	C	A	C	A	T	G	G
33	N	C	C	A	A	T	G	G
34	N	C	C	C	A	T	G	G
35	N	A	A	T	A	T	G	G
36	N	A	T	A	A	T	G	G
37	N	A	T	T	A	T	G	G
38	N	T	A	A	A	T	G	G
39	N	T	A	T	A	T	G	G
40	N	T	T	A	A	T	G	G
41	N	T	T	T	A	T	G	G
42	N	C	T	T	A	T	G	G
43	N	T	C	T	A	T	G	G
44	N	T	T	C	A	T	G	G
45	C	A	C	C	A	T	G	G
46	N	N	C	C	A	T	G	G
47	C	G	C	C	A	T	G	G

In some embodiments, the 3' end of a heterologous coding sequence may be operably linked to a termination sequence including, for example, a polyadenylation site, exemplified by, but not limited to, a nopaline synthase polyadenylation site and/or a octopine T-DNA gene 7 polyadenylation site. A polyadenylation site may be provided by the heterologous gene or coding sequence according to some embodiments. A nucleic acid, according to some embodiments, may comprise a 5' untranslated region (5' UTR), a 3' untranslated region (3' UTR), and/or combinations thereof. For example, a nucleic acid may comprise (e.g., in a 5' to 3' direction) an expression control sequence, a 5' UTR, a coding sequence (e.g., a transgene), a 3' UTR, and/or a termination sequence.

Microorganisms

The present disclosure relates, in some embodiments, to a microorganism comprising an expression control sequence. For example, a microorganism may comprise a bacteria, a yeast, and/or a virus. In some embodiments, an expression control sequence may comprise a SCBV promoter. A microorganism may comprise an expression control sequence and a coding sequence operably linked to the expression control sequence. Examples of microorganisms may include, without limitation, *Agrobacterium tumefaciens*, *Escherichia coli*, a lepidopteran cell line, a Rice tungro bacilliform virus, a *Commelin yellow mosaic virus*, a Banana streak virus, a Taro bacilliform virus, and/or baculovirus. An expression control sequence may be present on a genomic nucleic acid and/or an extra-genomic nucleic acid.

Plants

The present disclosure relates, in some embodiments, to a plant cell (e.g., an embryonic cell, a stem cell, a callous cell), a tissue, and/or a plant comprising an expression control

sequence. A plant and/or plant cell may be selected from a monocot and/or a dicot in some embodiments. Examples of a monocot may include, without limitation, sugarcane, miscanthus, a miscanthus x sugarcane hybrid, switch grass, oats, wheat, barley, maize, rice, banana, yucca, onion, asparagus, and/or sorghum. Examples of a dicot may include, without limitation, coffee, tomato, pepper, tobacco, lima bean, *Arabidopsis*, rubber, orange, grapefruit, potato, grapefruit, potato, squash, peas, and/or sugar beet. A plant cell may be included in a plant tissue, a plant organ, and/or a whole plant

in some embodiments. A plant cell in a tissue, organ, and/or whole plant may be adjacent, according to some embodiments, to one or more isogenic cells and/or one or more heterogenic cells. In some embodiments, a plant may include primary transformants and/or progeny thereof. A plant comprising an expression control sequence may further comprise a transgene operably linked to the expression control sequence, in some embodiments. A transgene may be expressed, according to some embodiments, in a plant comprising an expression control sequence in all (e.g., substantially all) organs, tissues, and/or cell types including, without limitation, stalks, leaves, roots, seeds, flowers, fruit, meristem, parenchyma, storage parenchyma, collenchyma, sclerenchyma, epidermis, mesophyll, bundle sheath, guard cells, protoxylem, metaxylem, phloem, phloem companion,

and/or combinations thereof. In some embodiments, a transgene and/or its gene product may be located in and/or translocated to one or more organelles (e.g., vacuoles, chloroplasts, mitochondria, plastids).

Expression Systems

The present disclosure relates, according to some embodiments, to a system for expression of (e.g., to high levels) of a nucleic acid sequence (e.g., comprising one or more coding sequences). For example, an expression system may be comprised in plants to be used as a biofactory for high-value

proteins. Without being limited to any particular mechanism of action, an expression system may benefit from additive and/or synergistic expression control sequence activities, transcriptional synergism, and/or reduced silencing of an introduced coding sequence (e.g., transgene), a phenomenon frequently observed in plants when the same promoters are used to express the same or different transgenes, and constituting a major risk for the economic exploitation of plants as biofactories. Plants comprising an expression system may retain desirable (e.g., high) expression levels through one or

more consecutive generations of transgenic plants.

In some embodiments, an expression system may comprise two or more expression control sequences (e.g., promoters)

each operably linked to a respective number of clones of a single coding sequence. According to some embodiments, two, three, four, five, or more expression control sequences (e.g., promoters) may be operably linked to two, three, four, five, or more clones of a single coding sequence. Each expression control sequence independently may be constitutive and/or regulated (e.g., tissue-specific expression, developmentally-inducible expression, stress-inducible expression, defense-inducible expression, and/or drought-inducible expression) according to some embodiments. In some embodiments, each clone of a coding sequence may be identical to one or more of the other clones. Copies of a coding sequence, according to some embodiments, may differ from one another somewhat, for example, where one copy may be codon optimized for one family, genus, and/or species while another may be optimized for a different family, genus, and/or species, or not codon optimized at all. Each expression control sequence-coding sequence clone independently may be present (e.g., in a microorganism and/or plant) on an expression vector, on a genomic nucleic acid, and/or on an extra-genomic nucleic acid in some embodiments. Each expression control sequence-coding sequence clone independently, in some embodiments, may further comprise one or more terminators.

The present disclosure relates, according to some embodiments, to transgenic plants of sugarcane, a high biomass producer and sugar accumulator, which are generated from explants transformed with an expression system (e.g., a multiple promoter-one transgene system). Transgenic sugarcane plants according to some embodiments of the disclosure were observed expressing high levels (up to 6.0 mg per kg of total stalk fresh weight—to ~1% total soluble protein or TSP) of extractable active bovine stomach lyzozyme (BvLz) an antimicrobial protein. The high BvLz expression levels are stable in consecutive generations of transgenic plants, allowing for the economic production and purification of the corresponding protein.

The present disclosure relates, in some embodiments, to methods for producing the multiple promoter-one transgene expression vectors and the transgenic plants. Methods may be used, for example, to transform different varieties of sugarcane by co-bombarding a target explant tissue (e.g., embryogenic callus or leaf roll disc) with the BvLz transgene encoding a protein normally present in bovine stomach and that is codon optimized for expression in monocotyledonous plants, under the control of multiple promoters from separate vectors.

Methods

According to some embodiments, the present disclosure relates to methods for transforming and/or transfecting a plant with a nucleic acid comprising an expression control sequence. For example, a method may comprise contacting a cell (e.g., a yeast cell and/or a plant cell) with a nucleic acid comprising an expression control sequence. Contacting a nucleic acid with a cell may comprise, in some embodiments, co-cultivating the target cell with a bacteria (e.g., *Agrobacterium*) comprising the nucleic acid (e.g., in a binary vector), electroporating the cell in the presence of the nucleic acid, infecting the cell with a virus (baculovirus) comprising the nucleic acid, bombarding the cell (e.g., a cell in a leaf, stem, and/or callus) with particles comprising the nucleic acid, agitating the cell in a solution comprising the nucleic acid and one or more whiskers (e.g., silicone carbide whiskers), and/or chemically inducing the cell to take up extracellular DNA. In some embodiments, contacting a nucleic acid with a cell may comprise contacting the nucleic acid with a plant leaf disc and/or a plant protoplast.

The disclosure relates, in some embodiments, to methods for expressing a nucleic acid sequence (e.g., comprising one or more coding sequences) in a cell. For example, a method may comprise contacting a cell (e.g., a yeast cell and/or a plant cell) with a nucleic acid comprising an expression control sequence and a coding sequence operably linked to the expression control sequence under conditions that permit expression of the coding sequence. Expression, according to some embodiments, may be constitutive, conditional, native (e.g., in the normal time and/or tissue), and/or ectopic. In some embodiments, a method may further comprise expressing a nucleic acid sequence in a plant (e.g., a monocot and/or a dicot). A method may include harvesting (e.g., partially purifying) from a plant a gene product of a nucleic acid sequence (e.g., an exogenous sequence) expressed in the plant, according to some embodiments.

In some embodiments, the present disclosure relates to methods for isolating an expression control sequence operable in at least one monocot and/or at least one dicot. For example, a method may comprise screening a library (e.g., a plant genomic library, a bacterial artificial chromosome library, a plant virus genomic library) with a probe comprising a nucleic acid having a nucleic acid sequence of SEQ ID NO: 1, a complement thereof, and/or a portion thereof (e.g., under stringent hybridization conditions). A method may comprise amplifying an expression control sequence from a library (e.g., using a polymerase chain reaction) using one or more primers derived from a nucleic acid sequence of SEQ ID NO: 1, a complement thereof, and/or a portion thereof. Operability of a candidate expression control sequence in at least one monocot and/or at least one dicot may be confirmed, in some embodiments, by forming a transcriptional and/or translational fusion of a candidate expression control sequence with a coding sequence expressible in the at least one monocot and/or the at least one dicot to form an expression cassette, transferring the expression cassette into the at least one monocot and/or the at least one dicot, and/or detecting expression of the coding sequence. An assay for detecting expression of the coding sequence may depend on the nature of the coding sequence. For example, a coding sequence may comprise a reporter gene (e.g., an autofluorescent protein, chloramphenicol acetyl transferase and β-glucuronidase (GUS)). Standard assays are available to sensitively detect a reporter enzyme in a transgenic organism.

The present disclosure relates, according to some embodiments, to methods for isolating an expression control sequence operable in at least one monocot and/or at least one dicot. For example, a method may comprise selecting one or more primers from about 15 to about 40 nucleotides in length and corresponding to (but not necessarily identical to) sequences at or near the 5' and/or 3' ends of SEQ ID NO: 1, contacting the one or more primers with an amplification library (e.g., a partial or complete viral genomic library, a partial or complete plant genomic library) and a nucleic acid polymerase under conditions that permit amplification of an expression control sequence. A plant genomic library, according to some embodiments, may comprise nucleic acids isolated from a virus-infected and/or virus-free plant. In some embodiments, a method may comprise screening a library with a probe comprising SEQ ID NO:1 or a fragment thereof. One or more candidate expression control sequences (e.g., amplification products) may be cloned into an expression vector in a position to drive expression of a coding sequence (e.g., GUS, an autofluorescent protein). Operability of the amplification products may be assessed, for example, by contacting a plant cell with such expression vectors under conditions that permit expression of the coding sequence (e.g.,

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microprojectile bombardment, *Agrobacterium*-mediated transformation) and examining the plant cell for the appearance of a gene product of the coding sequence (e.g., the encoded protein).

The present disclosure, in some embodiments, relates to methods of increasing expression levels of a coding sequence in at least one monocot and/or at least one dicot. For example, an expression cassette and/or expression vector may be introduced into a plant in order to effect expression of a coding sequence. According to some embodiments, a method of producing a plant with increased levels of a product of a sucrose accumulating gene and/or a defense gene may comprise transforming a plant cell with an expression vector and/or expression cassette comprising an expression control sequence operably linked to a sucrose accumulating gene or a defense gene and regenerating a plant with increased levels of the product of the sucrose accumulating gene or defense gene. In some embodiments of the present disclosure, a transgenic sugarcane line may be produced in which sugar metabolism is altered to increase stem dry weight (e.g., more than about 50% sucrose, more than about 60% sucrose, more than about 70% sucrose). A transgenic sugarcane line may be produced, according to some embodiments, with enhanced bioinsecticidal activity (e.g., for protection against stem boring insects, which may be the most destructive pests).

The present disclosure, in some embodiments, relates to methods of decreasing expression levels of a coding sequence (e.g., a native plant sequence, a viral sequence) in at least one monocot and/or at least one dicot. For example, a method may comprise contacting at least one monocot cell and/or at least one dicot cell with an expression vector comprising an expression control sequence and an antisense sequence that is complementary to at least a portion of the coding sequence and operably linked to the expression control sequence. In some embodiments, a method may comprise contacting at least one monocot cell and/or at least one dicot cell with an RNA interference (RNAi) expression vector comprising an expression control sequence and a nucleic acid sequence which is an inverted repeat of the native plant gene, the expression level of which is to be reduced and/or silenced, and operably linked to the expression control sequence. A method may comprise, in some embodiments, contacting at least one monocot cell and/or at least one dicot cell with a cosuppression expression vector comprising an expression control sequence and a nucleic acid sequence coding for the native plant gene operably linked to the expression control sequence.

Embryonic calli and other susceptible tissues, in some embodiments, may be inoculated with a "disarmed" foreign DNA-containing *A. tumefaciens*, cultured for a number of days, and transferred to antibiotic-containing medium. Transformed shoots may be selected after rooting in medium containing the appropriate antibiotic, and transferred to soil. Transgenic plants may be pollinated and seeds from these plants may be collected and grown on antibiotic medium.

Expression of a heterologous or reporter gene in tissues, developing seeds, young seedlings and mature plants may be monitored, according to some embodiments, by immunological, histochemical, mRNA expression or activity assays. Choice of expression assay for the expression cassette may depend upon the nature of the heterologous coding sequence. For example, RNA gel blot analysis may be used to assess transcription if appropriate nucleotide probes are available. If antibodies to the polypeptide encoded by the heterologous gene are available, western analysis and immunohistochemical localization may be used to assess the production and

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localization of the polypeptide. Depending upon the heterologous gene, appropriate biochemical assays may be used.

The present disclosure further relates to methods for isolating and/or purifying ("purifying") a gene product (e.g., a nucleic acid and/or a protein) from a plant. For example, a method may comprise providing a plant comprising a nucleic acid having an expression control sequence and a coding sequence operably linked to the expression control sequence, wherein the coding sequence encodes a gene product of interest. A method may comprise, according to some embodiments, producing a transgenic protein in a plant, extracting juice containing the transgenic protein from the plant, cleaning the juice to remove particulate matter, and/or transmitting the juice through at least one membrane to produce two fractions, one of the fractions containing the transgenic protein. In some embodiments, a transgenic protein may comprise a lectin, an enzyme, a vaccine, a bacterial lytic peptide, a bacterial lytic protein, an antimicrobial peptide, an antimicrobial peptide protein, an antiviral peptide, an antiviral protein, an insecticidal peptide, an insecticidal protein, a therapeutic peptide, and a therapeutic protein.

As will be understood by those skilled in the art who have the benefit of the instant disclosure, other equivalent or alternative compositions, devices, methods, and systems for expressing a nucleic acid sequence in at least one monocot and/or at least one dicot can be envisioned without departing from the description contained herein. Accordingly, the manner of carrying out the disclosure as shown and described is to be construed as illustrative only.

Persons skilled in the art may make various changes in the shape, size, number, and/or arrangement of parts without departing from the scope of the instant disclosure. For example, the position and number of expression control sequences may be varied. Each disclosed method and method step may be performed in association with any other disclosed method or method step and in any order. Also, where ranges have been provided, the disclosed endpoints may be treated as exact and/or approximations as desired or demanded by the particular embodiment. Where the endpoints are approximate, the degree of flexibility may vary in proportion to the order of magnitude of the range. For example, on one hand, a range endpoint of about 50 in the context of a range of about 5 to about 50 may include 50.5, but not 52.5 or 55 and, on the other hand, a range endpoint of about 50 in the context of a range of about 0.5 to 50 may include 55, but not 60 or 75. In addition, it may be desirable, in some embodiments, to mix and match range endpoints. Also, in some embodiments, each figure disclosed (e.g., in one or more of the Examples and/or Drawings) may form the basis of a range (e.g., disclosed value +/- about 10%, disclosed value +/- about 100%) and/or a range endpoint. Persons skilled in the art may make various changes in methods of preparing and using a composition, device, and/or system of the disclosure. For example, a composition, device, and/or system may be prepared and or used as appropriate for animal and/or human use (e.g., with regard to sanitary, infectivity, safety, toxicity, biometric, and other considerations).

These equivalents and alternatives along with obvious changes and modifications are intended to be included within the scope of the present disclosure. Accordingly, the foregoing disclosure is intended to be illustrative, but not limiting, of the scope of the disclosure as illustrated by the following claims.

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EXAMPLES

Some specific example embodiments of the disclosure may be illustrated by one or more of the examples provided herein.

Example 1

SCBV Infection in Sugarcane Fields in the Mid Rio Grande Valley, Tex.

Leaves were harvested from haphazardly selected sugarcane plants in fields in the mid Rio Grande Valley of Texas. The incidence of SCBV infection was examined by Southern blotting after DNA extraction from the harvested sugarcane leaves (Table 2). The 32 P-dCTP-labelled DNA probe for Southern blots was prepared from the SCBV fragment of ~1.4 Kb encompassing SCBV ORF1, ORF2 and the 5' 450 nt of ORF3, which was cloned by PCR using sugarcane DNA prepared from CP72-1210. The Southern results showed that of fourteen sugarcane varieties/clones tested, eleven varieties/clones were SCBV positive, which indicates that SCBV infection is prevalent in the fields in the Mid Rio Grande Valley.

TABLE 2

SCBV incidence in commercial sugarcane variety.		
Variety/clone	Infectivity*	Plants that tested positive for SCBV/total plants tested
CP72-1210	100%	(6/6)
TCP87-3308	100%	(6/6)
TCP89-3505	100%	(6/6)
TCP04-4688	83.30%	(5/6)
TCP05-4732	100%	(6/6)
TCP05-4738	100%	(6/6)
TCP05-4747	100%	(6/6)
TCP05-4760	16.70%	(1/6)
TCP05-4784	100%	(6/6)
TCP98-4454	0%	(0/6)
NC0310	0%	(0/6)
91	0%	(0/6)
385	100%	(6/6)
1903	100%	(6/6)

*The results are based on Southern blots.

Example 2

Cloning and Sequencing of a SCBV Promoter (SCBV21)

Total genomic DNA was isolated from the leaf tissue of SCBV-positive sugarcane cultivar CP72-1210. The DNA concentration was adjusted to ~100 ng/ μ l, and ~250 ng of DNA was used for PCR reactions. The primer sequence information was provided by Dr. Guohui Zhou from Southern China Agricultural University, Guangzhou, China, who has cloned a Southern China isolate of SCBV. Primer names and sequences are as follows: P-2 (5'-acg ccg taa cac gta gtc cta agg t-3'; SEQ ID NO: 11), P-W3F (5'-gac atc aaa tgg ttg tat cc-3'; SEQ ID NO: 12), P-W4F (5'-aca ccg cat tca gag tga ag-3'; SEQ ID NO: 13) and P-W1R (5'-ccg cat taa cgt tct cc-3'; SEQ ID NO: 14). All PCR reactions were performed in 20 μ l of reaction mixture using Taq DNA polymerase (NEB) following the manufacturer's recommendation. The primer set, P-2 and P-W3F, was used for the first PCR reaction using the following PCR parameters for pre-amplification of SCBV genome containing its promoter sequence: 1 cycle at 94° C. for 4 min, 10 cycles each at 94° C. for 30 sec, at 48° C. for 30

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sec, and at 72° C. for 5 min. Then, 5 μ l of the first PCR reaction mixture was used as a template for the second PCR reaction with a primer set, P-W1R and P-W4F by the following PCR program: 1 cycle at 94° C. for 4 min, 35 cycles each at 94° C. for 30 sec, at 52° C. for 30 sec, and at 72° C. for 4 min, and 1 cycle at 72° C. for 5 min. The PCR reaction mixture was analyzed by electrophoresis on 1% agarose gels. The size of the obtained PCR product was ~2 kb which was cloned into the pGEM T-Easy vector (Promega). The nucleotide sequence of the cloned product was analyzed by sequencing, which confirmed that the cloned fragment has homology with SCBV ORF3. After the sequence alignment with other SCBV promoter sequences, two primers, SCBV/Prom/F (5'-GAA GAA CAG CAT GCT GAA CAT CTG TGG AAG ATG C-3'; SEQ ID NO: 15) and SCBV/Prom/R (5'-CAA ACT TGC TCA AAT GAT CAT GTG GTG AAC TAC CGA TG-3'; SEQ ID NO: 16) were designed from the conserved regions. The PCR condition with these two primers was: 1 cycle at 94° C. for 4 min, 35 cycles each at 94° C. for 30 sec, at 52° C. for 30 sec and at 72° C. for 2 min, and 1 cycle at 72° C. for 5 min. The PCR product was analyzed on 1% agarose gels, and the PCR product was cloned into pGEM T-Easy, and the cloned sequence was confirmed by sequencing. The cloned PCR product was named as pGEM/SCBV21 (FIG. 1).

Example 3

SCBV21 Promoter Activity

To test the promoter activity of the cloned SCBV21, it was subcloned upstream of the β -glucuronidase (GUS) gene to construct pBI/SCBV21-GUS. (FIG. 2). The promoter activity of SCBV21 was tested by bombarding DNA-coated tungsten particles onto the onion epidermal layers using a gene gun. GUS expression was confirmed by histochemical GUS assays 2 days after bombardment (FIG. 3).

Example 4

Sequence Comparison of SCBV Promoters from Different SCBV Isolates

The sequence of SCBV21 was compared with two SCBV isolates, SCBV Ireng Maleng (IM) and SCBV Morocco (Mor). Table 3 shows that SCBV21 has 87% and 71% identity with SCBV-IM and SCBV-Mor isolates, respectively.

TABLE 3

Sequence comparisons of SCBV21 to two other SCBV isolates		
	SCBV-IM-AJ277091*	SCBV-Mor M89923*
SCBV21	87%	71%

*NCBI Genebank accession number.

**Sequence identity (%): The sequence identity (%) was obtained by BLASTn search with SCBV21 in NCBI GeneBank

Example 5

SCBV21-Driven GUS Transgene Expression in Transgenic Sugarcane

Transgenic sugarcane was generated with the DNA construct, pBI/SCBV21-GUS (FIG. 1), and the GUS transgene expression level of this transgenic line was compared with other transgenic lines of which GUS transgene expression

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was driven by CaMV 35S promoter or a modified maize Ubi promoter that lacks a heat shock element (mUbil-no hse) (FIG. 4). GUS expression levels in SCBV21/GUS transgenic lines is about four to six times higher than in 35S/GUS transgenic lines, while mUbil-no hse/GUS transgenic lines displayed the highest GUS expression level which is six to ten times more than that of the SCBV21/GUS lines (FIG. 4).

Example 6

SCBV21 Directs GUS Expression in Sorghum, Tobacco and Lima Bean Seed

The promoter activity of SCBV21 was transiently tested in another monocot, sorghum, and two dicot species, tobacco and Lima bean by bombarding DNA-coated tungsten particles onto prepared tissue samples (FIG. 6). The results showed that SCBV21 functions as a promoter regardless of tissue samples (leaf or seed) and of plant species (monocot or dicot) (FIG. 6).

Example 7

Relative Expression Levels with Various Promoters: GUS and EYFP Transient Expression in Leaf Tissue

Young leaf segments were cultured in MS_{0.6} solid media (Murashige and Skoog, 1962), B5G 1 mg/L, 0.6 mg/L 2,4-D, 500 mg/L casein hydrolysate, 20 g/L sucrose and 7 g/L Agar for 4 days and leaf rolls were kept for 10 days or 28 days before bombardment. Plasmid DNA was precipitated onto tungsten particles (1.1 µm, Bio-Rad) at a concentration of 4 µg (for GUS construct) or 1 µg (for EYFP construct) DNA per mg of tungsten using calcium chloride and spermidine.

Example 8

Relative Expression Levels with Various Promoters: GUS Histochemical Assay

After 48 hrs post-bombardment, leaf segments were transferred to 0.1% X-Gluc staining solution containing 0.1% (v/v) Triton X-100, and 0.1 M sodium phosphate buffer (pH 7.0). Tissues were then incubated overnight (24 hr) at 37° C. After staining, chlorophyll was removed from tissue by immersing in 70% (v/v) ethanol and changed twice. Tissues were observed for GUS staining, and photographed using an OLYMPUS D71 camera Connected on a SZX7 stereoscopic microscope (Japan).

Example 9

Relative Expression Levels with Various Promoters: GUS Activity Quantitative Assay

Quantitative fluorometric GUS assays were performed by the modified procedure of Jefferson (1987). 500 mg of plant tissue were weighed and ground in liquid nitrogen and then transferred to 1.5 mL microcentrifuge tube with 750 µL GUS extraction buffer containing 50 mM sodium phosphate buffer (pH 7.0), 10 mM 2-mercaptoethanol, 10 mM EDTA (pH 8.0) and 0.1% (v/v) Triton X-100. After briefly vortexing, the tubes were incubated on ice for 1 hr and centrifuged at 12000 g for 10 min at 4° C. An aliquot of the supernatant was used for protein concentration determination and GUS activity assays. Protein concentrations were determined by the Lowry assay method based on the instruction manual of the DC

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protein assay kit (Bio-Rad). Fluorometric enzymatic GUS assay were carried out for leaf samples by adding 10 µL protein extracts and 15 µL GUS extraction buffer to 25 µL MUG (4 mM) assay buffer. 25 µL protein extracts were used for the reaction with 25 µL MUG solution for root samples. After incubation for 1 hr at 37° C., 950 mL 0.2 M Na₂CO₃ solutions were added to stop the reaction. The optical density was read at 455 nm after excitation at 365 nm on a VersaFluor™ Fluorometer (BIO-RAD). Protein extracts of untransformed plants were used for the negative control samples and a serial dilution of 4-methylumbellifereone (MU, Sigma) solutions in GUS extraction buffer were used as standards.

Example 10

Relative Expression Levels with Various Promoters: EYFP IMAGE Collection and Analysis

Images of 4080×3072 pixels and 256 gray levels for red, green and blue channels were collected every 6 hrs post-bombardment for at less 240 hrs. EYFP expression was quantified using the ImageJ software (Rasband 1997-2009) according to the revised method described by Chiera et al (2007 and 2008). Each series of images was imported, resized to 800×600 pixels and aligned by Adobe Imageready CS (8.0.1 version). After alignment, the serie of images was exported as a “mov” file. The “mov” file was opened by the ImageJ software and an area comprising 400×300 pixels containing the highest number of expressing cells was cropped from the series of images and then was saved as an “avi” file for quantification analysis of EYFP. Each series of images in the “avi” file was split into red, green and blue channels. A 20×20 pixel area without EYFP expression cells was selected in the background of green channel for determination of background gray value and was subtracted from sequential images to remove the background fluorescence. After adjusting the threshold levels, the focus number values, mean grayscale values and total area values were generated by the procedures of the macros that were kindly presented by Chiera and Hernandez-Garcia. The “Total Expression” value was calculated by multiplying a mean grayscale value per pixel by the total area.

Example 11

Relative Expression Levels with Various Promoters: Protoplast Isolation and Transfection

50 Protoplasts were isolated from Callus using a modified method of Chen (1987) and Yoo et al (2007). Briefly, Callus cultures were cultured on a rotary shaker (250 mL flasks; 100 rpm) by weekly subculture (1:5 dilution) for 2 to 3 months in a MS₃ liquid medium (Murashige and Skoog, 1962), B5G 1 mg/L, 3.0 mg/L 2,4-D, 500 mg/L casein hydrolysate and 20 g/L sucrose. The fresh suspension cells (subcultured for 2 or 3 days) were harvested digested overnight in enzyme solution (20 mM MES (pH 5.7), 2.0% Cellulysin® Cellulase (EMD Biosciences, USA), 0.1% PECTOLYASE Y-23 (Duchefa Biochemie, USA), 0.4 M mannitol, 20 mM KCl, 10 mM CaCl₂, and 0.1% BSA). Protoplasts were collected and washed in W5 solution twice and pelleting at 100 g for 2 min. After the second wash, protoplasts were resuspended in MMG solution (4 mM MES-KOH, pH5.7, 0.4 M mannitol, 15 mM MgCl₂) to reach a final concentration of 1 to 2×10⁶ protoplasts/mL. 100 µL of protoplasts were transferred into a 2-mL round-bottom microcentrifuge tube and mixed gently

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with the plasmid DNA (10 µg in 10 µL). Equivalent volumes of deionized, sterile water (mock-transfection) were used as control transfections. Transfection was initiated by the addition of 110 µL of PEG-calcium solution (40% PEG-4000, 0.2 M mannitol, 100 mM CaCl₂). Protoplasts were mixed with PEG-calcium solution by gently tapping the tube and incubating for 10 min at room temperature. Transfection was terminated by diluting the mixture with 440 µL of W5 solution. Transfected protoplasts were collected by centrifugation for 2 min at 100 g and resuspended in 250 µL of W5 solution. EYFP or GUS expression analysis was investigated after protoplasts were kept in the dark for 16 hrs at room temperature. Protoplasts were harvested by centrifugation at 100 g for 2 min, and then removing the supernatant and stored at -80° C. until GUS activity analysis. Adding 100 µL of GUS extraction buffer to the frozen protoplasts and mixing vigorously by vortexing for 2 s to rupture the protoplasts. After keeping on ice for 5 min, centrifuged at 1000 g for 2 min. Taking 25 µL of the protoplasts lysate into 25 µL 4 mM MUG assay buffer and incubated for 60 min at 37° C.

Example 12

Relative Expression Levels with Various Promoters:
Statistical Analysis

The relative expression levels of various promoters are shown in Tables 4-10. The data were collected from 2 to 4 independent experiments and 6 to 10 replicates in every experiment. The GLM procedure of Statistical Analysis System (8.0 version, SAS Institute, USA) was used for statistical analysis. Student-Newman-Keuls (SNK) Test was performed for multiple comparisons of the mean.

TABLE 4

GUS transient expression in sugarcane leaf segments_spot number*			
name	spot number	Std err	Significant Difference
mUbil-no hse/GUS	250	34	A
Ubi/GUS	63	13	B
SCBV21/GUS	36	10	BC
35S-GUS	4	1	C

*Images were taken by microscope (x15).

TABLE 5

EYFP transient expression in sugarcane leaf segments_Foci number*			
name	Foci number	Std err	Significant Difference
mUbil-no hse/EYFP	351	19	BC
Ubi/EYFP	384	29	BC
SCBV21/EYFP	514	27	A
35S-EYFP	318	12	C
E35S/EYFP	511	38	A

*Data were collected from the 48 hrs timepoint post-bombardment. Images were taken by Fluorescence microscope (x15) with YFP filter.

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TABLE 6

EYFP transient expression in sugarcane leaf segments_total expression*			
name	Total expression	Std err	Significant Difference
mUbil-no hse/EYFP	385	39	C
Ubi/EYFP	454	51	C
SCBV21/EYFP	701	87	B
35S-EYFP	316	21	C
E35S/EYFP	894	134	A

*Total expression is measured as mean Gray scale per pixel × total area × 1000. Data were collected from the 48 hrs timepoint post-bombardment. Images were taken by Fluorescence microscope (x15) with YFP filter.

TABLE 7

GUS transient expression in sugarcane protoplasts_GUS activity*			
name	GUS activity	Std err	Significant Difference
mUbil-no hse/GUS	36.99	1.64	A
Ubi/GUS	11.51	0.69	B
SCBV21/GUS	3.16	0.21	C
35S/GUS	0.87	0.09	C

*p-mole 4-MU/ug protein per minute

TABLE 8

EYFP transient expression in sugarcane protoplasts_Foci number*			
name	Foci number	Std err	Significant Difference
mUbil-no hse/EYFP	21	2	B
Ubi/EYFP	21	2	B
SCBV21/EYFP	31	3	A
35S-EYFP	17	2	B
E35S/EYFP	36	4	A

*Images were taken by Fluorescence microscope (x85.5) with YFP filter.

TABLE 9

GUS expression in transgenic sugarcane leaves_GUS activity*			
name	GUS activity	Std err	Significant Difference
mUbi1-no hse/GUS	46.60	2.56	A
SCBV21/GUS	8.21	0.45	B
35S/GUS	1.32	0.20	C

Data form two independent experiments.

mUbi1-no hse/GUS: 2 events, 5 plants;

SCBV21/GUS: 2 events, 6 plants;

35S/GUS: 6 events, 18 plants.

*p-mole 4-MU/ug protein per minute

TABLE 10

GUS expression in transgenic sugarcane stems_GUS activity*			
name	GUS activity	Std err	Significant Difference
mUbi1-no hse/GUS	23.61	2.61	A
SCBV21/GUS	6.75	1.32	B
35S/GUS	3.83	1.54	C

Data form two independent experiments.

mUbi1-no hse/GUS: 5 plants, 1 event;

SCBV21/GUS, 1 plant, 1 event;

35S/GUS, 3 plants, 1 event.

*p-mole 4-MU/ug protein per minute

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Example 13

Expression Pattern of SCBV21

Expression of GUS under the control of SCBV21 in stalks, leaves, and roots of in the transgenic sugarcane of Example 5 is shown in FIGS. 7A, 7B, and 7C, respectively. Staining is observed in all three tissues. FIG. 7A shows a sectional view of the opposite halves of a radially sectioned stalk segment (left, marked a1), an isometric view of two stalk segments, each including a leaf node with the leaves trimmed away (upper right, marked a2), and a substantially plan view of a stalk segment and transverse stalk sections (lower right, marked a3). FIG. 7B shows two leaves and a leaf sheath from a single node. FIG. 7C shows shoot roots from a single transgenic plant with the highest expression in the region around the ground meristem.

In addition, expression levels of SCBV21 in various cell types were observed. For example, micrographs (FIGS. 8A-8D) of transgenic SCBV21/GUS stalks showed strong staining (using the GUS staining protocol of Example 9) of storage parenchyma and the vascular system. In these images, xylem (x), phloem (p), and storage parenchyma (pa) are marked. Transgenic SCBV21/GUS stalks also showed strong staining of sclerenchyma.

Example 14

Identification of Potential Transcription Start Sites in SCBV21

The cloned SCBV promoter sequence of 1816 bp (SEQ ID NO:1) was analyzed with Promoter Finder (available through the Berkeley *Drosophila* Genome Project at fruitfly dot org slash seq_tools slash promoter dot html) to identify potential transcription start sites. Promoter Finder predicted two potential transcription start sites, TSS1 (nucleotides 1055-1104 of SEQ ID NO:1) and TSS2 (nucleotides 1737-1786 of SEQ ID NO:1).

Example 15

Generation of Deletion Mutants of SCBV21

All deletion mutants in this Example were generated from pSK-SCBV21-EYFP-Nos (FIG. 9A; SEQ ID NO:19). The restriction enzyme sites used for deletions were indicated on the map. TSS1 and TSS2 are shown with horizontal hash lines. The approximate positions of primers to generate deletion mutants were indicated with filled arrowheads. XhoI site in forward primers (F1 and F2; SEQ ID NOS: 34 and 35, respectively) and NcoI site in reverse primers (R1 and R2; SEQ ID NOS: 36 and 37, respectively) were incorporated for cloning purposes.

Deletion mutant B was generated by deleting the region between StuI and NcoI sites from pSK-SCBV21-EYFP-Nos (FIG. 7A). First, pSK-SCBV21-EYFP-Nos was double digested with StuI and NcoI, followed by Klenow reaction to make blunt ends of digested fragments. Then, the digested fragment of 4964 bp was eluted from agarose gel for blunt-end ligation. The nucleotide sequence of the ligation junction was confirmed by sequencing. The plasmid map of Mutant B is shown in FIG. 9B. In FIG. 9B, the deleted region of SCBV21 is indicated with a black bar and the remaining fragment of SCBV21 is shown with the crossed-line fill pattern.

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To generate deletion mutant C, the region between StuI and the 3' end of SCBV21 was PCR amplified from pSK-SCBV21-EYFP-Nos with the primer F1 (SEQ ID NO:34) and R1 (SEQ ID NO:36) (FIG. 9A). The primer R1 was designed to remove 19 nucleotides present in pSK-SCBV21-EYFP-Nos between the start codon of EYFP and the 3' end of SCBV21 (FIG. 9A). This sequence was derived from the multicloning site of pGEM T-Easy vector. The 805 bp PCR product (mutant C fragment of SCBV21) was cloned into pGEM T-Easy (Promega) vector, and the nucleotide sequence of mutant C fragment was confirmed by sequencing. XhoI and NcoI double digestion, whose enzyme sites were flanking 5' and 3' end of the mutant C fragment, respectively, excised the mutant C fragment from pGEM T-Easy vector. The XhoI-NcoI fragment of pSK-SCBV21-EYFP-Nos was replaced with the mutant C fragment to generate Mutant C (FIG. 9C). In FIG. 9C, the deleted region of SCBV21 is indicated with a black bar and the remaining fragment of SCBV21 is shown with the crossed-line fill pattern. TSS1 and TSS2 are also indicated in the map.

To generate deletion mutant D, the 3' 710 bp of SCBV21 was PCR amplified with primer F2 (SEQ ID NO:35) and R1(SEQ ID NO:36) from pSK-SCBV21-EYFP-Nos (FIG. 9A). The PCR product (mutant D fragment of SCBV21) was cloned into pGEM T-Easy vector, and the nucleotide sequence of mutant D fragment was confirmed by sequencing. Mutant D was generated by the same procedure used to make Mutant C. XhoI and NcoI double digestion, whose enzyme sites were flanking 5' and 3' end of the mutant D fragment, respectively, excised the mutant D fragment from pGEM T-Easy vector. The XhoI-NcoI fragment of pSK-SCBV21-EYFP-Nos was replaced with the mutant D fragment to generate Mutant D (FIG. 9D). In FIG. 9D, the deleted region of SCBV21 is indicated with a black bar and the remaining fragment of SCBV21 is shown with the crossed-line fill pattern. TSS2 is also indicated in the map.

To generate deletion mutant E, the region between nt 1722 and nt 2440 was PCR amplified with primer F1 (SEQ ID NO:34) and R2 (SEQ ID NO:37) from pSK-SCBV21-EYFP-Nos (FIG. 9A). The PCR fragment (mutant E fragment) was cloned into pGEM T-Easy vector, and the nucleotide sequence of mutant E fragment was confirmed by sequencing. Mutant E was generated by the same procedure used to make Mutant C and D. The mutant E fragment was excised from pGEM T-Easy vector by XhoI and NcoI double digestion, whose enzyme sites were flanking both 5' and 3' end of the mutant E fragment. The XhoI-NcoI fragment of pSK-SCBV21-EYFP-Nos was replaced with the mutant E fragment to generate Mutant E (FIG. 9E). In FIG. 9E, the deleted region E1 and E2 of SCBV21 is indicated with a black bar and the remaining fragment of SCBV21 is shown with the crossed-line fill pattern. TSS1 is also indicated in the map.

To generate deletion mutant F, the region between nt 1815 and nt 2440 was PCR amplified with primer F2 (SEQ ID NO:35) and R2 (SEQ ID NO:37) from pSK-SCBV21-EYFP-Nos (FIG. 9A). The PCR fragment (mutant F fragment) was cloned into pGEM T-Easy vector, and the nucleotide sequence of mutant F fragment was confirmed by sequencing. Mutant F was generated by the same procedure used to make the aforementioned three mutants, Mutant C, D and E. The mutant F fragment was excised from pGEM T-Easy vector by XhoI and NcoI double digestion, whose enzyme sites were flanking 5' and 3' end of the mutant F fragment, respectively. The XhoI-NcoI fragment of pSK-SCBV21-EYFP-Nos was replaced with the mutant F fragment to generate Mutant F (FIG. 9F). In FIG. 9F, the deleted region of SCBV21 is

indicated with a black bar and the remaining fragment of SCBV21 is shown with the crossed-line fill pattern.

Example 16

Transient Expression Assay on Sugarcane Leaf Sections

Target sugarcane leaf tissue for transient EYFP expression assay was prepared from commercial sugarcane hybrid CP72-1210. Actively growing top portions of stalks, including the first 2-3 nodes from top, were harvested from field grown sugarcane. After removing all fully expanded leaves until the first visible dewlap was exposed, the sugarcane top was sterilized in 10% bleach for 20 min. The outermost 2-3 layers of green leaf sheaths above first node were removed, then the next 1-2 layers of leaf sheath were sectioned in 10 mm×20 mm size after removing mid rib. The prepared target tissue sections were placed adaxial side down and kept on MS solid media for 3 days in the dark. Each tissue section was transferred onto a new MS medium plate and used for particle bombardment with DNA-coated 1.1 μm-tungsten particles that were prepared by the manufacturer's instruction (Bioread).

For particle bombardment, 500 ug of tungsten particles coated with 500 ng of DNA was placed on a microcarrier filter, then the filter was installed at the tip of a nozzle releasing 110 psi helium gas in a vacuum chamber. Each target tissue on a MS medium plate was placed 7 cm below the tip of microcarrier filter in a vacuum chamber. The DNA coated tungsten particles were bombarded on the target tissue at 110 psi under 26 inch-Hg vacuum pressure. After bombardment, the target tissue was kept in the dark for 2 days. The expression of EYFP was investigated under a fluorescence microscope with EYFP or GFP filter. Results are shown in Table 11 and FIGS. 11A-11F.

TABLE 11

EYFP expression in transgenic sugarcane leaves					
Construct	Promoter size (bp)	TSS1	TSS2	YFP Expression	FIG.
A. SCBV21-EYFP-Nos	1816	Yes	Yes	+++	11A
B. SCBV21 Δnt1014-nt1837-EYFP-Nos	1013	No	No	-	11B
C. SCBV21 Δnt1010-EYFP-Nos	805	Yes	Yes	+++	11C
D. SCBV21 Δnt1104-EYFP-Nos	710	No	Yes	+++	11D
E. SCBV21 Δnt1010 Δnt1732-nt1837-EYFP-Nos	721	Yes	No	+/-	11E
F. SCBV21 Δnt1104 Δnt1732-nt1837-EYFP-Nos	626	No	No	+/-	11F

Yellow Fluorescent Protein (YFP) was observed in tissue bombarded with SCBV21-EYFP-Nos (unmodified), SCBV21 Δnt1-nt1010-EYFP-Nos (deletion C), and SCBV21 Δnt1-nt1104-EYFP-Nos (deletion D) as shown in FIG. 11A, FIG. 11C, FIG. 11D, respectively. Little or no YFP was observed in tissue bombarded with SCBV21 Δnt1014-nt1837-EYFP-Nos (deletion B), SCBV21 Δnt1-nt1010 Δnt1732-nt1837-EYFP-Nos (deletion E), or SCBV21 Δnt1104 Δnt1732-nt1837-EYFP-Nos (deletion F) as shown in FIG. 11B, FIG. 11E, and FIG. 11F, respectively.

Example 17

Transient Expression Assay on *Nicotiana Tabacum* Leaf Sections

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For the transient EYFP expression assay on *N. tabacum*, the leaves of 45-day-old *N. tabacum* grown in a Magenta box were collected and placed adaxial side down on MS-medium supplemented with 0.1 M mannitol and 0.2 M sorbitol for 4 hours in the dark before bombardment. The prepared target tissue was bombarded with 500 μg of 1.1 μm-tungsten particles coated with 500 ng of DNA at 60 psi under 26 inch-Hg vacuum pressure. After keeping the bombarded target tissues on MS-medium supplemented with 0.1M mannitol and 0.2 M sorbitol for about 12 hours in the dark, the target tissues were transferred and kept on MS-medium for 24 hours. The YFP expression was examined under a fluorescence microscope with GFP filter. The results are summarized in Table 12 below.

10 Results are shown in Table 12. Yellow Fluorescent Protein (YFP) was observed in tissue bombarded with SCBV21-EYFP-Nos (unmodified), SCBV21 Δnt1-nt1010-EYFP-Nos (deletion C), and SCBV21 Δnt1-nt1104-EYFP-Nos (deletion D). Little or no YFP was observed in tissue bombarded with

15 SCBV21 Δnt1014-nt1837-EYFP-Nos (deletion B), SCBV21 Δnt1-nt1010 Δnt1732-nt1837-EYFP-Nos (deletion E), or SCBV21 Δnt1-nt1104 Δnt1732-nt1837-EYFP-Nos (deletion F). Thus, the expression pattern paralleled that seen for monocots in Example 16.

TABLE 12

EYFP expression in transgenic tobacco leaves					
Construct	Promoter size (bp)	TSS1	TSS2	YFP Expression	
A. SCBV21-EYFP-Nos	1816	Yes	Yes	+++	
B. SCBV21 Δnt1014-nt1837-EYFP-Nos	1013	No	No	-	
C. SCBV21 Δnt1-nt1010-EYFP-Nos	805	Yes	Yes	+++	
D. SCBV21 Δnt1-nt1104-EYFP-Nos	710	No	Yes	+++	
E. SCBV21 Δnt1-nt1010 Δnt1732-nt1837-EYFP-Nos	721	Yes	No	+/-	
F. SCBV21 Δnt1-nt1104 Δnt1732-nt1837-EYFP-Nos	626	No	No	+/-	

Example 18

Multi-Promoter Expression of Bovine Stomach Lysozyme

Sugarcane (*Saccharum* spp.) has a great potential for the production of protein-based therapeutics. It has a fast growth cycle and an efficient carbon fixation pathway, produces a large biomass, and offers the prospect of inexpensive biopharmaceutical production. This example illustrates development of sugarcane as a recombinant expression system for the production of a mammalian enzyme (bovine stomach lysozyme) having broad-spectrum antimicrobial activity and a potential use in food, cosmetics and agriculture. Expression of this mammalian gene was enhanced in sugarcane by modulating transcription, transcript stability and translation. Expression vectors were generated using a synthetic gene that was codon optimized for expression in a plant monocot system (e.g., SEQ ID NO: 38). A single promoter as well as a multiple promoter system was used to drive expression. The

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5' and 3' untranslated regions of a virus that infects sugarcane were fused to the coding region of the gene to enhance translation. Embryogenic calli and leaf rolls of two commercial sugarcane varieties were transformed biolistically, and the phosphinothricin acetyl transferase (BAR) gene was used as a selectable marker. Immunoblot analysis as well as enzymatic activity assays of stably transformed sugarcane plants revealed the presence of intact bovine stomach lysozyme that accumulated at levels as high as 0.33 mg/kg in stalks of plants expressing it from a single promoter vector, and up to 6.0 mg/kg in stalks transgenic for co-expression of the BvLz gene from three different promoters in separate vectors. Each vector did not adversely affect the others as shown by copy number, steady-state mRNA levels and the presence of the functional enzyme. These results suggest that transcriptional synergism resulted through additive promoter activities and increased gene expression. A growth cycle study for an 11-month period showed a substantial increase in enzyme accumulation over time in the transgenic lines. This study suggests the commercial feasibility of producing a stable recombinant enzyme in transgenic sugarcane, and developing sugarcane as a biofactory for high value proteins.

Example 19

Single Promoter Expression of Bovine Stomach Lysozyme

A. Growth Cycle Study of Sugarcane Single Promoter BvLz Transgenic Lines: Monitoring BvLz Activity of the Transgenic Lines for 7-, 9- and 11-Month Period

A number of sugarcane BvLz transgenic lines were generated in accordance with this Example. These represent: (1) 36 lines with 74 plants that are transgenic for BvLzm (maize BvLz) under the control of the strong constitutive promoter of maize ubiquitin 1 with no heat shock element (pMUBi), and (2) 4 lines with 18 plants that are transgenic for pMUBi BvLzm and for P1HcPro, a suppressor of gene silencing isolated in our laboratory (U.S. Pat. No. 7,001,739). A total of 15 BvLz transgenic lines were selected for further characterization of their BvLz activity level.

To study the temporal accumulation of BvLz in sugarcane, the selected 15 BvLz transgenic lines were subjected to an 11-month greenhouse growth cycle study with harvests at 7-, 9- and 11-months. Stalks were harvested for the three time harvests, shredded and shipped frozen to the BioSeparations Laboratory at Texas A&M University (College Station). The juice from the 15 BvLz transgenic lines for the three different harvests was extracted by manual press and evaluated for BvLz by a standard turbidity assay. The extract was adjusted to pH 4.0, clarified by centrifugation, and passed over an SP-Sepharose cation exchange column for BvLz concentration. The BvLz activity was assayed in the concentrated extract.

Table 13 lists the BvLz activity (mg of BvLz per kg of harvested cane/stalk) of the 15 BvLz sugarcane transgenic lines for the 7-, 9- and 11-month harvests. (BvLz activity was assayed in 200 mL of stalk extract from the 7-month harvest, and 650-700 mL (one kg of cane/stalk) of stalk extract from the 11-month harvest.) In general, there was a substantial increase in BvLz yield at the 11-month harvest for the 15 transgenic lines. Nine out of 15 lines showed a two-fold increase in their BvLz activity level. A lower level of BvLz activity was detected in the 7-month harvested stalks.

Additional experiments were conducted to evaluate the efficiency of BvLz extraction and purification from the existing BvLz transgenic lines. These include the following:

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1. western analysis of BvLz in the flow-through of the column was performed, and no detectable amount of BvLz was observed.

2. western analysis of BvLz of the shredded stalks from the 15 BvLz transgenic lines was also performed. The BvLz activity data correlated very well with the BvLz level detected by the western analysis in the stalks.

Total soluble protein from leaf (40 µg) and stalk (2-5 µg) was analyzed by western blot, using a polyclonal anti-BvLz antibody. One kg of cane/stalk (650-700 mL extract) from the 11-month harvest was analyzed for BvLz activity. The BvLz expression level of the transgenic lines was also measured in the leaves of the same physiological age (fully expanded second-leaf stage) at the three different harvest times, western analysis showed that there was no difference in the BvLz level of leaves harvested at 7-, 9- and 11-months. Furthermore, the BvLz level of leaves of the transgenic lines correlated very well with that of stalks for the same harvest.

TABLE 13

	Transgenic line	mg BvLz/kg cane (stalk)		
		7-month	9-month	11-month
<u>Very high expresser</u>				
EM108	0.13	0.20	0.33	
EM114	0.12	0.18	0.32	
<u>High expresser</u>				
EM123	0.09	0.21	0.26	
EM67	0.12	0.19	0.29	
EM33	0.05	0.14	0.22	
<u>Medium expresser</u>				
EM112	0.05	0.09	0.20	
EM106	0.04	0.14	0.18	
EM97	0.12	0.11	0.15	
EM63	0.11	0.07	0.18	
EM52	0.07	0.04	0.15	
EM38	0.05	0.12	0.15	
<u>Low expresser</u>				
EM35	0.07	0.06	0.10	
<u>Very low expresser</u>				
EM129	0.04	0.05	0.08	

B. Agronomic Performance of Sugarcane Single Promoter BvLz Transgenic Lines

To assess whether sugarcane BvLz expressing lines incurred any growth penalty, the height of leaves and stalks, and the number of tillers were measured every two weeks for a period of three months.

Differences in agronomic performance of the sugarcane BvLz transgenic lines were independent of BvLz accumulation. There was no observable penalty in leaf height, stalk height and number of tillers for the BvLz transgenic lines. The growth pattern of the BvLz highly expressing lines, such as EM116 and EM123, was not affected.

Sprouting, however, was affected only for the first week of planting in some of the BvLz highly expressing lines such as EM116, EM123, EM112, EM114 and EM96. Medium BvLz expressers such as EM108, EM38 and EM33, as well as low BvLz expressers such as EM35 did not even sprout during the first week. BvLz transgenic lines were noted to sprout better during the second week of planting, with the exception of the two high expressers EM112 and 114, and the low expresser

EM35 (FIG. 3D). However, all BvLz expressing lines were able to fully sprout during the third week of planting (data not shown).

To investigate whether photosynthesis was limiting in the sugarcane BvLz transgenic lines, the level of three key photosynthetic enzymes, ribulose-1,5-biphosphate carboxylase-oxygenase (Rubisco, large subunit or RbcL), phosphoenolpyruvate carboxylase (PEPC) and pyruvate orthophosphate dikinase (PPDK), was analyzed in leaves by western blot. Total soluble protein (40 µg) from leaf extract was analyzed, using polyclonal anti-RbcL, anti-PEPC, or anti-PPDK antibody. western blots were scanned, and net intensity of RbcL, PPDK and PPDK bands was recorded.

The level of the three major photosynthetic enzymes was not affected by the BvLz expression level of the transgenic lines. The high, medium and low BvLz expressing lines displayed a good level of RbcL, PEPC and PPDK, which is comparable to that of the non-transformed plants. Net intensities of the scanned bands of RbcL, PEPC and PPDK were high in most of the BvLz transgenic lines. Each of the photosynthetic enzyme intensity level correlated very well with the BvLz expression level of the different lines.

Example 20

Multi-Promoter Expression of Bovine Stomach Lysozyme

The expression of a particular heterologous gene/transgene and subsequent production of its protein in plant cells are influenced by several factors. These include:

(1) Transcriptional factors such as transgene copy number, and promoter activity. Promoters, whether they are constitutive, tissue-specific (stem-specific in the case of sugarcane) or inducible, play a crucial role in controlling the production of heterologous proteins at a particular growth and developmental stage, or in a specific tissue. Two promoters, pSPRP and pSEF1 α , that constitutively express in sugarcane were isolated in our laboratory; these are from a sugarcane proline rich protein (SPRP) and an elongation factor 1 α (SEF1 α). Two stem-expressed and stress-inducible promoters, pJAS and pOMT, were also isolated; these are from a sugarcane jasmonate-inducible protein (or dirigent protein) (JAS) and an o-methyltransferase (OMT). The pSPRP, pSEF1 α , and pJAS were used together with the strong constitutive promoter pUbI from maize ubiquitin (with no heat shock element) (pMUbi), to drive the expression of the BvLz gene, either as a single or triple promoter combination.

(2) Post-transcriptional factors including mRNA splicing, mRNA stability, and translation.

- a. Untranslated regions for enhancement of translation, such as the 5' and 3' untranslated regions (UTR) of viruses infecting monocots were fused to the BvLz gene. These include the 5' and 3' UTRs of Sorghum mosaic virus (SrMV).
- b. Suppressors of post-transcriptional gene silencing (PTGS) were used in co-transformation with the BvLz construct. These include the P1/HC-Pro protein isolated from Sorghum mosaic virus, and the CTV P23 protein isolated from Citrus tristeza virus.

(3) Translational and Post-translational factors such as codon usage, protein stability, modification, trafficking and final compartmentalization.

A. Assembly of New Genetic Constructs

Several genetic constructs were assembled, using a combination of different constitutive and stem-regulated/inducible promoters to drive the expression of the BvLzm gene

(BvLz synthesized following the codon usage of maize) or the BvLzsc gene (BvLz synthesized following the codon usage specific to sugarcane). Untranslated regions of SrMV were also fused to BvLz gene to enhance its translation. Suppressors of gene silencing were also co-introduced into sugarcane with the BvLz gene to reduce its silencing.

B. Transformation of Sugarcane

Biostatic transformation: The new genetic constructs were introduced into sugarcane biolistically (direct gene transfer via microprojectile bombardment). Although the method of introducing DNA into cells by physical means (i.e. micro-projectile bombardment) has revolutionized the field of genetic transformation of crop plants, considerable variation may be seen in stability, integration and expression of the introduced transgene.

Agrobacterium-mediated transformation: This type of transformation exploits the nature of *Agrobacterium tumefaciens* to deliver a discrete segment of DNA into the recipient genome. New *Agrobacterium*-mediated transformations of sugarcane were initiated in our laboratory using a binary vector containing the BvLz gene under the control of the maize ubiquitin 1 constitutive promoter.

Target plant tissue transformed: Sugarcane callus and leaf rolls are being used in our regular transformation experiments. The transformation of leaf rolls followed by direct embryogenic regeneration to produce transgenic plants, has demonstrated an improvement on the current method of callus transformation. Plant regeneration through embryogenic callus cultures is labor-intensive, time-consuming, and has increased chances of somaclonal variation.

Sugarcane varieties: The commercial variety of sugarcane, CP72-1210, as well as other commercial varieties, such as TCP87-3388, TCP89-3505 and TCP99-4454, are being used for transformation with the new BvLz constructs. Over 3,000 new lines were generated and screened for expression levels that were higher than the best expressers of Example 19. Table 14 summarizes the different new BvLz constructs used for the new sugarcane transformations.

TABLE 14

BvLz constructs used for biostatic transformation of sugarcane.			
	Genetic construct	Variety	Target tissue
			No. of shoots
<u>Single promoter</u>			
45	1. pMUbi BvLzm	CP72-1210	Callus
50	2. pMUbi BvLzm/ pUbI P1HcPro	CP72-1210	Callus
55	3. pMUbi BvLzm SrMV 3'	TCP89-3505	Callus
60	4. pMUbi BvLzm/ pMCG ds SGS2	TCP87-3388	Callus
65	5. pMUbi BvLzm/ pMUbi CTVP23	TCP87-3388	Callus
	6. pSPRP BvLzm SrMV 3'	TCP89-3505	Callus
	7. pSEF1 α BvLzm SrMV 3'	TCP89-3505	Callus
<u>Triple promoter</u>			
65	1. pSPRP BvLzm SrMV 3'/ pSEF1 α BvLzm SrMV 3'/ pMUbi 5' SrMV BvLzsc SrMV 3'	CP72-1210	Callus
70	2. pSPRP (no 5'UTR) BvLzsc 5'/ SrMV 3'/ pSEF1 α BvLzm SrMV 3'/ pMUbi BvLzm SrMV 3'	CP72-1210	Callus
75	3. pSPRP BvLzm SrMV 3'/ pSEF1 α BvLzm SrMV 3'/ pJAS BvLzm SrMV 3'	CP72-1210	Callus
		TCP89-3505	Callus

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TABLE 14-continued

Genetic construct	Variety	Target tissue	No. of shoots
6. pSPRP BvLzm SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pJAS BvLzm	TCP87-3388	Leaf roll	1
7. pSPRP (no 5'UTR) BvLzsc 5' SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pJAS BvLzm	TCP87-3388	Leaf roll	1

Of the BvLz transgenic plants that were generated and further analyzed for their BvLz expression level, 23 displayed better expression levels than the best expressers of Example 19. These include pSPU (16 plants), pSPnU (1 plant), and pSPJ (6 plants) plants that are transgenic for BvLz under the control of a triple promoter (three promoters, each driving the expression of a separate copy (e.g., identical and/or substantially identical) of a single transgene (BvLz) in the same plant).

pSPU refers to plants that are transgenic for the BvLz gene whose expression is driven by the three constitutive promoters: pSEF1α (promoter for a sugarcane elongation factor 1α), pSPRP (promoter for a sugarcane proline rich protein) and pMUbi (promoter for maize ubiquitin 1). Plants were transformed with three genetic constructs: pSEF1α BvLzm SrMV 3', pSPRP BvLzm SrMV 3', and pMUbi 5' SrMV BvLzsc SrMV 3'. BvLzm refers to a synthetic BvLz gene with codons optimized for maize and BvLzsc refers to a synthetic BvLz gene with codons optimized for sugarcane. 5' and 3' SrMV refer to the 5' and 3' untranslated regions (UTR) of *Sorghum* mosaic virus (see Table 14).

pSPnU refers to plants that are transgenic for the BvLz gene whose expression is driven by the three constitutive promoters: pSEF1α, pSPRP with no 5'UTR, and pMUbi. Plants were transformed with three genetic constructs: pSEF1α BvLzm SrMV 3', pPRP (no 5'UTR) 5' SrMV BvLzsc SrMV 3', and pUbi BvLzm SrMV 3' (see Table 14).

pSPJ refers to plants that are transgenic for the BvLz gene whose expression is driven by two constitutive promoters, pSEF1α and pPRP, and one stem-regulated promoter, pJAS (promoter for jasmonate-inducible protein or dirigent protein). Plants were transformed with three genetic constructs: pSEF1α BvLzm SrMV 3', pSPRP BvLzm SrMV 3', and pJAS BvLzm SrMV 3' (see Table 14).

Genomic DNA gel blot analysis was used to determine the number of copies and integration events of the BvLz transgene in the newly generated pSPU and pSPJ plants. Multiple bands were detected in the genome of these plants, reflecting the insertion of multiple copies of BvLz transgene. The DNA gel blot analysis identified a total of 5 independent pSPU lines with 16 plants, one independent pSPnU with one plant, and 2 independent pSPJ lines with 6 plants.

The BvLz level of the pSPU and pSPJ plants generated was first evaluated in leaves by western blot analysis. Total soluble protein (40 µg) from leaves of 26 transgenic sugarcane plants was analyzed, using a polyclonal anti-BvLz antibody. Most of the plants displayed a higher BvLz expression level than the single promoter BvLz plants.

The BvLz expression level detected by western analysis in leaves of the pSPU and pSPJ plants was supported by the BvLz enzymatic activity in the stalks (Table 15). BvLz activity was assayed in 0.105 to 0.345 kg of stalks that are about 7-month-old. pSPU32E, pPSU19A and pSPJ10A plants accumulated the highest amounts of BvLz levels in their

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stalks. In general, the newly generated BvLz transgenic lines showed a higher increase in their BvLz expression level than the Example 19 lines. The BvLz expression level was improved in the new lines by using a triple promoter to drive BvLz expression.

TABLE 15

BvLz Expression in Sugarcane Stalks.		
	Transgenic Plant	BvLz (mg bvlz/kg cane)
	<u>pSPJ</u>	
10	10A	0.56
	14A	0.171
	14C	0.312
	14E	0.156
	14F	0.46
	<u>pSPU</u>	
15	30A	0.52
	32A	2.2
	32C	4.2
	32E	3.0
	32D	4.7

In conclusion, the best expressing lines in this Example had as much as 4.7 mg of BvLz per kg of fresh weight as compared to the BvLz recovered from the stalks of the single promoter highly expressing plants of Example 19, which had 0.33 mg of BvLz/kg of stalk.

Example 21

Expression of Bovine Stomach Lysozyme: pJSU BvLzm Plants: pJSU Triple BvLzm Plants:

Plants that are transgenic for BvLzm whose expression is driven under the control of a triple promoter (three promoters driving the expression of BvLz in the same plant). Two constitutive promoters, pSEF1α (promoter for a sugarcane elongation factor 1α gene) and pMUbi (promoter for maize ubiquitin 1 gene) were used as well as one stem-regulated promoter, pJAS (promoter for the gene coding for jasmonate-inducible protein).

Leaf rolls of sugarcane variety TCP98-4454 were transformed biologically (direct gene transfer via microprojectile bombardment) with three genetic constructs:

pJAS BvLzm/
pSEF1α BvLzm SrMV 3'/
pMUbi (no hse) BvLzm SrMV 3'

The resulting plants were assayed for BvLz expression by western blot analysis to confirm expression and by Southern analysis to evaluate construct copy number. A minimum of 6 independent events were observed.

The BvLz level of the newly generated pJSU plants was first evaluated in sugarcane leaves by western blot analysis. Total soluble protein (40 µg) from leaves of 26 transgenic sugarcane plants was analyzed, using a polyclonal anti-BvLz antibody. One plant, pSPU 32E, was used as a positive control generated in Example 20. It is a highly expressing BvLz plant, where the BvLzm gene is under the control of three constitutive promoters, pSEF1α, pSPRP and pMUbi. Strong BvLz expression was observed in the leaves of all pJSU plants tested.

The BvLz accumulation level of the newly generated pJSU plants was also determined in stalks by ELISA. Table 16 shows the BvLz activity of 17 plants that were analyzed at a 7-8 month-growth stage.

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TABLE 16

Transgenic plant	BVLZ activity (mg/kg cane)
pJSU	
18	5.1
19	4.6
44	2.9
54	6.0
70	3.50
73	3.0
74	3.20
75	2.55
76	2.47
84	3.44
85	3.51
87	3.07
pSPU^a	
32C	2.10-3.04

^apSPU 32C plant is used as a positive control generated in Example 20. It is a highly expressing BvLz plant, where BvLzm is under the control of three constitutive promoters, pSEFI α , pSPRP and pUbi.

Southern blot analysis was used to determine the number of copies and integration events of the BvLz transgene in the newly generated pJSU plants. Genomic DNA (15 μ g) for twenty BvLz transgenic plants was digested with HindIII, and hybridized with full-length BvLz cDNA. Multiple bands of the BvLz transgene were detected in the genome of these plants, reflecting the insertion of multiple copies of BvLz. The banding pattern revealed the presence of 4 independent transformation events. Event 1 is represented by plants 23, 27, 30 and 42, event 2 by plant 22, event 3 by plants 24, 25, 26, 28, 52, 53 and 54, and event 4 by plant 29.

A total of 35 pJSU BvLz highly expressing plants were analyzed, and the highest BvLz activity level detected among the analyzed plants was 6.0 mg/kg of stalk (~1% TSP), as compared to an average of 2.4 mg/kg obtained from pSPU 32C (reference BvLz plant described in Example 20). Thus, there is a 2.5-fold increase in BvLz activity.

Example 22

Inducibility of Bovine Stomach Lysozyme Expression

The effect of defense-inducing/stress-regulated hormones on enhancing the BvLz level of sugarcane triple promoter pJSU BvLz expressing lines was evaluated. Specifically, plants were sprayed (or leaf rolls from the top of the stalk were incubated in vitro) with the stress-regulated hormones, jasmonic acid (JA) and salicylic acid (SA) that are known to induce the pUbi and pJAS promoters that drive the expression of BvLz in the existing triple promoter BvLz sugarcane lines. Total soluble protein was extracted from leaves of treated plants (or upper leaf rolls incubated in vitro), and its BvLz level was detected by western analysis and enzymatic assay.

Leaf rolls from pJSU BvLz 53 and 66 plants were incubated on MS (Murashige and Skoog) media supplemented with SA (5 mM) or JA (25 mM) for 0, 24 and 40 h. Total soluble protein was extracted from each treatment and its BvLz expression and activity levels were determined. The BvLz activity level of the triple promoter pJSU BvLz expressing lines was induced by the stress-regulated hormones, SA and JA. BvLz activity was maximally induced by SA at 40 h

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(2.4-fold for pJSU 53 and 2.0-fold for pJSU 66), and by JA at 24 h (1.5-fold for pJSU 53 and 2.7-fold for pJSU 66) and 40 h (2.6-fold for pJSU 53 and 3.9-fold for pJSU 66).

The effect of nitrogen fertilization on enhancing the photosynthetic rate and hence the biomass of the existing triple promoter pJSU and pSPU highest BvLz expressing lines was also assessed. Nitrogen (N) is an essential component of fertilization programs for the production of high quality crops with increased protein content. The triple promoter BvLz expressing plants were started from seed sets till maturity, and fertilized with a low (1.43 g of Peters' solution 20-20-20 per plant; twice per week) and a high (2.38 g of Peters' solution 20-20-20 per plant; twice per week) nitrogen level for a period of 6 months. Stalks from the BvLz plants were collected at 2 and 6 months following fertilization, shredded, and their total BvLz yield was determined by the BioSeparation Laboratory.

Photosynthetic activity of the fertilized triple promoter BvLz expressing plants was also determined by measuring the chlorophyll fluorescence, which detects the photochemical efficiency of photosystem II and leaf greenness. The uptake of essential macronutrients by the triple promoter BvLz expressing plants was also determined following fertilization (Table 17).

Nitrogen fertilization is important in increasing the biomass and BvLz yield of the sugarcane triple promoter BvLz expressing lines. For instance, for the pSPU 32C line (CP-72-1210 variety), there is a 6.3-fold and a 2.3-fold increase in the stalk biomass and BvLz yield with high fertilization as compared to low fertilization at the 2 month- and 6 month-growth stage, respectively. Furthermore, there is a 7.5-fold and a 2.0-fold increase in the stalk biomass and BvLz yield of the pJSU 19 line (TCP98-4454 variety) with high fertilization as compared to low fertilization at the 2 month- and 6 month-growth stage, respectively.

Chlorophyll fluorescence of the triple promoter BvLz expressing plants is enhanced by fertilization. The fold-increase in chlorophyll fluorescence of these plants is in the range of 1.1-1.5 with high fertilization as compared to low fertilization.

TABLE 17

Nitrogen/Macronutrient Uptake of Triple-Promoter BvLz Plants.				
Sample ID	Mineral Uptake			50
	Nitrogen (%)	Phosphorus (ppm)	Magnesium (ppm)	
pSPU 32C	0.78	1539	1247	55
	1.50	3136	2999	
pJSU 18	1.06	2438	1181	60
	1.84	4053	2059	
pJSU 19	1.26	2178	1274	65
	1.66	3559	2113	
pJSU 54	1.51	2189	2236	
	1.87	2861	2704	

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As shown in Table 1, a higher uptake of nitrogen, phosphorus and magnesium by the leaves of the triple promoter BvLz expressing lines was recorded following high nitrogen fertilization (HF) as compared to low nitrogen fertilization (LF). It is evident that, for increasing cane and BvLz yields, the uptake of nitrogen as well as of that of phosphorus and magnesium is essential as all of them are closely interlinked with one another.

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Example 23

Multi-Promoter Expression of Bovine Stomach Lysozyme

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Quadruple promoter driving BvLz expression: Genetic constructs of BvLz driven separately by four different promoters (quadruple promoter system) were introduced biolistically into each of several sugarcane varieties (Table 18). Several seedlings have been tested to confirm BvLz activity.

TABLE 18

BvLz Constructs Used for Sugarcane Quad Promoter Plants.					
Genetic construct	Variety	Target tissue	Age of tissue (day)	No. of DNA shots	No. of seedlings
Quadruple promoter driving BvLz expression					
1. pSPRP BvLzm 3'SrMV 35ST/ pSEF1α BvLzm 35ST NOST/ pSCBV BvLzm 35ST NOST/ pMUbi 5'SrMV BvLzsc 3'SrMV 35ST/ pUbi BAR	CP72-1210	Leaf roll	18	57 (4 µg DNA per shot)	29
2. pSPRP BvLzm 35ST NOST/ pSEF1α BvLzm 35ST NOST/ pSCBV BvLzm 35ST NOST/ pJAS BvLzm NOST/ pUbi BAR	CP72-1210	Leaf roll	42	34 (4 µg DNA per shot)	1
	TCP98-4454	Leaf roll	10	34 (4 µg DNA per shot)	1
	TCP98-4454	Leaf roll	18	40 (3 µg DNA per shot)	
	TCP98-4454	Leaf roll	9	44 (2 µg DNA per shot)	
	CP84-1198	Leaf roll	17	47 (2 µg DNA per shot)	
3. pSPRP BvLzm 35ST NOST/ pSCBV BvLzm 35ST NOST/ pJAS BvLzm NOST/ pMUbi BvLzm 3'SrMV/ pUbi BAR	TCP98-4454	Leaf roll	17	34 (4 µg DNA per shot)	
4. pSPRP BvLzm 35ST NOST/ pSEF1α BvLzm 35ST NOST/ pJAS BvLzm NOST/ pMUbi BvLzm 3'SrMV 35ST/ pUbi BAR	TCP98-4454	Leaf roll	22	40 (2 µg DNA per shot)	
	TCP98-4454	Leaf roll	26	40 (1 µg DNA per shot)	
5. pSPRP BvLzm 35ST NOST/ pSEF1α BvLzm 35ST NOST/ pSCBV BvLzm 35ST NOST/ pMUbi BvLzm 35ST NOST/ pUbi BAR	CP84-1198	Leaf roll	17	47 (2 µg DNA per shot)	
		Callus	63	39 (2 µg DNA per shot)	
	TCP98-4454	Leaf roll	13	60 (2 µg DNA per shot)	3
6. pPRP BvLzm 3'SrMV 35ST/ pSEF1α BvLzm 3'SrMV 35ST/ pMUbi BvLzm 3'SrMV 35ST/ pJAS BvLzm 3'SrMV NOST/ pUbi BAR	CP84-1198	Leaf roll	32	48 shots (2 µg DNA per shot)	
	TCP98-4454	Leaf roll	31	50 shots (2 µg DNA per shot)	3
		Callus	44	40 shots (2 µg DNA per shot)	

TABLE 18-continued

BvLz Constructs Used for Sugarcane Quad Promoter Plants.					
Genetic construct	Variety	Target tissue	Age of tissue (day)	No. of DNA shots	No. of seedlings
BvLz stacking into existing BvLz transgenic events/lines					
1. pJSU lines (triple promoter BvLz lines) with pPRP BvLzm and pSCBV BvLzm, and pUBi NPTII (for selection of transformants)	TCP98-4454 transgenic for BvLz	Leaf roll			
pJSU 236/pPRP BvLzm/pSCBV BvLzm			15	21	
pJSU 242/pPRP BvLzm/pSCBV BvLzm			15	32	
pJSU 247/pPRP BvLzm/pSCBV BvLzm			15	22	
pJSU 248/pPRP BvLzm/pSCBV BvLzm			15	30	
pJSU 250/pPRP BvLzm/pSCBV BvLzm			15	16	
pJSU 258/pPRP BvLzm/pSCBV BvLzm			15	8	
pJSU 259/pPRP BvLzm/pSCBV BvLzm			15	12	
pJSU 76/pPRP BvLzm/pSCBV BvLzm			36	27	
pJSU 177/pPRP BvLzm/pSCBV BvLzm			36	7	
pJSU 191/pPRP BvLzm/pSCBV BvLzm			36	25	
pJSU 197/pPRP BvLzm/pSCBV BvLzm			36	16	

In the present work, explants (leaf roll or callus) were transformed biolistically with four genetic constructs, each containing the bovine lysozyme (BvLz) gene driven by a different promoter.

Promoters that drive gene expression: pSEF1 α is a constitutive promoter isolated from a sugarcane elongation factor 1 α gene, pPRP is a constitutive promoter isolated from a gene coding for a sugarcane proline rich protein, pSCBV is a stem-expressed promoter isolated from Sugarcane bacilliform virus, pJAS is a stem-expressed promoter isolated from a sugarcane jasmonate-inducible/diirigent gene, and pMUbI is a the commonly used constitutive promoter for monocots and is derived form the maize ubiquitin 1 gene.

Genes expressed: BvLzm refers to synthetic BvLz with codons optimized for maize, and BvLzsc to synthetic BvLz with codons optimized for sugarcane.

Terminators of transcription: 35ST refers to the 35S terminator derived from the 35S RNA of Cauliflower mosaic virus. NOST refers to the NOS terminator derived from the nopaline synthase gene (from the *Agrobacterium tumefaciens* Ti plasmid). It is a terminator of transcription. 35ST NOST refers to a double terminator that consists of 35ST and NOST. Recent research has proven that the fusion of a 35ST NOST double terminator to a transgene at its 3' end may have a significant effect on decreasing gene silencing and enhancing transgene expression.

Enhancers of translation: 5' and 3' SrMV refer to the 5' and 3' untranslated regions (UTR) of Sorghum mosaic virus protein. They are used for enhancement of translation.

Selectable markers for plant transformation: BAR refers to the bar gene, which is one of the most commonly used selectable markers for plant transformation. It codes for phosphinothrin acetyl transferase enzyme that detoxifies Bialaphos or phosphinothrin, the active ingredient of herbicides such as Basta and Finale. Selection for BAR gene activity can be achieved easily and at low cost by spraying plants with the herbicide. NPTII refers to the NPTII gene, which is one of the most widely used selectable markers for plant transformation. It codes for neomycin phosphotransferase (or aminoglycoside 3'-phosphotransferase) enzyme, which inactivates by phosphorylation a range of aminoglycoside antibiotics such as gentamicin.

pJSU refers to sugarcane plants that are transgenic for the BvLz gene whose expression is driven by the two constitutive promoters, pSEF1 α and pMUbI, and the stem-regulated promoter, pJAS. Plants were transformed with three genetic constructs: pJAS BvLzm, pSEF1 α BvLzm SrMV 3', and pMUbI BvLzm SrMV 3'.

Example 24

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Multi-Promoter Expression of Bovine Stomach Lysozyme

Several genetic constructs of BvLz were introduced biolistically into sweet and grain sorghum (Table 19). Several seedlings have been tested to confirm BvLz activity.

TABLE 19

BvLz Constructs Used for <i>Sorghum</i> Quad Promoter Plants.					
Genetic construct	Variety	Target tissue	Age of tissue (day)	No. of DNA shots	No. of seedlings
Single promoter driving BvLz expression					
1. pMUbI BvLzm 3'SrMV 35ST/ pUbI BAR (Selection of transformants on Bialaphos)	Rio-Sweet (Sweet <i>sorghum</i>) TX-430 (Grain <i>sorghum</i>)	Callus Callus	60 24	38 (2 μ g DNA per shot) 28 (2 μ g DNA per shot)	
2. pMUbI BvLzm 3'SrMV 35ST/ pUbI PMI (Selection of transformants on mannose)	Rio-Sweet (Sweet <i>sorghum</i>) TX-430 (Grain <i>sorghum</i>)	Callus Callus	96 76	28 (2 μ g DNA per shot) 28 (2 μ g DNA per shot)	

TABLE 19-continued

BvLz Constructs Used for <i>Sorghum</i> Quad Promoter Plants.					
Genetic construct	Variety	Target tissue	Age of tissue (day)	No. of DNA shots	No. of seedlings
Triple promoter driving BvLz expression					
1. pSPRP BvLzm 3'SrMV 35ST/ pSEF1α BvLzm 3'SrMV 35ST/ pMUb1 BvLzm 3'SrMV 35ST/ pUbi PMI	Ramada (Sweet sorghum)	Callus	11	15 (4 µg DNA per shot)	
	B-TX-623 (Grain sorghum)	Callus	134	30 (4 µg DNA per shot)	
Quadruple promoter driving BvLz expression					
1. pSPRP BvLzm 3'SrMV 35ST/ pSEF1α BvLzm 3'SrMV 35ST/ pMUb1 BvLzm 3'SrMV 35ST/ pJAS BvLzm 3'SrMV NOST/ pUbi PMI	TX-430 (Grain sorghum)	Callus	88	20 (2 µg DNA per shot)	

PMI refers to the *E. coli* phosphomannose isomerase gene (isolated by Getu Beyene for the present work on *sorghum* transformation). PMI is a versatile selectable marker for plant transformation. It is very efficient in sorghum transformationS.

Example 25

Harvesting Protein from Transgenic Plants

The potential for an example expression system, triple promoter plants, to serve as a biofactory was evaluated by purifying the expressed transgenic protein. The protocol was adapted from U.S. Application no. 2007/0130655. The transgenic sugarcane is first shredded and crushed once without maceration water and then twice more with 20% by weight water in a pilot scale Squire mill. Essentially, the cane stalk is shredded and then pressed through 3 rollers on the Squire mill with 3,000 pounds per square inch. This produces a mixture of about 70% water, 15% sucrose, and 10% fibre. The remaining 5% of the mixture consists of proteins and other sugars, salts and organic molecules. The juice containing the high value protein (BvLz) is then pumped to a purification skid and filtered through a set of vibrating (self cleaning) screens and enters a tank. This step removes the fibre. The first screen is 150 microns, and the second is 100 microns. The pH of the juice is adjusted to 5.2 and it is supplemented with 1 mM EDTA and 0.1% sodium sulphite to prevent oxidation and the formation of phenolics. From the tank the juice is permeated through a 0.2 micron cross flow filtration membrane. This step removes all the insoluble solids and high molecular weight soluble solids such as bacteria, starches and dextrans. The permeate, which contains sugar and the high value protein, enters a second tank and the retentate in the first tank is discarded. From the second tank, the juice is permeated through a 0.05 micron membrane. This step removes soluble molecules with a molecular weight greater than 150,000 kd. High value proteins with a molecular weight greater than 150,000 kd would be retained in the second tank, and could be further purified with the HPLC steps described below. The second permeate, which contains sugar and the high value proteins smaller than 150,000 W (BvLz in this example) enters a third tank and the retentate in the second tank is discarded. At this point we have a relatively clean sample

²⁵ from which all high molecular weight material has been removed, i.e., bacteria, starch, dextrans, and proteins with high molecular weights.

From the third tank, the sample is concentrated (~30 fold) ³⁰ on a 3 kDa membrane and then further purified by 2 cycles of high pressure liquid chromatography (HPLC). The first cycle uses SP Sepharose® ion exchange resin, while the second cycle uses a hydrophobic interaction resin. Preliminary runs produced protein.

³⁵ We have identified low molecular weight off membranes that can be used to concentrate the sample in the third tank. The water, sugars and other small molecules will flow through the membranes, but the high value protein will be ⁴⁰ concentrated in the third tank. This will greatly improve the performance of the HPLC steps. Further modifications using different initial extraction conditions, different ion exchange resins/membranes, affinity resins and HPLC columns can be used to enhance performance. Results for the first and second ⁴⁵ runs are shown in Tables 21 and 22, respectively.

TABLE 21

Isolated BvLz from Transgenic Plants					
Sample	Volume (L)	Total mg	Overall Yield (%)	Purification Yield (%)	
Starting Juice	212.0	1.6	339	100	
0.2 µm Permeate	181.7	0.5	96	28	
3 kDa Conc	4.73	9.1	43	13	
0.2 µm Filtrate	4.66	8.6	40	12	
SP Load*	4.01	8.6	35	10	100
HIC Pool	0.050	239	12	4	35
HIC Pool after dialysis**	0.082	172	14	4	41

*Represents portion of filtrate that was loaded on SP column

**Obtained after ammonium sulfate removal by activity as well as optical density at 280 nm before freeze-drying.

TABLE 22

Extracted BvLz from Triple Promoter BvLz Transgenic Plants						
Sample	Description	Activity		ELISA		5
		Volume (L)	mg	Recovery (%)	mg	
T1 S	Start (9 bins crushed)	124.9	92.4	100.0	66.8	100.0
T1 F	0.2 um Retentate	18.9	17.9	19.4	14.8	22.2
T3 Start	0.2 um Permeate	113.6	67.1	72.6	42.8	64.1
T4 Sample	3 kDa Permeate	109.8	1.8	1.9	2.7	4.1

TABLE 22-continued

Extracted BvLz from Triple Promoter BvLz Transgenic Plants						
Sample	Description	Activity		ELISA		5
		3 kDa Concentrate	6.6	mg	Recovery (%)	
Concentrate	Concentrate	11		11.9	24.6	36.9

Thus, it was possible to harvest up to 14 mg of BvLz to 99% purity from about 500 pounds of the triple promoter plants.

Example 26

Multi-Promoter Expression of Bovine Stomach Lysozyme

As shown in Tables 23 and 24, additional constructs with one or more promoters were prepared and used to transform sugarcane varieties.

TABLE 23

Transgenic BvLz Plants with One or More Promoters.				
Genetic construct	Variety	Target tissue	No. of plants	
<u>Single promoter driving BvLz expression</u>				
1. pMUBi (no hse) BvLzm/pUbi BAR	CP72-1210	Callus	74	
	CP72-1210	Callus		
	CP72-1210	Callus #	20	
2. pMUBi (no hse) BvLzm SrMV 3'/pUbi BAR	CP72-1210	Leaf roll	8	
	TCP89-3505	Callus	109	
3. pMUBi (no hse) 5' SrMV BvLzsc SrMV 3'/pUbi BAR	CP72-1210	Leaf roll	9	
4. pMUBi (no hse) BvLzm 35ST NOST/pUbi BAR	CP72-1210	Leaf roll	10	
	TCP89-4454	Leaf roll	28	
5. pSPRP BvLzm SrMV 3'/pUbi BAR	TCP89-3505	Callus	94	(Low expressers)
6. pSEF1α BvLzm SrMV 3'/pUbi BAR	TCP89-3505	Callus	1	
7. pSCBV BvLzm 35ST NOST/pUbi BAR	CP72-1210	Leaf roll		Green shoots
8. pSEF1α BvLzm 35ST NOST/pUbiBAR	CP72-1210	Leaf roll		1
<u>Single promoter driving BvLz expression in presence of a suppressor of gene silencing or programmed cell death</u>				
1. pMUBi (no hse) BvLzm/pMUBi (no hse) P1HcPro/pUbi BAR	CP72-1210	Callus	18	
	CP72-1210	Callus	76	
2. pMUBi (no hse) BvLzm SrMV 3'/pUbi (no hse) P1HcPro/pUbi BAR	CP72-1210	Callus	12	
3. pMUBi (no hse) 5' SrMV BvLzsc SrMV 3'/pMUBi (no hse) P1HcPro/pUbi BAR	CP72-1210	Callus	31	
	CP72-1210	Leaf roll	9	
4. pJAS BvLzm/pMUBi (no hse) P1HcPro/pUbi BAR	CP72-1210	Callus	25	
<u>Single promoter driving BvLz expression in presence of a suppressor of gene silencing or programmed cell death</u>				
5. pSPRP BvLzm SrMV 3'/pUbi (no hse) P1HcPro/pUbi BAR	CP72-1210	Callus	61	
6. pSPRP 5' SrMV BvLzsc SrMV 3'/pUbi (no hse) P1HcPro/pUbi BAR	CP72-1210	Callus	209	
7. pSEF1α BvLzm SrMV 3'/pUbi (no hse) P1HcPro/pUbi BAR	TCP89-3505	Callus	0	

TABLE 23-continued

Transgenic BvLz Plants with One or More Promoters.			
Genetic construct	Variety	Target tissue	No. of plants
8. pMUBi (no hse) BvLzm/ pMUBi (no hse) CTVP20/ pUbi BAR	TCP98-4454 CP72-1210	Callus Callus	3
9. pMUBi (no hse) BvLzm SrMV 3'/ pUbi (no hse) CTVP20/ pUbi BAR	CP72-1210	Leaf roll	11
10. pMUBi (no hse) 5' SrMV BvLzsc SrMV 3'/ pMUBi (no hse) CTVP20/ pUbi BAR	CP72-1210	Callus	21
11. pMUBi (no hse) BvLzm/ pMUBi (no hse) CTVP23/ pUbi BAR	TCP87-3388	Callus	0
12. pMUBi (no hse) BvLzm SrMV 3'/ pMUBi (no hse) CTVP23/ pUbi BAR	TCP01-4543 CP72-1210	Callus Callus	0 0
13. pMUBi (no hse) 5' SrMV BvLzsc SrMV 3'/ pMUBi (no hse) CTVP23/ pUbi BAR	TCP01-4543 CP72-1210	Callus Callus	0 0
14. pJAS BvLzsc/ pMUBi (no hse) CTVP23/ pUbi BAR	TCP01-4543	Callus	1
15. pJAS BVLZm SrMV 3'/ pMUBi (no hse) CTVP23/ pUbi BAR	CP72-1210	Callus	5
16. pSPRP BvLzm SrMV 3'/ pMUBi (no hse) CTVP23/ pUbi BAR Single promoter driving BvLz expression in presence of a suppressor of gene silencing or programmed cell death	CP72-1210	Callus	0
17. pSPRP 5' SrMV BvLzsc SrMV 3'/ pUbi (no hse) CTVP23/ pUbi BAR	CP72-1210	Callus	117
18. pJAS BvLzm SrMV 3'/ pMUBi (no hse) P1HcPro/ pMUBi (no hse) CTVP23/ pUbi BAR	CP72-1210	Callus	0
19. pSPRP BVLZm SrMV 3'/ pMUBi (no hse) P1HcPro/ pMUBi (no hse) CTVP23/ pUbi BAR	CP72-1210	Callus	62
20. pSEF1α BvLzm SrMV 3'/ pMUBi (no hse) P1HcPro/ pMUBi (no hse) CTVP23/ pUbi BAR	CP72-1210	Callus	1
21. pMUBi (no hse) BvLzm/ pMCG ds SGS2/ pUbi BAR	CP72-1210 TCP87-3388	Callus Callus	39 45
22. pUbi (no hse) BvLzm SrMV 3'/ pMCG ds SGS2/ pUbi BAR	CP72-1210 CP72-1210	Callus Leaf roll Callus	48 12
23. pUbi (no hse) 5' SrMV BvLzsc SrMV 3'/ pMCG ds SGS2/ pUbi BAR	CP72-1210	Leaf roll	66
24. pMUBi (no hse) BvLzm/ pMCG ds SGS3/ pUbi BAR	CP72-1210	Callus	223
pUbi (no hse) BvLzm SrMV 3'/ pMCG ds SGS3/ pUbi BAR	CP72-1210	Callus	148
26. pMUBi (no hse) 5' SrMV BvLzsc SrMV 3'/ pMCG ds SGS3/ pUbi BAR	CP72-1210	Callus	54
27. pMUBi (no hse) BzLzm SrMV 3'/ pMCG ds SGS2/ pMCG ds SGS3/ pUbi BAR	CP72-1210	Leaf roll	15
28. pMUBi (no hse) BvLzm SrMV 3'/ pMCG ds 14-3-3/ pUbi BAR	CP72-1210	Leaf roll	74

TABLE 23-continued

Transgenic BvLz Plants with One or More Promoters.			
Genetic construct	Variety	Target tissue	No. of plants
29. pMUBi (no hse) 5' SrMV BvLzsc SrMV 3'/ pMCG ds 14-3-3/ pUbi BAR	CP72-1210 TCP01-4543	Callus Callus	2 0
30. pMUBi (no hse) BvLzm/ pUbi (no hse) anti-RNaseH/ pUbi BAR	CP72-1210	Leaf roll Callus	12 4
31. pJAS BvLzm/ pMUBi (no hse) anti-RNaseH/ pUbi BAR Single promoter driving BvLz expression in presence of a suppressor of gene silencing or programmed cell death	CP72-1210	Callus	0
32. pMUBi (no hse) BvLzm SrMV 3'/ pPTN254 Bcl-xl/ pUbi BAR	CP72-1210	Leaf roll	2
33. pJAS BvLzm SrMV 3'/ pPTN254 Bcl-xl/ pUbi BAR	CP72-1210	Leaf roll	82
34. pSPRP BvLzm SrMV 3'/ pPTN254 Bcl-xl/ pUbi BAR	CP72-1210	Leaf roll	141
35. pSEF1α BvLzm SrMV 3'/ pPTN254 Bcl-xl/ pUbi BAR Double promoter driving BvLz expression	CP72-1210	Leaf roll	1
1. pJAS BvLzm SrMv 3'/ pMUBi (no hse) 5' SrMv BvLzsc SrMV 3'/ pUbi BAR	TCP01-4543	Callus	0
2. pSPRP (no 5'UTR) 5' SrMV BvLzsc SrMV 3'/ pMUBi (no hse) BvLzm SrMV 3'/ pUbi BAR	CP72-1210	Callus	239
3. pPRP (no 5'UTR) 5' SrMV BvLzsc SrMV 3'/ pJAS BvLzm SrMV 3'/ pUbi BAR Triple promoter driving BvLz expression	CP72-1210	Callus #	372
1. pSPRP BvLzm SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pMUBi (no hse) 5' SrMV BvLzsc SrMV 3'/ pUbi BAR	CP72-1210	Callus	16 are transgenic
2. pSPRP BvLzm SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pJAS BvLzm SrMV 3'/ pUbi BAR (Very low expressers)	CP72-1210	Callus	67
3. pSPRP (No 5'UTR) 5' SrMV BvLzsc SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pMUBi (no hse) BvLzm SrMV 3'/ pUbi BAR	CP72-1210 CP72-1210	Callus Leaf roll	43: one is transgenic 2
4. pSPRP BvLzm SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pJAS BvLzm SrMV 3'/ pUbi BAR	CP72-1210 CP72-1210	Callus Leaf roll	47: 6 are transgenic 249
5. pSPRP (No 5'UTR) BvLzsc 5' SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pJAS BvLzm SrMV 3'/ pUbi BAR	CP72-1210	Callus	3
6. pSPRP (No 5'UTR) BvLzsc 5' SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pJAS BvLzm/ pUbi BAR Triple promoter driving BvLz expression	TCP87-3388	Leaf roll	1
1. pSPRP BvLzm SrMV 3'/ pMUBi (no hse) BvLzm SrMV 3'/ pJAS BvLzm/ pUbi BAR	CP72-1210	Callus and Leaf roll	31 277
10. pJAS BvLzm/ pSEF BvLzm SrMV 3'/ pMUBi (no hse) BvLzm SrMV 3'/pUbi BAR	TCP98-4454	Leaf roll	365 plants: 286 are transgenic; 64 remain to be tested
11. pJAS BvLzm/ pSEF BvLzm SrMV 3'/ pMUBi (no hse) 5' SrMV BvLzm SrMV 3'/ pUbi BAR	CP72-1210	Leaf roll	96

TABLE 23-continued

Transgenic BvLz Plants with One or More Promoters.			
Genetic construct	Variety	Target tissue	No. of plants
12. pSCBV BvLzm 35ST NOST/ pUbi (no hse) BvLzm 35ST NOST/ pJAS BvLzm/ pUbiBAR	CP72-1210 TCP98-4454	Lead roll Leaf roll	Green shoots Green shoots
13. pSCBV BvLzm 35ST NOST/ pSEF BvLzm SrMV 3'/ pJAS BvLzm/ pUbiBAR	CP72-1210	Leaf roll	Green shoots
14. pSCBV BvLzm 35ST NOST/ pSEF BvLzm SrMV 3'/ pPRP BvLzm SrMV 3'/ pUbiBAR	CP72-1210	Leaf roll	Green shoots
<u>Quadruple promoter driving BvLz expression</u>			
1. pSPRP (no 5' UTR) 5' SrMV BvLzsc SrMV 3'/ pSEF1α BvLzm SrMV 3'/ pJAS BvLzm SrMV 3'/ pSCBV BvLzm 35ST NOST/ pUbi BAR	CP72-1210 TCP98-4454	Leaf roll Leaf roll	3 2
<u>Double transformant for BvLz</u>			
1. pMUBi (no hse) BvLzm/pUbi BAR (EM116 plant) and pJAS BvLzm/pUbi NPTII <i>Agrobacterium</i> -mediated delivery of BvLz	EM116 in CP72-1210	Leaf roll	153: 52 tested positive
1. pBIN161 BvLzm	CP72-1210 TCP87-3388 TCP98-4454	Callus Leaf roll Leaf roll	6 56 430
			52 tested positive

TABLE 24

Transgenic BvLz Plants with One or More Promoters.			
Genetic construct	Variety	Total # Plants Generated	Total # Independent Lines
Single promoter BvLz transgenic lines			
Pro _{MUBi(no hse)} :BvLz(m) 35ST	CP72-1210	67	30
Pro _{SPRP} :BvLz(m) 3'SrMV 35ST			
Pro _{SEF1α} :5'SrMv BvLz(sc) 3'SrMV 35ST			
Pro _{JAS} :BvLz(m) 35ST	CP72-1210	25	6
BvLz transgenic lines with 3 promoter stacks			
1. pSPU:BvLz line that contains: Pro _{SEF1α} :BvLz(m) 3'SrMV 35ST	CP72-1210	16	5
Pro _{SPRP} :BvLz(m) 3'SrMV 35ST			
Pro _{MUBi(no hse)(no 5'UTR)} :5'SrMv BvLz(sc) 3'SrMV 35ST			
2. pSP _n U:BvLz line that contains: Pro _{SEF1α} :BvLz(m) 3'SrMV 35ST	CP72-1210	1	1
Pro _{SPRP(no 5'UTR)} : 5'SrMV BvLz(sc) 3'SrMV 35ST			
Pro _{MUBi(no hse)} :BvLz(m) 3'SrMV 35ST			
3. pSPJ:BvLz line that contains: Pro _{SEF1α} :BvLz(m) 3'SrMV 35ST	CP72-1210	6	2
Pro _{SPRP} :BvLz(m) 3'SrMV 35ST			
Pro _{JAS} :BvLz(m) 3'SrMV 35ST			
4. pJSU:BvLz line that contains: Pro _{JAS} :BvLz(m) 35ST	CP98-4454	166	6
Pro _{SEF1α} :BvLz(m) 3'SrMV 35ST			
Pro _{MUBi(no hse)} :BvLz(m) 3'SrMV 35ST			
BvLz transgenic lines with 4 promoter stacks			
1. pJSPB:BvLz line that contains: Pro _{JAS} :BvLz(m) 35ST	CP72-1210 CP98-4454	1 3	1
Pro _{SEF1α} :BvLz(m) 35ST NOST			
Pro _{SPRP} :BvLz(m) 35ST NOST			
Pro _{SCBV21} :BvLz(m) 35ST NOST			

TABLE 24-continued

Transgenic BvLz Plants with One or More Promoters.			
Genetic construct	Variety	Total # Plants Generated	Total # Independent Lines
Single promoter BvLz transgenic lines			
2. pSPBU:BvLz line that contains: Pro _{SEF1α} :BvLz(m) 35ST NOST Pro _{SPRP} :BvLz(m) 35ST NOST Pro _{SCBV21} :BvLz(m) 35ST NOST Pro _{MUbi(no hse)} :BvLz(m) 35ST NOST	CP98-4454	14	2
3. pPSUJ:BvLz line that contains: Pro _{SPRP} :BvLz(m) 3'SrMV 35ST Pro _{SEF1α} :BvLz(m) 3'SrMV 35ST Pro _{MUbi(no hse)} :BvLz(m) 3'SrMV 35ST Pro _{JAS} :BvLz(m) 3'SrMV 35ST	CP98-4454	3	3
BvLz transgenic lines with 5 promoter stacks			
1. pJSU:BvLz existing line with Pro _{SPRP} :BvLz(m) 35ST NOST and Pro _{SCBV21} :BvLz(m) 35ST NOST	CP98-4454	57	9

TABLE 25

DNA Constructs for Examples	25
Pro _{MUbi(no hse)} :BvLz(m) 35ST	SEQ ID NO: 39
Pro _{MUbi(no hse)} :BvLz(m) 3'SrMV 35ST	SEQ ID NO: 40
Pro _{MUbi(no hse)} :5'SrMv BvLz(sc) 3'SrMV 35ST	SEQ ID NO: 41
Pro _{MUbi(no hse)} :BvLz(m) 35ST NOST	SEQ ID NO: 42
Pro _{SPRP} :BvLz(m) 3'SrMV 35ST	SEQ ID NO: 43
Pro _{SPRP} (no 5'UTR):5'SrMv BvLz(sc) 3'SrMV 35ST	SEQ ID NO: 44
Pro _{SPRP} :BvLz(m) 35ST NOST	SEQ ID NO: 45
Pro _{SEF1α} : BvLz(m) 3'SrMV 35ST	SEQ ID NO: 46
Pro _{SEF1α} : BvLz(m) 35ST NOST	SEQ ID NO: 47
Pro _{JAS} :BvLz(m) 35ST	SEQ ID NO: 48
Pro _{JAS} :BvLz(m) 3'SrMV 35ST	SEQ ID NO: 49
Pro _{SCBV21} :BvLz(m) 35ST NOST	SEQ ID NO: 50

BvLz(m): Synthetic bovine lysozyme gene with codons optimized for corn.

BvLz(sc): Synthetic bovine lysozyme gene with codons optimized for sugarcane.

Pro_{MUbi(no hse)}: Maize ubiquitin 1 promoter with no heat shock element. The ubiquitin 1 promoter is a constitutive promoter that is commonly used for monocots.Pro_{MUbi(no hse)(no 5'UTR)}: Maize ubiquitin 1 promoter with no heat shock element and no 5' untranslatable region (UTR).Pro_{SPRP}: Promoter isolated from the gene coding for a sugarcane proline-rich protein. It is a constitutive promoter.Pro_{SPRP}(no 5'UTR): The sugarcane proline rich protein promoter with no 5'UTR.Pro_{SEF1α}: Promoter isolated from the gene coding for a sugarcane elongation factor 1α protein laboratory. It is a constitutive promoter.Pro_{JAS}: Promoter isolated from the gene coding for a sugarcane jasmonate-inducible protein in. It is a stem-expressed stress-inducible promoter.Pro_{SCBV21}: Promoter isolated from Sugarcane bacilliform virus. 5' and 3' SrMV refer to the 5' and 3' untranslated regions (UTRs) of Sorghum mosaic virus protein. They are used for enhancement of translation.

35S: 35S terminator derived from the 35S RNA of Cauliflower mosaic virus. It is a terminator of transcription.

NOST: NOS terminator derived from the nopaline synthase gene of the *Agrobacterium tumefaciens* Ti plasmid. It is a terminator of transcription.

35S NOST: A double terminator that consists of 35ST and NOST. Recent published research has proven that the fusion of a 35ST NOST double terminator to a transgene at its 3' end will have a significant effect on decreasing gene silencing and enhancing transgene expression.

45

50

SEQUENCE LISTING

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cacctgttat cttagttgg tggatgttctt ttccggatc tggccaccc tacctttgtt	1680
ggccacgttg octatgttta gcacccatcg aagcatagcg ctggcttgtt gtgtgttccc	1740
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<210> SEQ ID NO 2
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette SCBV21-GUS-NOS
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (30)..(1845)
<223> OTHER INFORMATION: SCBV21 Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1893)..(3704)
<223> OTHER INFORMATION: GUS coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (3777)..(4030)
<223> OTHER INFORMATION: NOS

<400> SEQUENCE: 2

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tatagtctcc aagatagctt attttgcgtt agaacgtctc aagaccaagg aaggactgaa	240
aagctggctgtt gcaacactca attatgccatc aaatcacatc aaggatatgg gaaaactcc	300
tggaccctta tatcctaaaa cttcggaaaa gggggaaaaa ggattaaatt ctgaagatttgc	360
gaaaatgttgc acgagaatca agacaatgtt cagaaatcttgc cttatccacc	420
agaggatgttca tatattatca ttgaaacaga tgcttgcgtt actgggttgg gtcgttttgc	480
caaataatggaaag aaatccaaagg cagacccaaag aagctccgag ctccatgttgc gatatgttgc	540
tggggaaatttt gacaaacccaa aaggggatgtt tgatgcgtt aaatgttgcgtt taatgttgc	600
gtggggaaaaa atgagactctt tttatcttgc taaaaggaaatcactgttgc ggacatgttgc	660
tgcggcaata gagggatgttgc acaacaagag tggatgttgc acaccccttgc aaatccgttgc	720

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gataaggttt atggactata tcactggagc aggaccaaag attgtgattg agcatatcaa	780
aggaaaacac aatggctctgg cagatatact ctcagatgg aaagcaaaac tggcagaatc	840
accttcagaa gaagtggttt tacttgcgaa agctttaag gaagttgcat actatccctga	900
acacccgcaa gtgc当地aaac taattgaatg gggaaagcaa attcttgcatt catttcccaa	960
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<210> SEQ ID NO 3
 <211> LENGTH: 2877
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Expression cassette SCBV21-EYFP-NOS
 <220> FEATURE:
 <221> NAME/KEY: promoter
 <222> LOCATION: (37)..(1852)
 <223> OTHER INFORMATION: SCBV21 Promoter
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (1874)..(2602)
 <223> OTHER INFORMATION: EYFP coding sequence
 <220> FEATURE:
 <221> NAME/KEY: terminator
 <222> LOCATION: (2612)..(2865)
 <223> OTHER INFORMATION: NOS

<400> SEQUENCE: 3

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aagaacatataat agtctccaaat atagctgatt ttgatgttgc acgtctcaag accaaggaa	240
gactgaaaat ctggctggca acactcaattt atgccagaaa tcacatcaag gatatggaa	300
aactccttggcc acccttatat cctaaaactt cagaaaagg agcaaaaggaa ttaaaattctg	360
aagatggaa attaatcgc agaatcaaga caatggtcgaa aatctgcac aatctgacta	420
ttccaccaga ggtatgcataattatcattt aaacagatgc ttgtgcactt ggtgggggtt	480
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tgtatgggtt ggagaaaatg agactctttt atcttgcataaa aagggaaatc actgtgagga	660
cagatgtgc cgcaatagag aggttctaca acaagagtgt tgaacataaa ccctcagaaa	720
tccgttggat aaggtttgc gactatatac ctggagcagg accaaaggatt gtgattgagc	780

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tttagagtccc	gcaattatac	atthaatac	cgatagaaaa	caaaatatac	cgcgaaact	2820
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<210> SEQ_ID NO 4
 <211> LENGTH: 3038
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Expression cassette 35S-GUS-NOS
 <220> FEATURE:
 <221> NAME/KEY: promoter
 <222> LOCATION: (25)..(859)

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<223> OTHER INFORMATION: 35S Promoter
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (896) ..(2707)
 <223> OTHER INFORMATION: GUS coding sequence
 <220> FEATURE:
 <221> NAME/KEY: terminator
 <222> LOCATION: (2780) ..(3033)
 <223> OTHER INFORMATION: NOS
 <300> PUBLICATION INFORMATION:
 <308> DATABASE ACCESSION NUMBER: AF502128
 <309> DATABASE ENTRY DATE: 2002-06-05
 <313> RELEVANT RESIDUES IN SEQ ID NO: (1) .. (3038)

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atctccag	ga aatcaaatac	c ttcccaaga	ag gttaaaga	t tgca	gttcaaa agattcagga	180
ctaactgc	at caagaacaca	ca gaaaagata	t atttctca	g atcaga	agt actattccag	240
tatggacg	at tcaaggctt	g cttcacaaac	ca aggca	at a	tagatggatt ggagtctcta	300
aaaaggtag	t tcccactgaa	t ctaaaggcca	t ggagtcaa	g attca	ata gaggctaa	360
cagaactcg	cg taaaagact	g ggcac	at gtcata	ca g	cttacagag tcttacga	420
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aagatac	at ctc	agaagac	ca aagg	gaa	ttgagactt	540
gaaacctc	cttcc	tttccat	tg cc	ca g	t ctgtca	600
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cctctgc	cg t	ggatgtccc	aa agat	ggac	ccccaccc	720
aagacgtt	cc	cgtct	tca	aa agc	aa ggtatctcc	780
gggatgac	gc	acaatccc	tat	ccttgc	ctctatataa	840
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gctgtat	cg	tttgc	atc	cggt	tcattac	1200
ggaa	gtat	gttgc	ggc	gttgc	ggg	1260
tattgc	gg	aaaagtgt	atc	gttgc	atc	1320
tatccc	ccg	aaatgt	gttgc	tttgc	aaacgg	1380
tgatttctt	aactat	gccg	aatccat	cg	tttgc	1440
ctgggtgg	ac	gtatc	accgt	tttgc	gttgc	1500
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gattaacc	ac	aaaccgtt	cttact	gg	tttgc	1800
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<210> SEQ ID NO 5
<211> LENGTH: 1893
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette 35S-EYFP-NOS
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (25)..(859)
<223> OTHER INFORMATION: 35S Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (879)..(1607)
<223> OTHER INFORMATION: EYFP coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (1617)..(1870)
<223> OTHER INFORMATION: NOS

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aagacgttcc aaccacgtct tcaaagcaag tggattgatg tgatatctcc actgacgtaa	780
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ttagccaaga ccccaacgg aagegcgatc acatggctt gctggagtgc gtgaccggcg	1560
ccgggatcac tctcgccatg gacgagctgt acaagagatc tatctagcga gctcgatcgt	1620
tcaaacattt ggcaataaaag ttctttaaga ttgaatcctt ttggccgtct tgcgatgatt	1680
atcatataat ttctgttgaa ttacgttaag catgtataaa ttaacatgtt atgcgtgacg	1740
ttatattatgat gatgggtttt tatgattaga gtcccgaaat tatacatttt atacgcata	1800
aaaaacaaaa tatagcgcgc aaactaggat aaattatcgc ggcgggtgtc atctatgtt	1860
ctagatcggtt gaaattcctgc agccggggta tcc	1893

<210> SEQ ID NO 6
 <211> LENGTH: 1890
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Expression cassette E35S-EYFP-NOS
 <220> FEATURE:
 <221> NAME/KEY: promoter
 <222> LOCATION: (73)..(829)
 <223> OTHER INFORMATION: E35S Promoter
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (887)..(1615)
 <223> OTHER INFORMATION: EYFP coding sequence
 <220> FEATURE:
 <221> NAME/KEY: terminator
 <222> LOCATION: (1625)..(1878)
 <223> OTHER INFORMATION: NOS

<400> SEQUENCE: 6

aagcttgc tgcattaaatg aatcgccaa cgcgcggggg gaggcggtt gcgtattggc	60
tagagcagct tgccaaatcg tggaggcagc acactctcgct ctactccaaat aatataaa	120
atacagtctc agaagaccaa agggctattt agactttca acaaagggtt atatcgaa	180
acctccctcg attccattgc ccagctatct gtcacttcat caaaaggaca gtagaaaagg	240
aagggtggcac ctacaaatgc catcattgcg ataaaggaaa ggctatcgat caagatgcct	300
ctggccgacag tggtccaaa gatggacccc cacccacgag gaggcatcgat gaaaaagaag	360
acgttccaaac cacgtttca aagcaagtgg attgatgtt aacatggat gaggcacgaca	420

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ctctcgctca ctccaagaat atcaaagata cagtctcaga agaccaaagg gctattgaga	480
ctttcaaca aaggtaata tcggaaacc tcctcgatt ccattgccca gctatctgc	540
acttcatcaa aaggacagta gaaaaggaag gtggcaccta caaatgccat cattgcata	600
aaggaaaggc tatcgtaaa gatgcctcg ccgacagtgg tcccaaagat ggaccccac	660
ccacgaggag catcgtaaa aaagaagacg ttccaaaccac gtcttcaaag caagtggatt	720
gatgtgatat ctccactgac gtaagggatg acgcacaatc ccactatcct tcgcaagacc	780
cttccttotat ataaggaagt tcatttcatt tggagaggac acgctgaaat caccagtctc	840
tctctacaaa tctatctctc tcgattcgca gatctgtcga tgcaccatgg tgagcaaggg	900
cgaggagctg ttcacgggg tggtgcccat cctggtcag ctggacggcg acgtaaacgg	960
ccacaagttc agcgtgtccg gcgaggggcga gggcgatgccc acctacggca agctgaccct	1020
gaagttcatc tgcaccaccg gcaagctgcc cgtgcctgg cccacectcg tgaccacctt	1080
cggctacggc ctgcagtgtc tcgcccccta ccccgaccac atgaaggcgc acgacttctt	1140
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caactacaag accccggccg aggttaagtt cgaggggcgc accctggtga accgcatcga	1260
gctgaagggc atcgacttca aggaggacgg caacatcctg gggcacaagc tggagtacaa	1320
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cttcaagatc cgccacaaca tcgaggacgg cagcgtgcag ctcgcccacc actaccagca	1440
gaacacccccc atcggcgacg gccccgtct gctgcccgc aaccactacc tgagctacca	1500
gtccgcctg agcaaagacc ccaacgagaa ggcgcgtcac atggctctgc tggagttcgt	1560
gaccgcgcgc gggatcactc tcggcatgga cgagctgtac aagagatcta tctagcgagc	1620
tgcgcgttc aacatggta caataaagtt tcttaagatt gaatcctgtt gccggcttc	1680
cgatgattat catataattt ctgttgaatt acgttaagca tggataatt aacatgtaat	1740
gcatgacgtt atttatgaga tgggtttta tgatttaggt cccgcaattt tacatttaat	1800
acgcgtataga aaacaaaata tagcgcgca actaggataa attatcgccg cgggtgtcat	1860
ctatgttact agatcgggaa tggggatcc	1890

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<210> SEQ_ID NO 7
<211> LENGTH: 4178
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette Pr4-GUS-NOS
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(966)
<223> OTHER INFORMATION: Pr4 Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (668)..(668)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (671)..(671)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (681)..(681)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (967)..(1977)
<223> OTHER INFORMATION: First exon (5'UTR) and first intron
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2036)..(3847)

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<223> OTHER INFORMATION: GUS coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (3920)..(4173)
<223> OTHER INFORMATION: NOS

<400> SEQUENCE: 7

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attgc	atgttc	taagtataa	aaaattacca	catat	120
gtttttatc	tatcttata	cataatatta	aactttactc	tacgaataat	180
gtactacaat	aatatcagt	tttttagagaa	tcatataat	gaacagttag	240
aaggacaatt	gagtat	tttg acaacaggac	tctacagtt	tatctttt	300
gttctcc	tttttgc	aaatgttac	ctatataata	cttcatccat	360
catccattt	gggtttaggg	ttaatgg	ttatagacta	attttttag	420
ttattctatt	ttatgc	ttatgc	aaaaactc	tat	480
ataattt	atataat	atataat	atgtactaa	atataacaa	540
agaaat	aaaacta	aggaaat	aaacat	ttttgc	600
aaacgc	gcg	gacg	acgac	accagc	660
aagcga	anca	nacggc	acgg	natctc	720
tccgc	atcca	aaatttgc	gttgc	cttcc	780
ctc	cac	ggc	acggc	ctacgg	840
tc	cgt	ccg	ataaaa	ttcc	900
gtt	ccg	ccg	ataaa	cccc	960
ttcaagg	gttac	cc	ccat	cc	1020
ccgg	tcat	tttgc	tttgc	tttgc	1080
ttgtgtt	aga	tttgc	tttgc	tttgc	1140
tctgatt	gttgc	tttgc	tttgc	tttgc	1200
cgc	atc	tttgc	tttgc	tttgc	1260
ttcc	ttat	ttcaat	ttcaat	ttcaat	1320
tttt	tttgc	tttgc	tttgc	tttgc	1380
ttct	tttca	tttgc	tttgc	tttgc	1440
tatt	catat	tttgc	tttgc	tttgc	1500
gtt	gttgc	tttgc	tttgc	tttgc	1560
tgt	gggt	tttgc	tttgc	tttgc	1620
tac	tttgc	tttgc	tttgc	tttgc	1680
gag	tttgc	tttgc	tttgc	tttgc	1740
ctg	atgc	tttgc	tttgc	tttgc	1800
tat	cttgc	tttgc	tttgc	tttgc	1860
tgg	catat	tttgc	tttgc	tttgc	1920
gctt	gggt	tttgc	tttgc	tttgc	1980
gact	cttgc	tttgc	tttgc	tttgc	2040
acgt	cctgt	tttgc	tttgc	tttgc	2100
tctggat	cg	aaaactgt	gaattgat	gcgttgg	2160

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ccgggcaatt gctgtgccag gcagtttaa cgatcagtgc gccgatgcag atattcgtaa	2220
ttagcgggc aacgtctggt atcagcgcga agtctttata ccgaaagggtt gggcaggcca	2280
gctgtatcgat ctgcgttcg atgcggcac tcattacggc aaagtgtggg tcaataatca	2340
ggaagtgtatc gagcatcagg gcccgtatac gccatggaa gccgatgtca cggcgatgt	2400
tattgcggg aaaagtgtac gtatcacatgt ttgtgtgaac aacgaactga actggcagac	2460
tatccccccg ggaatgggtga ttaccgacga aaacggcaag aaaaaggcgt cttaatccca	2520
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gcaacccgggt gaagggttatac tctatgaact gtgcgtcaca gccaaggcc agacagatg	2820
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gattaaccac aaaccgttct actttactgg ctttggcgat catgaagatg cggacttgcg	2940
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ggccaaactcc taccgtaccc cgcattaccc ttacgctgaa gagatgtcg actgggcaga	3060
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cgtcgtcggtt gaaacgggtat ggaatttcgcg cgttttgcg acctcgcaag gcatattgcg	3720
cggtggcggtt aacaagaaag ggtatccac tcggcaccgc aaaccgaagt cggcgccctt	3780
tctgtgtcaaa acacgtggaa ctggcatgaa cttcggtgaa aaaccgcagc agggaggcaa	3840
acaatgaatc aacaactctc ctggcgccacc atcgtcggtt acagcgtcg gaaattgtatc	3900
cgagctcgaa ttcccccgat cgttcaaaca tttggcaata aagtttctta agattgaatc	3960
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taatttaacat gtaatgtatc acgttattta tgagatgggt ttttatgatt agagtccgc	4080
aattatacat ttaatacgca atagaaaaca aaatatacg cgcacaaacttag gataaaattat	4140
cgcgcgccggt gtcatactatg ttactagatc gggaaattc	4178

<210> SEQ ID NO 8
 <211> LENGTH: 3020
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Expression cassette Pr4-EYFP-NOS
 <220> FEATURE:
 <221> NAME/KEY: promoter
 <222> LOCATION: (1) .. (966)
 <223> OTHER INFORMATION: Pr4 Promoter
 <220> FEATURE:

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<221> NAME/KEY: misc_feature
<222> LOCATION: (668)..(668)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (671)..(671)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (681)..(681)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (967)..(1977)
<223> OTHER INFORMATION: First exon (5'UTR) and first intron
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2017)..(2745)
<223> OTHER INFORMATION: EYFP coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2755)..(3008)
<223> OTHER INFORMATION: NOS

<400> SEQUENCE: 8

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attgcattgtc taagttataaa aaaattacca catattttt ttgtcacact tgtttgaagt     120
gcagtttatac tatctttata cataatattta aactttactc tacgaataat ataatctata    180
gtactacaat aatatcagtg ttttagagaa tcatataaat gaacagtttag acatggctta    240
aaggacaatt gagtattttt acaacaggac tctacagttt tatcttttta gtgtgcattgt    300
gttctctttt tttttgcaa atagttcac ctatataataa cttcatccat tttttagtta    360
catccattta gggtttaggg ttaatggttt ttatagacta attttttagt tacatctatt    420
ttattctattt ttagcctcta aattaagaaa actaaaactc tatttttagtt tttttattna    480
ataatattaga tataaaatag aataaaataa agtgactaaa aattaaacaa ataccctta    540
agaaattnaa aaaactaagg aaacattttt ctgtttcgaa gtagataatg ccagectgtt    600
aacacccgtc gacgagtcta acggacacca accagcgaac cagcagcgtc gctggggcc    660
aagcgaanaca nacggcacgg natctctgtc gctgcctcca ccgttggact tgctccgtg    720
tcggcatcca gaaattgcgtt ggcggcaggc agacgtgagc cggcacaggc cggccctctc    780
ctcctctcac ggcacggcag ctacggggta ttcccttccc accgctccctt cgcttccctt    840
tcctcgccccg ccgtataataa tagacaccccc ctccacaccc tcttccccca acctcggtt    900
gttcggagcg cacacacaca caaccagatc tcccccaat ccacccgtcg gcaccccgcc    960
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gttgcgttgcgg gttttacttga tgcataataca gagatgtttt ntgcgttgcgtt gttgtatgt 1560
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tacctggtgtt atttataat tttggaaactg tatgtgtgtg tcatacatct tcataagttac	1680
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gactctagag gatctgatatac ctgatcagaa gacaccatgg tgagcaaggg cgaggagctg	2040
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cacaacgtct atatcatggc cgacaacgc aagaacggca tcaagggtgaa cttcaagatc	2520
cggccacaaca tcgaggacgg cagcgatgc ctcggccgacc actaccacgc acacacccccc	2580
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<210> SEQ ID NO 9
 <211> LENGTH: 4102
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Expression cassette Ubi-GUS-NOS
 <220> FEATURE:
 <221> NAME/KEY: promoter
 <222> LOCATION: (1)..(995)
 <223> OTHER INFORMATION: Ubi Promoter
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (996)..(2005)
 <223> OTHER INFORMATION: First exon (5'UTR) and first intron
 <220> FEATURE:
 <221> NAME/KEY: misc_feature
 <222> LOCATION: (2022)..(3833)
 <223> OTHER INFORMATION: GUS coding sequence
 <220> FEATURE:
 <221> NAME/KEY: terminator
 <222> LOCATION: (3843)..(4096)
 <223> OTHER INFORMATION: NOS

<400> SEQUENCE: 9

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cattgcattt ctaaggatata aaaaattacc acatattttt tttgtcacac ttgtttgaaag	120
tgcagtttat ctatctttat acatatattt aaactttact ctacgaataa tataatctat	180

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agtactacaa taatatacgt gtttagaga atcatataaa tgaacagtta gacatggct	240
aaaggacaat tgagtatttt gacaacagga ctctacagt ttatctttt agtgtgcatt	300
tgttctccctt ttttttgca aatagcttca cctatataat acttcatcca ttttattag	360
acatccattt agggtttagg gttaatggtt ttatagact aattttttt gtacatctat	420
tttattctat tttagctctt aaattaagaa aactaaaact ctattttagt ttttttattt	480
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cgtgagccgg cacggcaggg ggcctccctc tccctcactg gcacggcagc tacggggat	840
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tccacacccct ctttcccaa cctcgtgtt ttcggagcgc acacacacac aaccagatct	960
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actgtggat tggatgttcc tttttttttttt tttttttttttt tttttttttttt	2160
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atgttacttag atcggggat tc	4102

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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Expression cassette Ubi-EYFP-NOS
<220> FEATURE:
<221> NAME/KEY: promoter
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<223> OTHER INFORMATION: Ubi Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (996)..(2005)
<223> OTHER INFORMATION: First exon (5'UTR) and first intron
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2030)..(2758)
<223> OTHER INFORMATION: EYFP coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2768)..(3021)
<223> OTHER INFORMATION: NOS

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aaaggacaat tgatgtttt gacaacagga ctctacagg ttatctttt agtgtgcatt	300
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<210> SEQ ID NO 11
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 11

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<210> SEQ ID NO 12
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 12

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<210> SEQ ID NO 13
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P-W4F PCR primer

<400> SEQUENCE: 13

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<210> SEQ ID NO 14
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: P-W1R PCR primer

<400> SEQUENCE: 14

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<210> SEQ ID NO 15
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SCBV/Prom/F PCR primer

<400> SEQUENCE: 15

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<210> SEQ ID NO 16
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SCBV/Prom/R PCR pr

<400> SEQUENCE: 16

caaacttgc caaatgatca tgtggtaac taccgatg 38

<210> SEQ ID NO 17
<211> LENGTH: 1816
<212> TYPE: DNA
<213> ORGANISM: Sugarcane bacilliform virus
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1786)
<223> OTHER INFORMATION: SCBV21 Promoter
<220> FEATURE:
<221> NAME/KEY: misc_signal
<222> LOCATION: (1787)..(1787)
<223> OTHER INFORMATION: Transcription start site
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1813)..(1816)
<223> OTHER INFORMATION: n is a, c, t, or g

<400> SEQUENCE: 17

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ttggagataa tcagttagct gttcaagaac atatagtctc caagatagct gatggatg	180
aagaacgtct caagaccaag gaaggactga aaagctggct ggcaacactc aattatgcc	240
gaaatcacat caaggatatacg ggaaaactcc ttggaccctt atatcctaaa acttcagaaa	300
agggagcaaa aggattaaat tctgaagatt ggaaattaat cagcagaatc aagacaatgg	360
ttagaaatct gccaaatctg actattccac cagaggatgc atatattatc attgaaacag	420
atgcttgtgc aactgggtgg ggtgcagttt gcaaatggaa gaaatccaag gcagacc	480
gaagctccga gctcatatgt cgatatgcaa gtggaaatt tgacaaacca aaaggacat	540
gtgatgcaga aatctatgga gtaatgaatgg ggctggagaa aatgagactc ttttatctt	600
ataaaaaggaa aatcactgtg aggacagata gtgccgcaat agagaggttc tacaacaaga	660
gtgttgaaaca taaaccctca gaaatccgtt ggataaggtt tatggactat atcactgg	720
caggacaaa gattgtgatt gagcatatca aaggaaaaaca caatggctcg gcagatatcc	780
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ggccacgttg	octatgttta	gcaccta	aagcatagcg	ctcggttgtt	gtgtgttccc	1740
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catttgagca	agnnnn					1816

<210> SEQ ID NO 18
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<212> TYPE: DNA
<213> ORGANISM: Sugarcane bacilliform virus
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1786)
<223> OTHER INFORMATION: SCBV21 Promoter
<220> FEATURE:
<221> NAME/KEY: misc_signal
<222> LOCATION: (1787)..(1787)
<223> OTHER INFORMATION: Transcription start site
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1788)..(1826)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1856)..(1860)
<223> OTHER INFORMATION: n is a, c, t, or g
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1861)..(1863)
<223> OTHER INFORMATION: Start codon
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<222> LOCATION: (1864)..(1864)
<223> OTHER INFORMATION: n is g or t

<400> SEQUENCE: 18

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ttggagataa	tcaagttagct	gttcaagaac	atatagtctc	caagatagct	gattttgatg	180
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gtgatgcaga	aatctatgga	gtaatgaatg	ggctggagaa	aatgagactc	ttttatcttg	600
ataaaaggaa	aatcactgtg	aggacagata	gtgccgcaat	agagaggttc	tacaacaaga	660
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ggggaaaagca	aattcttgat	ccatccccca	agttcaagaa	ggacatgttt	aaaaaaactg	960
aacacatcat	gatggctagt	caagagccta	cactgcttgc	tggatgtaga	aggcctgcata	1020

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taaaacgacg	gccagtggac	gcgcgtata	cgactcaacta	tagggcgaat	tgggtaccgg	660
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 <223> OTHER INFORMATION: SCBV21 Promoter deletion B
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 <223> OTHER INFORMATION: EYFP coding sequence
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 <223> OTHER INFORMATION: NOS

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 <223> OTHER INFORMATION: pSK-SCBV21-EYFP-NOS Vector - deletion C
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 <221> NAME/KEY: promoter
 <222> LOCATION: (674) .. (1478)
 <223> OTHER INFORMATION: SCBV21 Promoter deletion C
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 <222> LOCATION: (718) .. (767)
 <223> OTHER INFORMATION: Transcription start site TSS1
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 <223> OTHER INFORMATION: Transcription start site TSS2
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 <222> LOCATION: (1481) .. (2209)
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 <222> LOCATION: (2219) .. (2472)
 <223> OTHER INFORMATION: NOS

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<211> LENGTH: 4626
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: pSK-SCBV21-EYFP-NOS Vector - deletion D
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (674) .. (1383)

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<223> OTHER INFORMATION: SCBV21 Promoter deletion D
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<220> FEATURE:
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<223> OTHER INFORMATION: EYFP coding sequence
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<223> OTHER INFORMATION: NOS

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ccaggaaccg taaaaaggcc ggcgttgcgg cgtttttccaa taggtccgc cccctgcac	2820
agcatcacaa aaatcgacgc tcaagtcaga ggtggcgaaa cccgcacagga ctataaaat	2880
accaggcgctt tcccccggta agctccctcg tgcgtctcc tttccgcacc ctggccgtt	2940
ccggataacctt gtccgcctt cttcccttgcg gaagcggtggc gtttctcat agctcagct	3000
gtaggatct cagttcggtt tagtgcgttc gtcacatcgat gggctgtgtg cacgaaaccc	3060
ccgttccagcc cgaccgcgtc gccttgcgtt gtaactatcg ttttgcgttcc aacccggtaa	3120

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gacacgactt atgcgccactg gcagcagccca ctggtaacag gattagcaga gcgaggtatg	3180
taggcgggtgc tacagagttc ttgaagtggtt ggctctaacta cggctacact agaaggacag	3240
tattttgtat ctgcgtctg ctgaagccag ttaccttcgg aaaaagagtt ggtagcttt	3300
gatccggcaa acaaaccacc gctggtagcg gtgggttttt tgtttgcag cagcagatta	3360
cgcgcagaaa aaaaggatct caagaagatc ctttgatctt ttctacgggg tctgacgctc	3420
agtggaaacgaa aaactcacgt taagggtttt tggtcatgag atttatcaaaa aggatcttca	3480
cctagatctt tttaaattaa aatgaagtt ttaaatcaat ctaaaagtata tatgagtaaa	3540
cttgggtctga cagttacca tgcttaatca gtgagggacc tatctcagcg atctgtctat	3600
ttcggttcatc catagttgcc tgactccccg tcgtgttagat aactacgata cggggaggc	3660
tacccatctgg ccccaagtgc gcaatgatac cgcgcagacc acgctcacccg gctccagatt	3720
tatcagcaat aaaccagcca gccggaaaggg ccgagcgcag aagtggtcct gcaactttat	3780
cgcgcctccat ccagtcttatt aatttgttgc gggaaagctag agtaagtagt tcggccagtt	3840
atagtttgcg caacgttgtt gccattgcta caggcatcg ggtgtcacgc tcgtgtttt	3900
gtatgggttc attcagctcc gggttccaaac gatcaaggcg agttacatga tccccatgt	3960
tgtcaaaaaa agcggttagc tccttcggc ctcggatcg tgcggatgt aagttggccg	4020
cagtgttatac actcatggtt atggcagcac tgcataattc tctttactgtc atgcccattcg	4080
taagatgctt ttctgtgact ggtgagttact caaccaagtc attctgagaa tagtgtatgc	4140
ggcgaccggat ttgcgtttgc ccggcgtcaaa tacggatataa taccggccca cataggcagaa	4200
cattttaaatgt gtcatttcatt ggaaacgtt cttcgcccccg aaaactctca aggatcttac	4260
cgcgtttagt atccagttcg atgttacccca ctgcgtgcacc caactgtatct tcaggatctt	4320
ttactttcac cagcgtttct gggttgcggaa aaacagggaaag gcaaaatgcc gcaaaaaagg	4380
gaataaggcc gacaacggaaa tggtaatac tcataactctt ctttttcaa tattttgaa	4440
gcatttatca gggattttgt ctcgtttcccg gatacatatt tgaatgtatt tagaaaaata	4500
aacaaatagg gttcccgcc acatccccca gaaaagtgcc ac	4542

<210> SEQ ID NO 25

<211> LENGTH: 1018

<212> TYPE: DNA

<213> ORGANISM: Sugarcane bacilliform virus

<400> SEQUENCE: 25

gaagaacagc atgcgttggaaat tgctacaga tatgcagaat gaatgggtt	60
atcttaagcc ctccaaatgt taaaattggat taaaaagagt tgactttctt ggttcaacaa	120
ttggagatata tcagtttagt gttcaagaac atatgtctc caagatagttt gatggat	180
aagaacgtctt caagaccaag gaaggactga aaagctggctt ggcaacactc aattatgcc	240
gaaatccat catggatatg ggaaaactcc ttggaccctt atatcctaaa acttcagaaa	300
aggggagcaaa aggattttat tctgttggat gggattttat cagcagaatc aagacaatgg	360
tcagaaatctt gccaatctt actattccac cagaggatgc atatattatc attgttacatc	420
atgcgttgc aactgggtgg ggtgcagttt gcaaatggaa gaaatccaaat gcagaccat	480
gaagctccga gtcattatgtt cgatgttccaa gttttttttt tgacaaacca aaaggatccat	540
gtgtatgcaga aatctatggat gtaatgttccaa ggttggat gaaatccaaat gcagaccat	600
ataaaaggaa aatctatgtt aggacagata gtgtccat aatgttccaaat gcagaccat	660
gtgttgaaca taaaacctca gaaatcccgat ggataaggtt tatggactat atcactggag	720

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caggaccaaa gattgtgatt gagcatatca aaggaaaaca caatggctg gcagatatcc 780
tctcaagatt gaaagcaaaa ctggcagaat cacccatcg agaagtggtt ttacttgcg 840
aagcttaaaa ggaagttgca tactatcctg aacacccgc a gtgcacaaa ctaattgaat 900
ggggaaagca aattcttgat ccatttcca agttcaagaa ggacatgtt gaaaaactg 960
aacacatcat gatggctagt caagagceta cactgcttg tggatgtaga aggcatgg 1018

<210> SEQ ID NO 26

<211> LENGTH: 811

<212> TYPE: DNA

<213> ORGANISM: Sugarcane bacilliform virus

<400> SEQUENCE: 26

aggcctgcat atcgttcac atctggcaca aaactcaacc caagcaggaa gttctataaa 60
tgtgcaatga acatgtgcca ctgctggat tggcagatc ttttagaaga atatgtccaa 120
gaacgaattg aagtgttcat gattgagaac tttgacaaga aaatggaaat tcaagatgt 180
ccaagtgacat caaatgctaa cattccagga aattttaaat ctcttgcaga tttgaagaag 240
gataaagaag ctaaaagctga atatcaagac atgcttgata atcategttc aagcattatt 300
gacagaccaa ggcacacaga tgaacacttc aagcctggat acatgtacac cgattccctg 360
cagaagatca aggaggacta cgcaagccca agacaggagg aaccaccatg agaagacatt 420
gagttctggat tatgcaagga agaagactac cacacagaag acctcaatac agaagatgca 480
gttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 540
ggaaggacaa ttatggagcg tggaggaccc atcaagcact cagaacgcga acctcaactt 600
tcggcgcacccatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 660
tacccatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 720
gtgtgttcccttccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 780
accacatgtat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 811

<210> SEQ ID NO 27

<211> LENGTH: 716

<212> TYPE: DNA

<213> ORGANISM: Sugarcane bacilliform virus

<400> SEQUENCE: 27

agatcttttta gaagaatatgc tccaagaacg aattgaagtgc ttcatgtatgc agaactttgt 60
caagaaaatgc ggaattcaag atgttaccaag tacatcaat gctaacatcc caggaaatttt 120
taaatctcttgc ccatgttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 180
tgataatcat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 240
tggatacatgc tacaccgatt ccctgcagaa gatcaaggagg gactacgcaac gcccacacaca 300
ggagggacca ccatgttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 360
agaagacccatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 420
gaggcgtatgc taagcaatgc tgcacggaa gacaattatgc gaggcgtggagg gacccatcaa 480
gcactcgaa cgcacccatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 540
gtcttttgcatgc ccatgttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatccat ctttttgcataatcc 600
tacgcaagca tagcgctcgg ctgggtgtgc ttccctctgc ctatataagg catggttgt 660
agactcttac actcatcggt agttcaccac atgatcattt gaggatgtt ccattgg 716

<210> SEQ ID NO 28

-continued

<211> LENGTH: 727
<212> TYPE: DNA
<213> ORGANISM: Sugarcane bacilliform virus

<400> SEQUENCE: 28

```

aggcctgcat atcgttcac atctggcaca aaactcaacc caagcaggaa gttctataaa      60
tgtgcataatga acatgtgcca ctgtcggtat tgggcagatc ttttagaaga atatgtccaa    120
gaacgaaattg aagtgttcat gattgagaac tttgacaaga aaatggaaat tcaagatgtaa    180
ccaaatgtacat caaatgctaa cattccagga aattttaaat ctcttgcaga tttgaagaag    240
gataaagaag ottaaagctga atatcaagac atgcttgata atcatcggtc aagcattatt    300
gacagaccaa ggccaacaga tgaacacttc aagcctggat acatgtacac cgattccctg    360
cagaagatca aggaggacta cgcaagccca agacaggagg aaccaccatg agaagacatt    420
gagttctggat tatgcaagga agaagactac cacacagaag acctcaatac agaagatgca    480
gttgatctta ctgacgtaag caatgacgat cagtggaggc gatcgtaagc aatgtgcac    540
ggaaggacaa ttatggagcg tggaggaccc atcaagcact cagaacgcga acctcaactt    600
tcggcgccag caccttgtat cttagtgg tgtgtgtctt ttccggcatc tgtgecacct    660
taccttgc ggcacggtt cctatgctta gcacctacgc aagcatagcg ctggctggat    720
gccatgg                                         727

```

<210> SEQ ID NO 29
<211> LENGTH: 632
<212> TYPE: DNA
<213> ORGANISM: Sugarcane bacilliform virus

<400> SEQUENCE: 29

```

agatctttta gaagaatatac tccaagaacg aattgaagtgc ttcatgatgc agaactttgc      60
caagaaaatg ggaattcaag atgtaccaag tacatcaaat gctaacatcc cagggaaattt    120
taaatcttgc gcaatgttgc agaaggataa agaagctaaa gctgaatatc aagacatgtt    180
tgataatcat cgttcaagca ttattgacag accaaggccca acagatgaac acttcaagcc    240
tggatacatg tacaccgatt ccctgcagaa gatcaaggag gactacgcac gcccaagaca    300
ggagggacca ccatgagaag acattgagtt ctggtttatgc aaggaagaag actaccacac    360
agaagaccc aatacagaag atgcagttgc tcttactgac gtaagcaatg acgatcagtgc    420
gaggcgatcg taagcaatgc tgacacggaa gacaattatg gagcgtggag gacccatcaa    480
gcactcgaa cgcgaacctc aactttcgcc gccagcacct tggatcttgc gttgggtgtt    540
gtcttttgc gcatctgtgc caccttacct ttgtcgccca cggtgcctat gcttagcacc    600
tacgcaagca tagcgctcggtt ctggtgccat gg                                         632

```

<210> SEQ ID NO 30
<211> LENGTH: 50
<212> TYPE: DNA
<213> ORGANISM: Sugarcane bacilliform virus
<220> FEATURE:
<221> NAME/KEY: misc_signal
<222> LOCATION: (41)..(41)
<223> OTHER INFORMATION: Transcription start site

<400> SEQUENCE: 30

```

caggaagttc tataaatgtgc caatgaacat gtgccactgc tggatttggg                                         50

```

<210> SEQ ID NO 31
<211> LENGTH: 50
<212> TYPE: DNA

-continued

<213> ORGANISM: Sugarcane bacilliform virus
<220> FEATURE:
<221> NAME/KEY: misc_signal
<222> LOCATION: (41)..(41)
<223> OTHER INFORMATION: Transcription start site

<400> SEQUENCE: 31

tccctctgcc tataataagc atgggtgtaa gactcttaca ctcatcggtta 50

<210> SEQ ID NO 32
<211> LENGTH: 853
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SCBV21 Promoter with TSS1 and TSS2
<220> FEATURE:
<221> NAME/KEY: misc_signal
<222> LOCATION: (45)..(94)
<223> OTHER INFORMATION: Transcription start site TSS1
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (727)..(776)
<223> OTHER INFORMATION: Transcription start site TSS2
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (806)..(845)
<223> OTHER INFORMATION: n is a, c, g, t, or absent
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (846)..(849)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (850)..(852)
<223> OTHER INFORMATION: Start codon
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (853)..(853)
<223> OTHER INFORMATION: n is g or t

<400> SEQUENCE: 32

aggcctgcat atcagttcac atctggcaca aaactcaacc caagcaggaa gttctataaa 60
tgcgtcaatga acatgtgcca ctgctggat tgggcagatc ttttagaaga atatgtccaa 120
gaacgaattt gaaatgttcat gattgagaac tttgacaaga aatggaaat tcaagatgtta 180
ccaaatgttcat ccaaattgttcaa cattccagga aattttaaat ctcttgcaaga tttgaagaag 240
gataaaagaag ctaaaagctga atatcaagac atgcttgata atcategttc aagcattatt 300
gacagacccaa ggccaaacaga tgaacacttc aagcctggat acatgtacac cgattccctg 360
cagaagatca aggaggacta cgcaagccca agacaggagg aaccaccatg agaagacatt 420
gagttctggat tatgcacggaa agaagactac cacacagaag acctcaatac agaagatgca 480
gttgcgttta ctgcgttcaag caatgcgtt cagtggaggc gatcgtaagc aatgtgcac 540
ggaaggacaa ttatggagcg tggaggaccc atcaagactt cagaacgcga acctcaactt 600
tcggcgccag caccttgtat ctttagttgg tttcggttctt ttgcgttcaatc tggtccacct 660
tacccatgttgc ggccacgtt cctatgttca gcacctacgc aagcatagcg ctggctgg 720
gtgtgttccc tctgcctata taaggcatgg ttgttaagact cttacactca tcggtagttc 780
accacatgttcat catttgagca agttttnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 840
nnnnnnnnnnna tgn 853

<210> SEQ ID NO 33
<211> LENGTH: 758
<212> TYPE: DNA
<213> ORGANISM: Sugarcane bacilliform virus
<220> FEATURE:

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<221> NAME/KEY: misc_signal
<222> LOCATION: (632)..(681)
<223> OTHER INFORMATION: Transcription start site TSS2
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (711)..(715)
<223> OTHER INFORMATION: n is a, t, c, or g
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (716)..(718)
<223> OTHER INFORMATION: Start codon
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (719)..(719)
<223> OTHER INFORMATION: n is g or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (720)..(758)
<223> OTHER INFORMATION: n is a, t, c, g or absent

<400> SEQUENCE: 33

agatctttta gaagaatatg tccaagaacg aattgaagtg ttcatgattg agaacttga      60
caagaaaatg gaaattcaag atgtaccaag tacatcaaat gctaacatc cagggaaattt    120
taaatctctt gcagatttga agaaggataa agaagctaaa gctgaatatc aagacatgct    180
tgataatcat cgttcaagca ttattgacag accaaggcca acagatgaac acttcaagcc    240
tggatacatg tacaccgatt ccctgcagaa gatcaaggag gactacgcaa gcccaagaca    300
ggaggaacca ccatgagaag acattgagtt ctggttatgc aaggaagaag actaccacac    360
agaagacctc aatacagaag atgcagttga tcttactgac gtaagcaatg acgatcatgt    420
gaggcgatcg taagcaatga tgcacggaag gacaattatg gagcgtggag gacccatcaa    480
gcactcgaaa cgcgAACCTC aacttcggc gccagcacct tgtatctta gttggtgtgt      540
gtcttttcg gcatctgtgc caccttacct ttgtcggcca cgttgcctat gcttagcacc    600
tacgcaagca tagcgctcggt ctggtgtgtg ttccctctgc ctatataagg catggttgt     660
agactcttac actcatcggt agttcaccac atgatcattt gagcaagttt nnnnnatgnn    720
nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnn          758

<210> SEQ ID NO 34
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward PCR primer F1

<400> SEQUENCE: 34

ttactcgagg cctgcataatc agttcacatc tgg                                33

<210> SEQ ID NO 35
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Forward PCR primer F2

<400> SEQUENCE: 35

ttactcgaga tcttttagaa gaatatgtcc aagaacg                                37

<210> SEQ ID NO 36
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Reverse PCR primer R1

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<400> SEQUENCE: 36

ttacccatgga aacttgctca aatgatcatg tggtaacta cc

42

<210> SEQ ID NO 37

<211> LENGTH: 36

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Reverse PCR primer R2

<400> SEQUENCE: 37

ttacccatggc accagcccgag cgctatgctt gcgtag

36

<210> SEQ ID NO 38

<211> LENGTH: 2855

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Expression cassette SCBV21-BvLz(m) -35S NOS

<220> FEATURE:

<221> NAME/KEY: promoter

<222> LOCATION: (1)..(1816)

<223> OTHER INFORMATION: SCBV21 Promoter

<220> FEATURE:

<221> NAME/KEY: misc_feature

<222> LOCATION: (1857)..(2300)

<223> OTHER INFORMATION: BvLz(m) coding sequence

<220> FEATURE:

<221> NAME/KEY: terminator

<222> LOCATION: (2375)..(2580)

<223> OTHER INFORMATION: 35S

<220> FEATURE:

<221> NAME/KEY: terminator

<222> LOCATION: (2603)..(2855)

<223> OTHER INFORMATION: NOS

<400> SEQUENCE: 38

gaagaacacgc atgctgaaca tctgtggaaatgctacaga tatgcaagaa gaatgggta 60

atcttaagcc cttccaagta taaaattggag taaaaagagt tgactttctt ggttcaacaa 120

ttggagataa tcagttagct gttcaagaac atatagtctc caagatagct gatggatg 180

aagaacgtct caagaccaag gaaggactga aaagctggct ggcaacacac 240

aaaaatcacat caaggatataa gggaaactcc ttggaccctt atatcataaa acttcagaaa 300

agggagcaaa aggattaaat tctgaagatt gaaatattaaat cagcagaatc aagacaatgg 360

tcagaatct gccaaatctg actattccac cagaggatgc atatattatc attgaaacag 420

atggcttgc aactgggttgg ggtgcagtt gcaaattggaa gaaatccaag gcagacccaa 480

gaagctccga gctcatatgt cgatatgcaat gtggaaatt tgacaaacca aaagggacat 540

gtgatgcaga aatctatgga gtaatgaatgg ggcgggaaaatggactc ttttatctt 600

ataaaaaggaa aatcaatgtt ggacagataa gtgccgaat agagaggatc tacaacaaga 660

gtgttgcata taaaccctca gaaatccgtt ggataagggtt tatggactat atcactggag 720

caggaccaaa gattgtgatt gagcatatca aaggaaacaa caatggctcg gcagatatcc 780

tctcaagattt gaaacaaaa ctggcagaat cacccatca agaaggatggtt ttacttgca 840

aagctttaaa ggaagttgcata tactatcctg aacacccgca agtgcacaaa ctaattgaat 900

ggggaaagca aattcttgtat ccattccca agttcaagaa ggacatgtt gaaaaactg 960

aacacatcat gatggcttagt caagagccata cactgcttggatgtaga aggctgcat 1020

atcagttcac atctggcaca aaactcaacc caagcaggaa gttctataaaa tgtgcata 1080

acatgtgcacatgtggat tggcagatc tttagaaga atatgtccaa gaacgaattg 1140

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aagtgttcat gattgagaac tttgacaaga aaatggaaat tcaagatgta ccaagtacat	1200
caaatacttccaa cattccagga aattttaaat ctcttcgaga tttgaagaag gataaagaag	1260
ctaaaggctga atatcaagac atgtttgata atcatcggtt aacgcattt gacagaccaa	1320
ggccaaacaga tgaacacttc aagcctggat acatgtacac cgattccctg cagaagatca	1380
aggaggacta cgcaagccca agacaggagg aaccacccat agaagacatt gagttcttgt	1440
tatgcaagga agaagactac cacacagaag acctcaatac agaagatgca gttgtatcca	1500
ctgacgtaag caatgacgat cagtgaggac gatcgtaagc aatgatgcac ggaaggacaa	1560
ttatggacgc tggaggaccc atcaagcact cagaacgcga acctcaactt tcggggccag	1620
cacccgttat ottagttgg tgtgtgttctt ttccggcatc tggtccacct tacctttgtc	1680
ggccacggtt octatgttta gcacccatgc aagcatagcg ctccggctgt gtgtgttccc	1740
tctgcctata taaggcatgg ttgttaagact cttacactca tcggtagttc accacatgt	1800
catttgagca agtttgaatc gaattccccgc ggccgcattt catctcgatcc ccaaatacg	1860
cgcccttggatcccttgc ttccgttcc tgcgttggc tggtcaggc aagggtgtcg	1920
aaagggtcgca actggctagg accctgaaga agctggccctt ggatggctac aagggtgtgt	1980
ccctggctaa ctggctgtgc ctgaccaagt gggaaatccctc ctacaacacc aaggctacca	2040
actacaaccc atccctccgaa tccaccgact acggcatctt ccagatcaac tccaaatgg	2100
ggtgcaacga tggcaagacc ccaaaccgtt tggatggctt ccacgtgttcc tgctccgagc	2160
tgtatggaaaa cgatatcgat aaggctgtgg ctgtgcgttta gcacatcgatcc tccggaaacagg	2220
gcacatccgc ctgggtggct tggaaatccc actgcaggaa tcacgtatgt tgcttctacg	2280
tggaaaggctt caccctgtga ttcaattcg gatccccccggc ggctgcaggaa attcgatatc	2340
aagcttatcg ataccgtcgaa ggggtccgca aaaatccacca gtctctctt acaaatactat	2400
ctctctctat ttttctccat aataatgtgtt gagtagttcc cagataaggaa aatttaggtt	2460
cttataagggt ttgcgttcatg tggttgcgttata aataagaaacc cttatgtatgtt atttgttattt	2520
gtaaaataact tctatataa aaatttctaa ttccctaaac caaaatccag tgacccgttgc	2580
ggcccgctcg acgaatttcc ccgatcggttcc aaacattgg caataaaggaa tcttaagatt	2640
gaatccgtt gccggcttgc cgatgattat catataattt ctgttgcattt acgttgcgttgc	2700
tgtataattt aacatgtaat gcatgacgtt atttatgaga tgggtttta tgatttaggt	2760
cccgcaattt tacatataat acgcgtataga aaacaaaata tagcgcgcacactaggataa	2820
attatcgccgc gcgggtgtcat ctatgttact agatc	2855

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<210> SEQ_ID NO 39
<211> LENGTH: 2745
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette MUbi(no hse)-BvLz(m)-35S
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1977)
<223> OTHER INFORMATION: MUbi (no hse) Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (668)..(668)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (671)..(671)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (681)..(681)

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<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1248)..(1248)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1315)..(1315)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1542)..(1542)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2032)..(2475)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2549)..(2745)
<223> OTHER INFORMATION: 35S

<400> SEQUENCE: 39

aagcttgcatt gcctgcagtgc agcgtgacc cggtcgtgcc cctctctaga gataatgagc      60
attgcattgtc taaggtaaa aaaattacca catattttt ttgtcacact tgtttgaagt      120
gcagtttatac tatctttata catatattta aactttactc tacgaataat ataatctata      180
gtactacaat aatatcagtgc tttagagaa tcatataat gaacagtttag acatggctta      240
aaggacaatt gagtattttt acaacaggac tctacagttt tatcttttta gtgtgcatt      300
gttctccccc ttttttgaa atagttcac ctatataata cttcatccat tttagttagta      360
catccattta gggttaggg ttaatggttt ttatagacta atttttttag tacatctatt      420
ttattctatt ttatgcattttt aattaagaaa actaaaactc tatttttagtt tttttttta      480
ataattttaga tataaaatag aataaaataa agtgactaaa attaaaacaa ataccctta      540
agaaataaaaaaa aaaactaagg aaacatttttt cttgtttcgaa gtagataatg ccagecttt      600
aacacccgtc gacgagtctt acggacacca accagcgaac cagcagcgtc gctggggcc      660
aagcgaanac nacggcacgg natctctgtc gctgcctcca ccgttggact tgctccgtg      720
tcggcatcca gaaatttgcgt ggcggcaggc agacgtgagc cggcacgagg cggccctcc      780
ctcctctcac ggcacggcag ctacggggaa ttcccttcccc accgctccctt cgcttccct      840
tcctcgcccg ccgtataaaaa tagacaccccc ctccacaccc tcttcccca acctcggtt      900
gttcggagcg cacacacaca caaccagatc tcccccaat ccacccgtcg gcaccccgcc      960
ttcaagggtac gcccgtcgcc ctcggggggcc cccctctctt accttctcta gatggcggt      1020
ccgggtccatgt gttaggggccc ggttagttcta cttctgttca tgtttgggtt agatccgtgt      1080
tttgtttaga tccgtgttgc tagcgttgcgt acacggatgc gacccgttacg tcagacacgt      1140
tctgattgttgc aacttgcacgg tggtttctttt tggggatcc tggggatggct ctggccgtc      1200
cgccagacggg atcgatttca tgatttttt tggttgcgtt catagggntt gggttgcctt      1260
ttccctttat ttcaatataat gcccgtccac ttgtttgttgc ggtcatctt tcatngctt      1320
tttttgcgtt ggttgcgtt atgtgggtctg gttgggggggt cgttcttagat cggagtagaa      1380
ttctgttca aactacctgg tggattttt aattttggat ctgtatgtgt gtgcataaca      1440
tattcatatgt tacgaatttgc agatgtatggat tggaaatatc gatcttaggtt aggtatacat      1500
gttgcgttgcgg gttttacttgc tgcatataaca gagatgtttt ntgcgttgcgtt gttgtatgt      1560
tgcgttgcgg tgggggggtc gttcatgttgcgt tcttagatggg agttagatatac tggttcaaac      1620
tacctgggtt atttataat tttggaaactg tatgtgtgtt tcatacatct tcatagttac      1680

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gagtttaaga tggatggaaa tatcgatcta ggataggat acatgttcat gtgggttta	1740
ctgatgcata tacatgtatgg catatgcgc atctattcat atgtctaac ctttagtacc	1800
tatctattat aataaacaag tatgtttat aattattttgc atcttgatat acttggatga	1860
tggcatatgc agcagctata tgtggatccc tttagccctgc ctttcatacg ctatcttt	1920
gttggtaact gtttcttttgc tcatgtctca cccgtttgtt tggtgttact tctgcaggc	1980
gactctagag gatctgatat ctgatcagaa gacaccatct cggatccaaa catggcggcc	2040
ctggtgatcc tgggttccct gttccctgtcc gtggctgtgc agggcaagggt gttcgaagg	2100
tgcgaactggc ottaggaccct gaagaagctg ggcctggatgc gatcacaagggg cgtgtccctg	2160
gctaactggc tgcgtctgac caagtggaa tcctcctaca acaccaaggc taccactac	2220
aaccatccct ccgaatccac cgactacggc atcttccaga tcaactccaa gtgggtggc	2280
aacgatggca agacccaaa cgctgtggat ggctgccacg tgcctgctc cgagctgatg	2340
gaaaacgata tcgctaaggc tggcttggaa gtcccactgc agggatcacg atgtgtccctc ctacgtggaa	2400
accgcctggg tggcttggaa gtcccactgc agggatcacg atgtgtccctc ctacgtggaa	2460
ggctgcaccc tgcgtattcga attcggatcc cccgggtgc aggaattcga tatcaagctt	2520
atcgatacccg tcgaggggtc cgcaaaaatc accagtctct ctctacaaat ctatctct	2580
ctatcttcttcc cagaataat gtgtgagtag ttcccgata agggatatttgc gtttcttata	2640
gggtttcgct catgtgttga gcatataaga aacccttagt atgtatttgt atttgtaaaa	2700
tacttctatc aataaaaatctt ctaattccata aaacccaaat ccagt	2745

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<210> SEQ ID NO 40
<211> LENGTH: 2977
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette MUbi (no hse) -BvLz (m) -3'SrMV
    UTR-35S
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1977)
<223> OTHER INFORMATION: MUbi (no hse) Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (668)..(668)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (671)..(671)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (681)..(681)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1248)..(1248)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1315)..(1315)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1542)..(1542)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2032)..(2475)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2494)..(2720)
<223> OTHER INFORMATION: 3' Untranslated Region of SrMV

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<220> FEATURE:	
<221> NAME/KEY:	terminator
<222> LOCATION:	(2781)..(2977)
<223> OTHER INFORMATION:	35S terminator
<400> SEQUENCE:	40
aagcttgc	60
atgc	120
gca	180
gtac	240
aagg	300
gttc	360
catcc	420
ttattc	480
ataatt	540
agaaatt	600
aaacgc	660
aaggca	720
tccctc	780
tcggat	840
tcgt	900
gttcgg	960
ttcaagg	1020
ccgg	1080
ttgtgtt	1140
tctcgat	1200
cgc	1260
ttccctt	1320
ttttgtt	1380
ttctgtt	1440
tattcat	1500
gttgatg	1560
tgtgg	1620
tacctgg	1680
gagttt	1740
ctgatgc	1800
tatctatt	1860
tggcatat	1920
gcttgg	1980
gactct	2040
ctgg	2100
tgcgaact	2160
gctaact	2220

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aaccatcct ccgaatccac cgactacggc atcttccaga tcaactccaa gtgggtgc	2280
aacgatggca agaccccaa cgctgtggat ggctgccacg tgcctgcgc cgagctgatg	2340
gaaaacata tcgctaaggc tgtggcttgc gctaagcaca tcgtgtccga acagggcatc	2400
accgcctggg tggcttggaa gtcccactgc agggatcacg atgtgtccctc ctacgtggaa	2460
ggctgcaccc tgtgattcga attcgatcc cccgatcttc attgcagttt ttAAAGTATT	2520
ttatatatTTT actatTCAG tgagggtctc ctccttagt attatatATG tactTCAGAA	2580
atAGTAGTCA ttctgcaggg gagtgaggtt cacCTCCAAc CCTATGGTA CTATTCTTA	2640
ctagcgtcga actacattac ggacaccctg ttgtgtgggtt ctaccacgag tcaggagctg	2700
cgagtattgt agcaagagaa gaattgggct gcaggaattc gatATCAAGC ttATCGATAC	2760
cgtcgagggg tccgcaaaaa tcaccagtct ctctctacaat atctatctct ctctatTTT	2820
ctccagaata atgtgtgagt agtcccaga taaggaaatt agggttctta tagggTTcg	2880
ctcatgtgtt gagcatataa gaaaccctta gtatgtattt gtatTTgtAA aataacttcta	2940
tcaataaaat ttctaattcc taaaacccaa atccagt	2977

<210> SEQ_ID NO 41
<211> LENGTH: 3114
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette MUbi(no hse)-5'SrMV
 UTR-BvLz(sc)-3'SrMV UTR-35S
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1977)
<223> OTHER INFORMATION: MUbi(no hse) Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (668)..(668)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (671)..(671)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (681)..(681)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (682)..(682)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1248)..(1248)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1315)..(1315)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1542)..(1542)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2028)..(2168)
<223> OTHER INFORMATION: 5' Untranslated Region of SrMV
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2169)..(2612)
<223> OTHER INFORMATION: BvLz (sc) coding sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2613)..(2847)
<223> OTHER INFORMATION: 3' Untranslated Region of SrMV
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2918)..(3114)
<223> OTHER INFORMATION: 35S

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<400> SEQUENCE: 41

aagcttgc	atgcgctgacc	cggtcgtgcc	cctctctaga	gataatgagc	60			
attgc	atgttcataa	aaaattacca	catattttt	ttgtcacact	tgtttgaagt	120		
geagtttatc	tatcttata	cataattta	aactttactc	tacgaaataat	ataatctata	180		
gtactacaat	aatatcagt	ttttagagaa	tcatataat	gaacagttag	acatggct	240		
aaggacaatt	gagtatttg	acaacaggac	tctacagtt	tatctttta	gtgtcatgt	300		
gttctc	tttttgcaa	atagttcac	ctatataata	cttcatccat	tttattagta	360		
catccat	tttttaggg	ttaatgg	ttatagacta	attttttag	tacatctatt	420		
ttattctatt	ttagectcta	aattaagaaa	actaaaactc	tattttagtt	tttttattta	480		
ataattt	atataatag	aataaaataa	agtgactaaa	aattaaacaa	ataccctt	540		
agaaattt	aaaactaagg	aaacattt	cttgttcga	gtagataat	ccagectgtt	600		
aaacgcgc	gacgagtcta	acggacacca	accagcga	cagcagcgtc	gctcgccc	660		
aagcga	anca	nacggcacgg	natctctgtc	gctgcctcca	ccgttggact	tgctccgctg	720	
tccgc	atcca	gaaattgcgt	ggcgccaggc	agacgtgagc	eggcaegagg	cgccctcc	780	
ctc	c	ggcacggcag	ctacggggga	ttcccttccc	accgctc	cgtttccct	840	
tcc	tcg	ccgcgtat	aaaaataaa	tagacacccc	ctccacaccc	tcttccccca	acctctgtt	900
gtt	cgg	gagcg	cacacacaca	caaccagatc	tccccaaat	ccacccgtcg	gcacctcc	960
ttc	aaagg	gtac	gttac	cccttttctc	accttctcta	gatcgcc	gtt	1020
cgg	gc	atgc	tttgcgt	tttgcgt	tttgcgt	tttgcgt	tttgcgt	1080
ttt	gtt	gat	gttac	atgcgtatc	gacctgtac	tca	gacac	1140
tct	gtt	gtt	gtt	atcttcttt	tggggatcc	tggggatgg	ctatccgtt	1200
cg	ca	gc	atgc	tttca	tgtttttt	tgtttttt	tttgcgtt	1260
ttt	c	c	c	ttcaatata	gcgtg	tttgcgt	tttgcgt	1320
ttt	ttt	ttt	ttt	tttgcgt	tttgcgt	tttgcgt	tttgcgt	1380
ttt	ttt	ttt	ttt	tttgcgt	tttgcgt	tttgcgt	tttgcgt	1440
tat	tat	tat	tat	tat	tat	tat	tat	1500
gtt	gtt	gtt	gtt	gtt	gtt	gtt	gtt	1560
tgt	tgt	tgt	tgt	tgt	tgt	tgt	tgt	1620
tac	tac	tac	tac	tac	tac	tac	tac	1680
gag	gat	gat	gat	gat	gat	gat	gat	1740
ctg	atg	atg	atg	atg	atg	atg	atg	1800
tat	tat	tat	tat	tat	tat	tat	tat	1860
tgg	cat	atg	atg	atg	atg	atg	atg	1920
gtt	gtt	gtt	gtt	gtt	gtt	gtt	gtt	1980
gac	tct	act	act	act	act	act	act	2040
tca	acaca	aca	aca	aca	aca	aca	aca	2100
tccaa	atcga	at	at	at	at	at	at	2160
cag	caa	acat	acat	acat	acat	acat	acat	2220
aaa	agg	ttt	cg	cg	cg	cg	cg	2280
aca	agg	gt	at	at	at	at	at	2340

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ccaaaggccac	gaactataac	ccctcctccg	agtctaccga	ttacgggatc	ttccagatta	2400
actccaatgt	gtgggtcaac	gacggaaaga	cccctaaccgc	ggtgacggt	tgccacgtgt	2460
cctgctccga	gcttatggag	aacgatattg	ccaaggcggt	tgcgtgcgcc	aagcatattg	2520
tgtccgagca	gggtatcacc	gcgtgggtcg	cctggaagag	ccactgcagg	gaccacgtg	2580
tgagcagcta	cgtggagggc	tgcacccctct	gatgtactga	gatcttcatt	gcagtttta	2640
aagtatttta	tatatttact	atttcagtga	gggtctccct	cotttagtatt	atatatgtac	2700
ttcagaaata	gtagtcattc	tgcagggag	tgaggttac	ctccaaacct	atggttacta	2760
tttcttacta	gcgtcgaact	acattacgga	caccctgttg	tgtggttcta	ccacgagtc	2820
ggagctgcga	gtattgtage	aagagaagaa	ttcggatccc	ccgggctgca	ggaattcgat	2880
atcaagctta	tcgataccgt	cgaggggtcc	gcaaaaatca	ccagtctctc	tctacaatc	2940
tatctctctc	tattttctc	cagaataatg	tgtgagtagt	tcggagataa	gggaatttagg	3000
gttctttag	ggtttcgctc	atgtgttag	catataagaa	acccttagt	tgtatttcta	3060
tttgtaaaat	acttctatca	ataaaatttc	taattcctaa	aaccaaatac	cagt	3114

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<210> SEQ ID NO 42
<211> LENGTH: 3020
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette MUBi(no hse)-BvLz(m)-35S
      NOS
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1977)
<223> OTHER INFORMATION: MUBi(no hse) Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (668)..(668)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (671)..(671)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (681)..(681)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1248)..(1248)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1315)..(1315)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1542)..(1542)
<223> OTHER INFORMATION: n is a, c, g, or t
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2032)..(2475)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2549)..(2745)
<223> OTHER INFORMATION: 35S
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2768)..(3020)
<223> OTHER INFORMATION: NOS

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<400> SEQUENCE: 42						
aagcttgcatt	gcctgcagt	cagcgtgacc	cggtcggtcc	cctctctaga	gataatgagc	60
attgcattgtc	taagttataa	aaaattacca	catatttttt	ttgtcacact	tgtttgaagt	120

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gcagtttac tatctttata catatattt aactttactc tacgaataat ataatctata	180
gtactacaat aatatcagt ttttagagaa tcatataat gaacagttag acatggcta	240
aaggacaatt gaggatttt acaacaggac tctacagtt tatctttta gtgtgcgt	300
gttctccctt tttttgcaa atagttcac ctatataata cttcatccat tttattagta	360
catccattt gggtttaggg ttaatggtt ttatagacta attttttagt tacatctatt	420
ttattctatt ttagcctcta aattaagaaa actaaaactc tatttttagt tttttattha	480
ataattttaga tataaaatag aataaaataa agtgactaaa aattaaacaa ataccctta	540
agaaattaaa aaaactaagg aaacattttt ctgtttcga gtagataatg ccagectgtt	600
aaacgcccgc gacgagtcta acggacacca accagcgaac cagcagcgtc gcgtcgcc	660
aagcgaanca nacggcacgg natctctgtc gctgcctcca cgggtggact tgctccgtg	720
tccgcacatcca gaaattgcgt ggcggcaggc agacgtgagc cggcacgagg cggcctcc	780
cctctctcac ggcacggcag ctacggggga ttcccttccc accgctccctt cgcttccct	840
tcctcgcccc cctgtataaaa tagacaccccc ctccacaccc tctttccca acctcggtt	900
gttcggagcg cacacacaca caaccagatc tcccccaaat ccacccgtcg gcacccgc	960
ttcaaggatc gccgctcg tcctccccc cccctctct accttcctta gatggcggt	1020
ccgggtccatg gttagggccc ggtagttcta ctctgttca tgtttgcgtt agatccgtgt	1080
ttgtgttaga tccgtgtcg tagcgttgc acacggatgc gacctgtacg tcagacacgt	1140
tctgattgt aacttgcag tgtttcttt tggggatcc tggggatggct ctacccgttc	1200
cgcacacggg atcgatttca tgatttttt tgtttgcgtt catagggntt ggtttgcct	1260
ttccctttat ttcaatataat gccgtgccac ttgttgtcg ggtcatctt tcatngctt	1320
tttttgtctt gggtgtatg atgtggctcg gttggcggt cgttctagat cggagtagaa	1380
ttctgttca aactacctgg tggattttt aattttggat ctgtatgtgt tgccataca	1440
tattcatatg tacgaattga agatgatgga tggaaatatac gatcttagat aggtatacat	1500
gttgatgcgg gttttactga tgcataataca gagatgtttt ntctcgctt gttgtatga	1560
tgtgggtgttgg ttggcggtc gttcattcgt tctagatcgg agtagaatac tgttcaaac	1620
tacctgggtt atttataat ttggaaactg tatgtgtgtc tcatacatct tcatatgtac	1680
gagttttaaga tggatggaaa tatcgatcta ggataggtat acatgttgc gtgggttta	1740
ctgatgcata tacatgtatgg catatgcgc atctattcat atgtcttaac cttggatacc	1800
tatctattat aataaacaag tatgttttat aatttttttgc atcttgcata acattggatga	1860
tggcatatgc agcagctata tgtggatttt tttagccctt ctttgcatacg ctatttttt	1920
gtttggtaact gtttttttgc tgcgtgtca cccgtttgtt tggtgttaact tctgcaggc	1980
gactctagag gatctgatata ctgatcgaa gacaccatct cggatccaaa catggcgcc	2040
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<210> SEQ ID NO 43
<211> LENGTH: 4020
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette SPRP(no 5'UTR)-5'SrMV
 UTR-BvLz(sc)-3'SrMV UTR-35S
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1) ..(2900)
<223> OTHER INFORMATION: SPRP(no 5'UTR) promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2934)..(3074)
<223> OTHER INFORMATION: 5' Untranslated Region of SrMV
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3075)..(3518)
<223> OTHER INFORMATION: BvLz (sc) coding sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3519)..(3753)
<223> OTHER INFORMATION: 3' Untranslated Region of SrMV
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (3824)..(4020)
<223> OTHER INFORMATION: 35S

<400> SEQUENCE: 43

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gagtagtgtg caagtgtgaa agcgtcggtt ttttatttga aaaaacaaaa aaatgcgcag	840
tatattaagg gacatcctaa ttaagaggct aagagcaaat gcacaacagt gtactccacg	900

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caagactcaa cacaacacaa caagacacag caaagcaact tatattgca cgc当地	3000
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<210> SEQ ID NO 44
<211> LENGTH: 3993
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette SPRP-BvLz(m)-3'SrMV UTR-35S
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(3016)
<223> OTHER INFORMATION: SPRP Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3048)..(3491)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3510)..(3736)
<223> OTHER INFORMATION: 3' Untranslated Region of SrMV
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (3797)..(3993)
<223> OTHER INFORMATION: 35S

<400> SEQUENCE: 44

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agggagcata tattgacact caaatgggc ctctgcctat taaataggc ccatgacagt	300
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155**-continued****156**

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cagaaaagga	catcgccgtt	ggaacaagg	acgagtgac	cactccggcc	acgcccgtacc	1080
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<210> SEQ ID NO 45
<211> LENGTH: 4045
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette SPRP-BvLz(m)-35S NOS
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(3016)
<223> OTHER INFORMATION: SPRP Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3048)..(3491)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (3565)..(3761)
<223> OTHER INFORMATION: 35SEsp
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (3793)..(4045)
<223> OTHER INFORMATION: NOS

<400> SEQUENCE: 45

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tatattaagg	gacatcctaa	ttaagaggct	aagagcaaat	gcacaacagt	gtactccacg	900

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159**160**

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agcatgccc当地 gtcaacacaca agctaaatgc cttttacccg tcccgtgtat catcaaagat	2580
taacaaacctt acctgtcagg cagcagcgta tatgtgaaac aagaaatgga tggaaagatgc	2640
cgtgagaaag taaaggatgaa agatacgtgc tactgtatctc cggtgaatag caataaacac	2700
gggcttagct gtttacccctt cgttgc当地 agccatggcca aacgtgtaaa gcagcttattt	2760
tttttaatg agagagtttgc当地 aaggcagctac ttatgtggcc agacagccca tccacgccc当地	2820
caaagctgct tggctctc当地 ggc当地 tccgatccatggcca ggc当地 acatccac	2880
atccacacac acaacacacgacttactcc当地 actaccaaca gctgctctag agaaagagag	2940
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ggagcagccgacttactcc当地 ctagccgaat tcctgc当地 cc当地 tccaaacatg gccc当地	3060
tgatcctgggg cttccctgttc ctgtccgtgg ctgtgc当地 agggatgtc gaaaggatgc当地	3120
aactggcttag gaccctgaag aagctggcc当地 tggatggcttca agggccgttccctggcttca	3180
actggctgttccctggcttca agggccgttccctggcttca acttactcc当地 aactacaacc	3240
catccctccgaa atccacccgacttactcc当地 tacggcatct tccagatcaa ctccaaatgg tggatgc当地	3300

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atggcaagac cccaaacgct gtggatggct gccacgtgtc ctgctccgag ctgatggaaa	3360
acgatatcgta taaggctgtg gcttgcgcta agcacatcgat gtccgaacag ggcatacccg	3420
cctgggtggc ttggaaagtcc cactgcaggatc acacatcgatgt gtcctctac gtggaaaggct	3480
geaccctgtg attcgaattc ggatcccccg ggctgcaggatc aatcgatatac aagtttatcg	3540
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ttttctccag aataatgtgt gtagtagttcc cagataaggaaatttagggtt ttataggg	3660
ttagctcatg ttttgagcat ataagaaacc ctttagtatgt atttgtattt gtaaaataact	3720
tctatcaata aaatttctaa ttccctaaac caaaatccag tgacctgcag gggccgcctcg	3780
acgaatttcc ccgatcggttc aaacatttgg caataaagttt tottaagatt gaatctgtt	3840
ggcgggtttt cgatgattt catataattt ctgttgaattt acgttaagca tgtaataattt	3900
aacatgtaat gcatgacgtt atttatgaga tgggttttta tgatttagagt cccgttattt	3960
tacatttat acgcgtataga aaacaaaata tagcgcgcaaa actaggataaa attatcgccc	4020
gggggtgtcat ctatgttact agatc	4045

<210> SEQ ID NO 46
<211> LENGTH: 2936
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette SEF1alpha-BvLz(m) -3'SrMV
 UTR-35S
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (1)..(1959)
<223> OTHER INFORMATION: SEF1alpha Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1991)..(2434)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2453)..(2679)
<223> OTHER INFORMATION: 3' Untranslated Region of SrMV
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2740)..(2936)
<223> OTHER INFORMATION: 35S

<400> SEQUENCE: 46

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gtcataaattc taaaaaagtc aaacgtctta taatttaaaa tggagggagg actttctaac	180
tcataccact tggcccttg ctgggttatta attactacat acaaagacca aaattgaata	240
gcacaaacttg attctcaaac caactatattt atcaaaatct atgctttgtt ccatttccaa	300
gcaaggaaaa ttagttgtga acgtgcaag tagtaaaggaa ccccttcca aaaggagac	360
gagccccat tggtaggaca aaaaaatctt tagtataattt gttttttttaaaatgttat	420
ataattctac tccatataatg acattaaatgt gtaactggta tctaaatgtc taagacata	480
acaaggatcaa aattcaaaact tattaaatctt aggtcccccg taccttccaa ctctttctgc	540
agtttaattt gtcacaaacg cctcccttctt tttttttttt ttccgtgtatc cctgtgcag	600
tataccaaaa tttaattttt ttttagcgtac caaaatgtt tttcaattt aattttcatg	660
tttcattttt gttttactac gtggatccaa ccatataacta cgtataacaag agcaactcca	720
agagatttgg taaaatttgcgtac tggtaatgg tggatgttccattatgtaaatagaaag	780
tctatctaaa atgtgtatgtt taaaaccat ccttactaaa ttggaaaaca caaatagcaa	840

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gtaggactcg ctagggaaat atggccageg agaatggtag gatacgacg tagaaaaaat	900
aaaaaccaat atagacagct gtttaatgt tttttaag aacattagct gtaatcgct	960
ttactataac attttgctca tggccacgag gcaggggtcg ggtcttgggt cttttttt	1020
ttgaaaacttgc attactcgcc taacaaatcg ttagcaacgg agtctatcca	1080
tataaaaaac aatagtatgt gttagtcgaa tgctgtttt gttcatttgt ggccatgaa	1140
gtgtttttt tggcccaat agcccattca tccatgcctg aaccctaggg cgcttcctt	1200
ataaaaaaccc agctccattc tggttcataa ccccaacacg cagtcggccg ccgcagaccc	1260
ggagtagccg acgcgcgcgc accgtatect cagatcagcg gcgagccgta accaagcaac	1320
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tcgctgtgtc ttgagccgtt ttgtttgtat actagatcta ctgggttgt cgctatgt	1440
tgatgagacg agccgattca tgcactggat ttctaatcaa gtgttggttc cgccgctgct	1500
acctctatattt agtgcataatg tatgaattt gttcagttt acaactgattt tgtcgagcca	1560
taaattataa ccgttgggt gttctagact agatccagtt tccgatctat gatattacgt	1620
ggctgaggca cttaactctg ttttgtgtgt aagaactgag ccgattcatg tgctggagta	1680
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gttgattgtt tgattactgg tttatccatataattttt tctaaactgtt tttatctgt	1920
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ggatccaaac atggggggcc tggtgatctt gggcttcgtt ttccctgtccg tggctgtgca	2040
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ctacaaggccgc gtgtccctgg ctaactggct gtgcctgacc aagtggaaat cctccatcaa	2160
caccaaggctt accaactaca acccatccctc cgaatccacc gactacggca tcttcagat	2220
caactccaaatggtggtgc acgtggcaaa gaccccaaaat gctgtggatg gctgcacgt	2280
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gggttcttat tgggtttccgc tcatgtgttg agcatataag aaacccttagt tatgtat	2880
tatttgtaaa atacttctat caataaaattt tctaattccctt aaaaccaaaa tccagtt	2936

<210> SEQ ID NO 47
 <211> LENGTH: 2988
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Expression cassette SEFialpha-BvLz(m)-35S NOS
 <220> FEATURE:
 <221> NAME/KEY: promoter

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<222> LOCATION: (1)..(1959)
<223> OTHER INFORMATION: SEF1alpha Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1991)..(2434)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2508)..(2704)
<223> OTHER INFORMATION: 3' S
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (2736)..(2988)
<223> OTHER INFORMATION: NOS

<400> SEQUENCE: 47

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gtcataaaatc taaaaaaagtc aaacgtctta taatttaaaa tggagggagg actttcta 180
tcataccact tgccccctt ctggtttata attactacat acaaagacca aaattgaata 240
gccaaacttg attctcaaac caactatccc atcaaaatct atgctttgt ccatttcaa 300
gcaagggaaa ttagttgtga acgtgcaag tagtaaagga ccccttcca aaaggagac 360
gagccccat ttttaggaca aaaaaatctt tagtatatta gtttttatt taaagtctat 420
ataattctac tccatatacg acattaaatgt gtaactggta tctaaagatc taagacata 480
acaagcataa aattcaaaact tattaaatctt aggtcccccg tacctccaa ctcttctgc 540
agtttaattc gtcacaaacg cctccctt tttttttt ttccgtgtta cctgtgcag 600
tataccaaaa tttaatttt tttgagcgac caaaataacct tttcgaattt aattttcatg 660
tttcattttt gttttactac gtggtatcca ccatatacta cgtataacaag agcaactcca 720
agagatttgg taaaattaga tgctaaattt tgagattttt ccattatgtt aaatagaaag 780
tctatctaa atgttagatt taaaaccag cctaactaa ttggaaaaca caaatagcaa 840
gttaggactcg ctagggaaat atggccagcg agaatggtag gataccgc tagaaaaat 900
aaaaaccaat atagacagct gttgtatgt ttttttaaag aacatttagt gtaatcgct 960
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ttgaaacttg gtaaaacttc attactcgcc taacaaatcg ttagcaacgg agtctatcca 1080
tataaaaaac aatagtatgt gtaggtcgaa tgctttttt gttcattttt ggccatgaa 1140
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ataaaaaaccc agctccattc tgttctcaa ccccaacacg cagtcggccg ccgcagaccg 1260
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acctctattt agtgtctatg tatgaattt gttcgatgtt acaactgatt tgtcgagcca 1560
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cagttccga tctctgattt acctgcgtt ggtacttcgtt ctttggat tccctgtcctt 1860
gttgattgtt tgattactgg tttatccaa tatattttt tctaaactgtt tttatctgt 1920

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attttatgt aagcagcagt gtagcggttc ctttgcggcc aattctgtca gccccatctc	1980
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ggccaagggt ttccaaagggt gcgaactggc taggaccctg aagaagctgg gcctggatgg	2100
ctacaagggc gtgtccctgg ctaactggct gtgcctgacc aagtggaaat cctctacaa	2160
caccaaggct accaactaca acccatcctc cgaatccacc gactacggca tcttccagat	2220
caactccaag tggtggtgca acgtggcaa gacccaaac gctgtggatg gctgccacgt	2280
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cgtgtccgaa cagggcatca ccgcctgggt ggcttggaaat tcccaactgca gggatcacga	2400
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ggaaatttagg gttttatag ggttccgttc atgtgtttag catataagaa acccttagta	2640
tgtatgttta tttgtaaaat acttcttatca ataaaatttc taattcctaa aaccaaaatc	2700
cagtgacctg cagggccgc tcgacgaatt tcccgatcg ttccaaacatt tggcaataaa	2760
gtttcttaag attgaatctt gttccggc ttgcgtatgtatcatataa ttctgttga	2820
attacgttaa gcatgtataa attaacatgt aatgcgtac gtttattatg agatgggtt	2880
ttatgatttag agtcccgcaa ttatacattt aatacgcgt agaaaacaaa atatagcgcg	2940
caaactagga taaattatcg cgcgcgggtt catctatgtt actagatc	2988

<210> SEQ ID NO 48
<211> LENGTH: 3621
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette JAS-BvLz (m) -35S
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (54)..(2681)
<223> OTHER INFORMATION: JAS Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2692)..(3135)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (3425)..(3621)
<223> OTHER INFORMATION: 35S

<400> SEQUENCE: 48

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atgcccataatgc caccggcccc taagtcaacc tgcggggatgg gtagcttcgc cagggtcaga	180
gtcgccggcgtt caccctggta gacccgttcc ctgtatgaccc ggggggttgcg ctccatgaag	240
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tgcattgcacc tagccgccttgc gcagcgatctt cctgtatgtat cacctgcgtc gcctccagct	360
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tctcttttctt tggccatctt ttggatattt cacaatgtc cccctacaaa tgataatca	540
ccaaaactca tggagcttgc tagttataaa ctcttaattctt aagtttgggtt tttatgtttag	600
tggattttgtt gtgaaagttt gttgggtttagaa ataggatgtt aggaccgcac acaagatccc	660

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ccacacttag cccttgctc atcctcgagt aaagttcaag gactaagggtg gaacatctcc	720
tcaaatggta cgatgcctgc atataagttt ttcagaagcc cacctataca tgtgaacttt	780
gaagtgtcta ccacgcctac ttgggtgggt gagaaatggaa acagatcaga atccagtcata	840
ctttacctct cttgtttaga taacttgggt tttttaagg ttttcaatt taaaacatag	900
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tgtttgcct ctttcatec tacttctaat atttcttttggagttt ggttagggaaat	1020
aaaaaggaaag catacttgca ttgcataatgt tactaagtca aaaaccaaataat ctgaggagaa	1080
gcaagtcata caatctgatc aagatgtgca agtgtgtggaa tatgtggatt aagataactc	1140
ctgtttatcc atgctctcct ccttaataaaa ctttagaggg catggcaatc tttgcattggg	1200
ccttcatcgag ctcatcgat gtctaaagcat ggagctcatc attttatataa gcatgggtat	1260
accaaaaatta ctcctttga gcatgtttat atttaggggg acgttttacc tggtgaggta	1320
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ttgatgatgg acaatattga tgagggtgact gacaaatgat tgaaggctt aaaggagatt	1440
gagaaggata aatctacaat aaaaatgtaa agaagaaagc attcaaagtg tgagatctgg	1500
tgtggaaagac tattttgcct cttgggggtaa aaagacaaca agtttagtaa gtggcctcaa	1560
aattgggagg gccccatgcaaa gattgttaaa gtaattgtttt tggattgacg gaggcatttc	1620
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cccaattttt ttccctttcc aaccctaattt atagtttcc ttttgcctct aggacaaattt	1980
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aaaaacgata tcgctaaggc tggatgttccctt gctaaactggc tcaactccaa gttggctgtgc	3060

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accgcctggg tggcttggaa gtcccactgc agggatcagc atgtgtcc tcacgtggaa	3120
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ttttatatat ttactatssc agtgagggtc tccctcctta gtattatata tgtaatttcag	3240
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tactagcgta gaactacatt acggacaccc tgggtgtgg ttctaccacg agtcaggagc	3360
tgcgagtatt gtagcaagag aagaatttac aagcttacg ataccgtcga ggggtccgca	3420
aaaatcacca gtctctctct acaaatttat ctctctctat ttttctccag aataatgtgt	3480
gagtagttcc cagataaggg aattagggtt cttatagggt ttcgctcatg tggtgagcat	3540
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ttcctaaac caaaatccag t	3621

<210> SEQ_ID NO 49
<211> LENGTH: 3855
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Expression cassette JAS-BvLz(m) -3'SrMV UTR-35S
<220> FEATURE:
<221> NAME/KEY: promoter
<222> LOCATION: (54)..(2681)
<223> OTHER INFORMATION: JAS Promoter
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2694)..(3137)
<223> OTHER INFORMATION: BvLz (m) coding sequence
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (3158)..(3384)
<223> OTHER INFORMATION: 3' Untranslated Region of SrMV
<220> FEATURE:
<221> NAME/KEY: terminator
<222> LOCATION: (3659)..(3855)
<223> OTHER INFORMATION: 35S

<400> SEQUENCE: 49	
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tgcattgcacc tagcgcctg gcagcgagct cctgtatgtat cacctgcgtc gcctccagct	360
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What is claimed is:

1. An isolated nucleic acid comprising:
 - (a) an expression control sequence having promoter activity in at least one monocot and at least one dicot, wherein the expression control sequence is selected from the group consisting of the sequence of nucleotides 1-1786 of SEQ ID NO: 1; the sequence of SEQ ID NO: 1, the sequence of SEQ ID NO: 17, the sequence of SEQ ID NO: 26, and the sequence of SEQ ID NO: 27 and
 - (b) an expressible nucleic acid, wherein the expressible nucleic acid is heterologous to the expression control sequence.
2. An isolated nucleic acid according to claim 1, wherein the expressible nucleic acid comprises a nucleic acid sequence selected from the group consisting of: a human sequence, a non-human animal sequence, a plant sequence, a

yeast sequence, a bacterial sequence, a viral sequence, an artificial sequence, an antisense sequence thereof, and combinations thereof.

3. An isolated nucleic acid according to claim 1, wherein the expressible nucleic acid alters carbon metabolism in the plant cell when expressed or transcribed.

4. An isolated nucleic acid according to claim 1, wherein the expressible nucleic acid encodes an insecticide effective against at least one stem-boring insect.

5. An expression vector comprising, in a 5' to 3' direction: a sugarcane bacilliform virus (SCBV) promoter having the sequence of SEQ ID NO: 1; an expressible nucleic acid; and a 3' termination sequence, wherein the SCBV promoter has promoter activity sufficient to express the expressible nucleic acid in at least one monocot and at least one dicot, and wherein the expressible nucleic acid is heterologous to the sequence of SEQ ID NO: 1.

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6. An expression vector according to claim 5, wherein the expressible nucleic acid comprises a nucleic acid sequence selected from the group consisting of: a human sequence, a non-human animal sequence, a plant sequence, a yeast sequence, a bacterial sequence, a viral sequence, an artificial sequence, an antisense sequence thereof, and combinations thereof.

7. A bacterial cell comprising the expression vector according to claim 5.

8. A plant cell comprising the expression vector according to claim 5.

9. An expression vector according to claim 5 further comprising a linker 3' of the expression control sequence, 5' of the expressible nucleic acid, and having a length of from about 1 to about 200 nucleotides.

10. A bacterial cell comprising an expression vector having:

a SCBV promoter having a sequence selected from the group consisting of the sequence of nucleotides 1-1786 of SEQ ID NO: 1, the sequence of SEQ ID NO: 1, the sequence of SEQ ID NO: 17, the sequence of SEQ ID NO: 26, and the sequence of SEQ ID NO: 27; an expressible nucleic acid; and

a 3' termination sequence, wherein the SCBV promoter has promoter activity sufficient to express the expressible exogenous nucleic acid in at least one monocot and at least one dicot.

11. A plant cell comprising an expression vector having: a promoter having a SCBV sequence selected from the group consisting of the sequence of nucleotides 1-1786 of SEQ ID NO: 1, the sequence of SEQ ID NO: 1, the sequence of SEQ ID NO: 17, the sequence of SEQ ID NO: 26, and the sequence of SEQ ID NO: 27; an expressible nucleic acid operably linked to the promoter;

and a 3' termination sequence, wherein the promoter has promoter activity sufficient to express the expressible nucleic acid in at least one monocot and at least one dicot, and wherein the expressible nucleic acid is heterologous to the sequence of the promoter.

12. A plant cell according to claim 11, wherein the expressible nucleic acid comprises a nucleic acid sequence selected from the group consisting of: a human sequence, a non-human animal sequence, a plant sequence, a yeast sequence, a bacterial sequence, a viral sequence, an artificial sequence, an antisense sequence thereof, and combinations thereof.

13. A plant cell according to claim 11, wherein the expressible nucleic acid alters carbon metabolism in the plant cell when expressed or transcribed.

14. A plant cell according to claim 11, wherein the expressible nucleic acid encodes an insecticide effective against at least one stem-boring insect.

15. A plant regenerated from the plant cell according to claim 11.

16. A plant cell according to claim 14, wherein the plant cell is a monocot plant cell.

17. A plant cell according to claim 14, wherein the plant cell is selected from the group consisting of sugarcane cell, miscanthus cell, a miscanthus x sugarcane hybrid cell, a switch grass cell, an oats cell, a wheat cell, a barley cell, a maize cell, a rice cell, a banana cell, a yucca cell, an onion cell, an asparagus cell, a sorghum cell, and cells of hybrids thereof.

18. A plant cell according to claim 14, wherein the plant cell is a dicot plant cell.

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19. A plant cell according to claim 14, wherein the plant cell is selected from the group consisting of a coffee cell, a tomato cell, a pepper cell, a tobacco cell, a lima bean cell, an *Arabidopsis* cell, a rubber cell, an orange cell, a grapefruit cell, a potato cell, a grapefruit cell, a potato cell, a squash cell, a pea cell, and a sugar beet cell.

20. A plant comprising an expression vector having: a promoter having a SCBV sequence-selected from the group consisting of the sequence of nucleotides 1-1786 of SEQ ID NO: 1, the sequence of SEQ ID NO: 1, the sequence of SEQ ID NO: 17, the sequence of SEQ ID NO: 26, and the sequence of SEQ ID NO: 27; an expressible nucleic acid operably linked to the promoter; and a 3' termination sequence, wherein the promoter has promoter activity sufficient to express the expressible nucleic acid in at least one monocot and at least one dicot, and wherein the expressible nucleic acid is heterologous to the sequence of the promoter.

21. A plant according to claim 20, wherein the expressible nucleic acid alters carbon metabolism in the plant cell when expressed or transcribed.

22. A plant according to claim 20, wherein the expressible nucleic acid encodes an insecticide effective against at least one stem-boring insect.

23. A plant according to claim 20, wherein the plant is a monocot.

24. A plant according to claim 20, wherein the plant is selected from the group consisting of sugarcane, miscanthus, a miscanthus x sugarcane hybrid, switch grass, oats, wheat, barley, maize, rice, banana, yucca, onion, asparagus, sorghum, and hybrids thereof.

25. A plant according to claim 20, wherein the plant is a dicot.

26. A plant according to claim 20, wherein the plant is selected from the group consisting of coffee, tomato, pepper, tobacco, lima bean, *Arabidopsis*, rubber, orange, grapefruit, potato, squash, peas, and sugar beet.

27. A method for constitutively expressing an expressible nucleic acid in a plant, the method comprising:

contacting an expression cassette or expression vector with the cytosol of a cell of the plant, wherein the expression cassette or expression vector comprises (i) the expressible nucleic acid, (ii) a SCBV promoter comprising a SCBV sequence-selected from the group consisting of the sequence of nucleotides 1-1786 of SEQ ID NO: 1, the sequence of SEQ ID NO: 1, the sequence of SEQ ID NO: 17, the sequence of SEQ ID NO: 26, and the sequence of SEQ ID NO: 27 and operable to drive expression of the expressible nucleic acid, and (iii) a 3' termination sequence operably linked to the expressible nucleic acid, and

wherein the plant is selected from the group consisting of a monocot and a dicot.

28. A method according to claim 27, wherein the contacting further comprises biolistically bombarding the cell with a particle comprising the expression cassette.

29. A method according to claim 27, wherein the contacting further comprises co-cultivating the cell with a *Agrobacterium* cell comprising the expression cassette.

30. A method according to claim 27, wherein the plant is a monocot.

31. A method according to claim 27, wherein the plant is selected from the group consisting of sugarcane, miscanthus,

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a miscanthus x sugarcane hybrid, switch grass, oats, wheat, barley, maize, rice, banana, yucca, onion, asparagus, sorghum, and hybrids thereof.

32. A method according to claim **27**, wherein the plant is a dicot.

33. A method according to claim **27**, wherein the plant is selected from the group consisting of coffee, tomato, pepper, tobacco, lima bean, *Arabidopsis*, rubber, orange, grapefruit, potato, squash, peas, and sugar beet.

34. A method of directing constitutive expression of a nucleic acid in a plant comprising:

transforming the plant with an expression nucleic acid, the expression nucleic acid comprising a promoter having its sequence selected from the group consisting of the sequence of nucleotides 1-1786 of SEQ ID NO: 1, the sequence of SEQ ID NO: 1, the sequence of SEQ ID NO: 17, the sequence of SEQ ID NO: 26, and the sequence of SEQ ID NO: 27, an expressible nucleic acid, and a 3' termination sequence,

wherein the plant is selected from the group consisting of a monocot and a dicot.

35. A method according to claim **34**, wherein the expression nucleic acid comprises an expression vector.

36. A method according to claim **34**, wherein transforming further comprises biolistically bombarding the plant with a particle comprising the expression cassette.

37. A method according to claim **34**, wherein transforming further comprises co-cultivating the plant with a *Agrobacterium* cell comprising the expression cassette.

38. A method according to claim **34**, further comprising transforming an embryonic callus.

39. A method according to claim **34** further comprising regenerating a plant from the embryonic callus.

40. A method according to claim **34**, further comprising transforming a plant cell.

41. A method according to claim **40**, further comprising breeding progeny of the transformed plant.

42. A method according to claim **34**, wherein the plant is a monocot.

43. A method according to claim **34**, wherein the plant is selected from the group consisting of sugarcane, miscanthus, a miscanthus x sugarcane hybrid, switch grass, oats, wheat, barley, maize, rice, banana, yucca, onion, asparagus, sorghum, and hybrids thereof.

44. A method according to claim **34**, wherein the plant is a dicot.

45. A method according to claim **34**, wherein the plant is selected from the group consisting of coffee, tomato, pepper, tobacco, lima bean, *Arabidopsis*, rubber, orange, grapefruit, potato, squash, peas, and sugar beet.

46. An isolated nucleic acid comprising:

(a) an expression control sequence having promoter activity in at least one monocot and at least one dicot, wherein

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the expression control sequence has the sequence of nucleotides 632-716 of SEQ ID NO: 33 and

(b) an expressible nucleic acid, wherein the expressible nucleic acid is heterologous to the expression control sequence.

47. An isolated nucleic acid according to claim **46**, wherein the expression control sequence is at least about 0.75 kb.

48. An isolated nucleic acid comprising:

(a) an expression control sequence having promoter activity in at least one monocot and one dicot, wherein the expression control sequence has the sequence of SEQ ID NO: 33 and

(b) an expressible nucleic acid, wherein the expressible nucleic acid is heterologous to the expression control sequence.

49. An isolated nucleic acid comprising in a 5' to 3' direction:

(a) an expression control sequence, having a 3' end and having promoter activity in at least one monocot and at least one dicot, and

(b) an expressible nucleic acid having a 5' end, wherein

the expression control sequence comprises the sequence of nucleotides 1-1786 of SEQ ID NO: 18, the 3' end of the expression control sequence and the 5' end of the expressible nucleic acid together comprise the nucleotide sequence AAAATGG, and the expressible nucleic acid is heterologous to the expression control sequence.

50. An isolated nucleic acid according to claim **49**, wherein the 3' end of the expression control sequence comprises the nucleic acid sequence of nucleotides 1787-1860 of SEQ ID NO: 18 and the 5' end of the expressible nucleic acid comprises the nucleic acid sequence of nucleotides 1861-1864 of SEQ ID NO: 18.

51. An isolated nucleic acid comprising:

(a) an expression control sequence having promoter activity in at least one monocot and at least one dicot, wherein the expression control sequence is selected from the group consisting of the sequence of SEQ ID NO: 18, the sequence of SEQ ID NO: 26, the sequence of SEQ ID NO: 27, the sequence of SEQ ID NO: 32, and the sequence of SEQ ID NO: 33, and

(b) an expressible exogenous nucleic acid,

wherein the expression control sequence has promoter activity in at least one monocot and at least one dicot and the expressible nucleic acid is heterologous to the expression control sequence.

52. An isolated nucleic acid according to claim **51**, wherein the nucleic acid further comprises a 3' terminator.

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