



(86) Date de dépôt PCT/PCT Filing Date: 2011/07/07
(87) Date publication PCT/PCT Publication Date: 2012/01/12
(45) Date de délivrance/Issue Date: 2019/10/22
(85) Entrée phase nationale/National Entry: 2012/12/28
(86) N° demande PCT/PCT Application No.: US 2011/043217
(87) N° publication PCT/PCT Publication No.: 2012/006439
(30) Priorité/Priority: 2010/07/07 (US61/362,222)

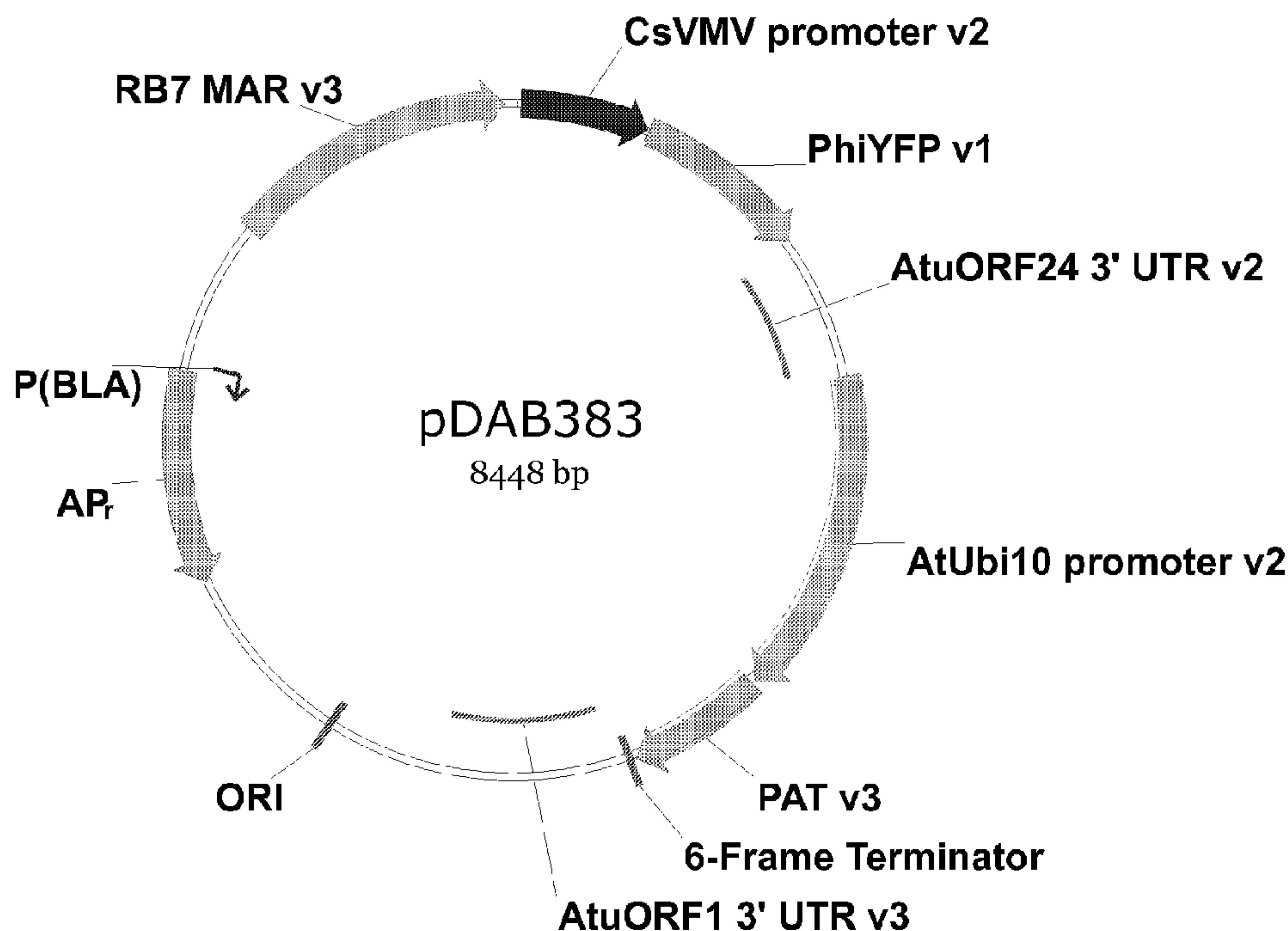
(51) Cl.Int./Int.Cl. *C12N 15/87* (2006.01),
B82Y 5/00 (2011.01), *C12N 15/82* (2006.01)

(72) Inventeurs/Inventors:
YAU, KERRM Y., US;
SAMUEL, JAYAKUMAR PON, US;
BURROUGHS, FRANK G., US;
SAMBOJU, NARASIMHA CHARY, US;
WEBB, STEVEN R., US

(73) Propriétaire/Owner:
DOW AGROSCIENCES LLC, US

(74) Agent: SMART & BIGGAR

(54) Titre : PRODUCTION DE CASSETTE D'ADN LINEAIRE FONCTIONNALISE ET SA DIFFUSION DANS DES
PLANTES ASSISTEE PAR DES POINTS QUANTIQUES/NANOPARTICULES
(54) Title: PRODUCTION OF FUNCTIONALIZED LINEAR DNA CASSETTE AND QUANTUM DOT/NANOPARTICLE
MEDIATED DELIVERY IN PLANTS



(57) **Abrégé/Abstract:**

Methods for introducing a functionalized linear nucleic acid cassette molecule of interest into a plant cell comprising a cell wall include use of nanoparticles. In some embodiments, the cell comprising a cell wall is a cultured plant cell. Methods include genetically or otherwise modifying plant cells and for treating or preventing disease in any plant, especially crop plants. Transgenic plants include a nucleic acid molecule of interest produced by regeneration of whole plants from plant cells transformed with functionalized linear nucleic acid cassette molecules.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 January 2012 (12.01.2012)

(10) International Publication Number
WO 2012/006439 A3

(51) International Patent Classification:

C12N 15/87 (2006.01) *C12Q 1/68* (2006.01)
C12N 15/82 (2006.01) *B82Y 5/00* (2011.01)
A01H 5/00 (2006.01)

(21) International Application Number:

PCT/US2011/043217

(22) International Filing Date:

7 July 2011 (07.07.2011)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/362,222 7 July 2010 (07.07.2010) US

(71) Applicant (for all designated States except US): **DOW AGROSCIENCES LLC** [US/US]; 9330 Zionsville Road, Indianapolis, Indiana 46268 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **YAU, Kerm Y.** [CA/US]; 14482 Baldwin Lane, Carmel, Indiana 46032 (US). **SAMUEL, Jayakumar Pon** [US/US]; 3336 Kilkenny Circle, Carmel, Indiana 46032 (US). **BURROUGHS, Frank G.** [US/US]; 929 Queensbury Drive, Noblesville, Indiana 46062 (US). **SAMBOJU, Narasimha Chary** [IN/US]; 12914 University Crescent Rd, Apt 2d, Carmel, Indiana 46032 (US). **WEBB, Steven R.** [CA/US]; 15416 Bloomfield Court, Westfield, Indiana 46074 (US).

(74) Agent: **CATAXINOS, Edgar R.**; TraskBritt, P.O. Box 2550, Salt Lake City, Utah 84110 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))

Published:

— with international search report (Art. 21(3))

[Continued on next page]

(54) Title: PRODUCTION OF FUNCTIONALIZED LINEAR DNA CASSETTE AND QUANTUM DOT/NANOPARTICLE MEDIATED DELIVERY IN PLANTS

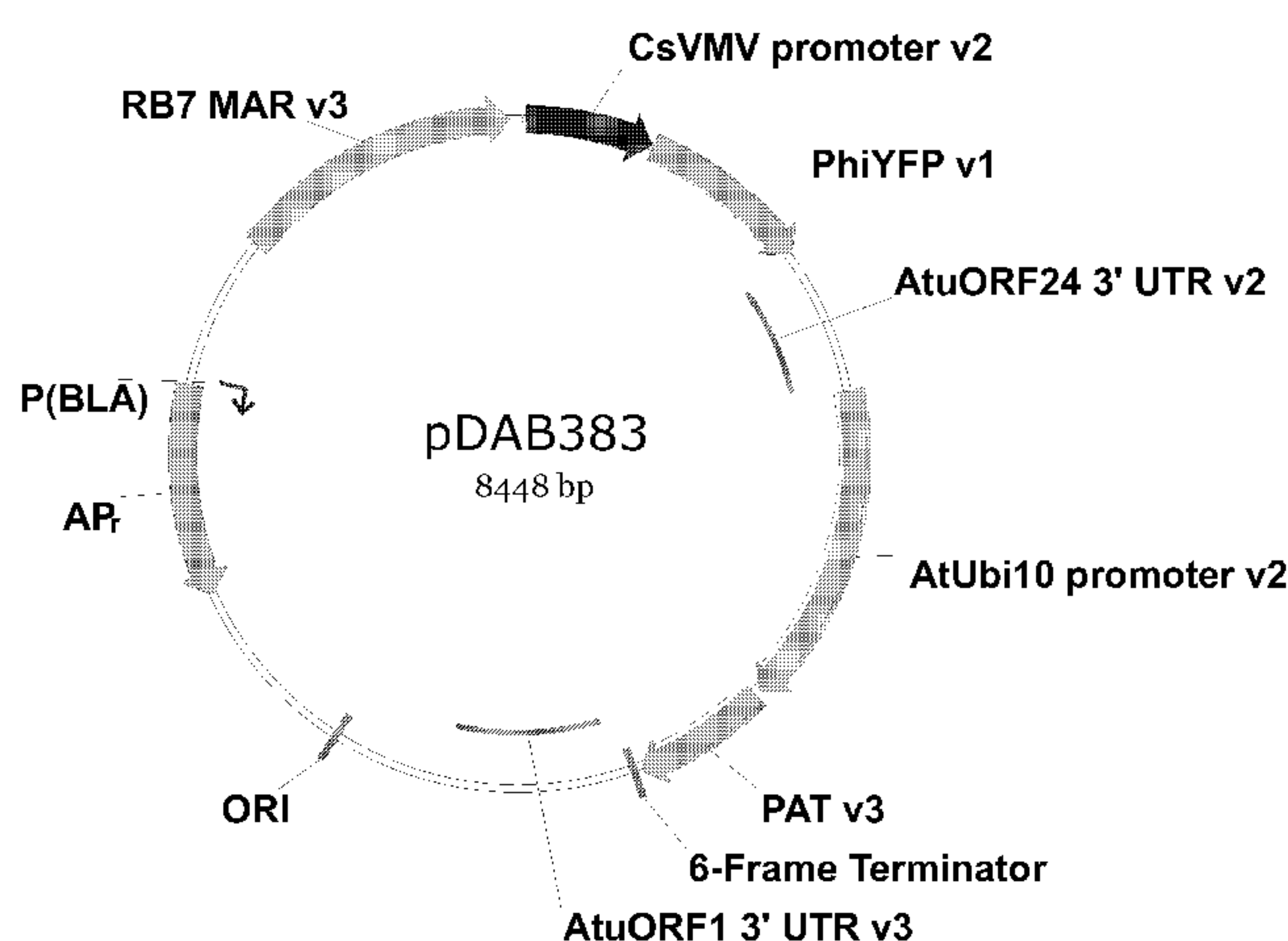


Fig. 1

(57) Abstract: Methods for introducing a functionalized linear nucleic acid cassette molecule of interest into a plant cell comprising a cell wall include use of nanoparticles. In some embodiments, the cell comprising a cell wall is a cultured plant cell. Methods include genetically or otherwise modifying plant cells and for treating or preventing disease in any plant, especially crop plants. Transgenic plants include a nucleic acid molecule of interest produced by regeneration of whole plants from plant cells transformed with functionalized linear nucleic acid cassette molecules.

WO 2012/006439 A3



-
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*
- *with sequence listing part of description (Rule 5.2(a))*
- (88) Date of publication of the international search report:**
29 March 2012

-1-

PRODUCTION OF FUNCTIONALIZED LINEAR DNA CASSETTE AND
QUANTUM DOT/NANOPARTICLE MEDIATED DELIVERY IN PLANTS

5

PRIORITY CLAIM

This application claims the benefit of the filing date of United States Provisional Patent Application Serial Number 61/362,222 filed July 7, 2010, for “PRODUCTION OF FUNCTIONALIZED LINEAR DNA CASSETTE AND QUANTUM DOT/NANOPARTICLE MEDIATED DELIVERY IN PLANTS.”

10

TECHNICAL FIELD

The present invention relates to methods using nanoparticles to non-invasively deliver functionalized linear nucleic acid cassette molecules into plant cells having an intact cell wall.

15

BACKGROUND

Nanoparticles have unique properties that have been exploited to deliver DNA to specific animal cells. It has been found that when certain DNA-coated nanoparticles are incubated with cells not having a cell wall, the cells take up the nanoparticles and begin
20 expressing genes encoded on the DNA. Semi-conductor nanoparticles (*e.g.*, quantum dots (“QDs”)) within the size range of 3-5 nm have also been used as carriers to deliver molecules into cells. DNA and proteins can be linked to certain ligands attached to the QD surface. *See, e.g.*, Patolsky, *et al.* (2003) J. Am. Chem. Soc. 125:13918. Carboxylic acid- or amine-coated QDs can be cross-linked to molecules containing a thiol group, *see e.g.*,
25 Dubertret *et al.* (2002) Science 298:1759; Akerman, *et al.* (2002) Proc. Natl. Acad. Sci. U.S.A. 99:12617; Mitchell, *et al.* (1999) J. Am. Chem. Soc. 121:8122, or an N-hydroxysuccinimyl (“NHS”) ester group, by using standard bioconjugation protocols. *See e.g.*, Pinaud, *et al.* (2004) J. Am. Chem. Soc. 126:6115; Bruchez, *et al.* (1998) Science 281:2013. An alternative way to attach molecules to the surface of QDs is *via* conjugation
30 of streptavidin-coated QDs to biotinylated proteins, oligonucleotides, or antibodies. *See,*

-2-

e.g., Dahan, *et al.* (2003) *Science* 302:442; Pinaud, *et al.* (2004) *J. Am. Chem. Soc.* 126:6115; Wu, *et al.* (2003) *Nature Biotechnol.* 21:41; Jaiswal, *et al.* (2003) *Nature Biotechnol.* 21:47; and Mansson, *et al.* (2004) *Biochem. Biophys. Res. Commun.* 314:529.

Delivery of foreign nucleic acid molecules to plants is challenging due to the presence of plant cell walls. Current methods rely on invasive delivery for genetic transformation of plants. In plant cells, the cell wall is a barrier against the delivery of exogenously applied molecules. Many invasive cell delivery methods, for example, biolistic delivery (gene gun), microinjection, electroporation, and *Agrobacterium*-mediated transformation, have been employed to achieve gene and small molecule delivery into walled plant cells, but delivery of proteins has only been achieved by microinjection. Where nanoparticle delivery of nucleic acid molecules to plant cells is desired, the cell wall is stripped before the addition of the particles to protoplasts of plant. See, e.g., Torney, *et al.* (2007) *Nature Nanotechnol.* 2:295-300.

Moreover, conventional plant transformation techniques, such as *Agrobacterium*-mediated transformation, require the use of a recombinant plasmid. These conventional techniques, therefore, result undesirably in the integration of the bacterial vector backbone sequence into the host genome along with the attached exogenous genes. *See, e.g.*, Kohli *et al.* (1999) *Plant J.* 17:591-601; and Meza *et al.* (2002) *Nucleic Acids Res.* 30(20):4556-66. The presence of the vector backbone sequence in the transplant serves no purpose in biolistic transfer procedures. Furthermore, the vector backbone sequences have a tendency to stimulate illegitimate recombination by providing AT-rich sequences as recombination hotspots during the formation of secondary structures. Muller *et al.* (1999) *J. Mol. Biol.* 291:29-46. Vector backbone sequences may additionally produce new lengths of “filler” DNA homologous to flanking plant genomic DNA, which may escape into the environment. Kohli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:7203-8; Pawlowski and Somers (2000) *Proc. Natl. Acad. Sci. U.S.A.* 95:12106-10; Svitashchev *et al.* (2002) *Plant J.* 32:433-45.

Transformation with transgene cassettes using particle bombardment has had only limited success, in tissue culture, and in rice (*Oryza sativa*) and potato (*Solanum tuberosum*). Fu *et al.* (2000) *Transgenic Res.* 9:11-9; Loc *et al.* (2002) *Mol. Breeding*

-3-

9:231-44; Romano *et al.* (2003) Transgenic Res. 12:461-73; and Agrawal *et al.* (2005) Mol. Breeding 16:247-60. These biolistic techniques have been suggested to generate a larger proportion of transgenic rice and potato with simple integration patterns. Two groups of linear gene constructs (*gus* and *bar*, and *1Ax1* and *bar*) lacking vector backbone sequences have been independently transferred into the elite wheat (*Triticum aestivum* L.) variety EM12 by particle bombardment, and genetically stable transgenic plants with low copy number transgene integration were recovered. Yao *et al.* (2006) J. Exp. Botany 57(14):3737-46. Transformation frequency by biolistic bombardment was observed to be between 0.2 and 0.6. *Id.* It has been suggested that three possible elements (*i.e.*, reducing the amount of concatemerization prior to transgene integration; limiting the occurrence of transgene rearrangements; and preventing homologous interactions between different transgenes during integration events) work together to generate simple intact transgenic loci represented by simple hybridization patterns. Agrawal *et al.* (2005), *supra*.

Particle bombardment and Whiskers™ (See U.S. Patent Nos. 5,464,765 and 5,302,523), together with restriction enzyme-digested DNA fragments, is the only route of delivering linear DNA cassettes to plant cells having intact cell walls at this time.

DISCLOSURE OF THE INVENTION

Described herein are methods and compositions for use of nanoparticles and linearized nucleic acid molecules for introducing a molecule of interest into a plant cell having a cell wall. Some embodiments of methods of the disclosure may be used to produce a stably-transformed genetically modified fertile plant. In some embodiments, the distinctive properties of functionalized linear nucleic acid cassette molecules allow the delivery of specific gene sequences of interest without undesirable nucleic acid sequences, for example and without limitation, vector backbone sequences.

In embodiments, several different types of nanoparticles may be used to transform plant cells having a cell wall. In some embodiments, nanoparticles may be PEGylated with functionalized linear nucleic acid cassette molecules. In particular embodiments, the nanoparticles may be semi-conductor nanoparticles, such as quantum dots ("QDs"); or gold nanoparticles. In other embodiments, the functionalized linear nucleic acid cassette

-4-

molecules may be linearized plasmid DNA. In alternative embodiments, the functionalized linear nucleic acid cassette molecules may comprise sequences encoding Phosphinothricin-N-acetyltransferase (PAT) and/or Yellow fluorescence protein (YFP).

Also disclosed are methods for introducing a molecule of interest into a plant cell
5 having a cell wall, wherein the methods may comprise providing the plant cell having a cell wall; coating the surface of nanoparticles with at least one functionalized linear nucleic acid cassette molecule of interest; placing the plant cell having a cell wall and the nanoparticles coated with the functionalized linear nucleic acid cassette molecule(s) of interest in contact with each other; and allowing uptake of the nanoparticle and the
10 functionalized linear nucleic acid cassette molecule(s) of interest into the plant cell comprising a cell wall. In particular embodiments, a functionalized linear nucleic acid cassette molecule of interest may be a biotinylated linearized double-stranded DNA molecule comprising a gene of interest. In further embodiments, a functionalized linear nucleic acid cassette molecule of interest may be a chemically-unmodified double-stranded
15 DNA molecule comprising a gene of interest. In particular embodiments, a nanoparticle may be a QD-streptavidin nanoparticle. Functionalized linear nucleic acid cassette molecules may be conjugated to nanoparticles using a variety of reagents employing different functional groups. In some embodiments, nanoparticles may be surface functionalized with proteins and/or other molecules; e.g., pesticides that possess
20 compatible functional groups. In some embodiments, more than one type of molecule may be conjugated to the surface of a nanoparticle. Thus, in particular embodiments, cell-penetrating pesticides and linearized nucleic acid cassette molecules may be co-functionalized onto the surface of nanoparticles, for example, to facilitate the targeted delivery of biomolecules.

25 Further disclosed are methods for introgressing a trait into a plant. In some embodiments, the method may comprise providing a plant cell; coating the surface of nanoparticles with a means for expressing the trait in the plant; placing the plant cell and the nanoparticles coated with means for expressing the trait in the plant in contact with each other; allowing uptake of the nanoparticle and the means for expressing the trait in
30 the plant into the plant cell to produce a transformed plant cell; regenerating a whole plant

-5-

from the transformed plant cell; and propagating the plant. In some embodiments, a trait that may be introgressed according to methods of the invention includes a trait selected from, without limitation: male sterility; herbicide resistance; insect resistance; and resistance to bacterial disease, fungal disease, and/or viral disease.

5 Also disclosed are methods of the invention may be used for *in planta* transformation of a plant. In some embodiments, the plant may be selected from plants of the genus, *Arabidopsis*, for example, *A. thaliana*. In particular embodiments, a plant transformed by *in planta* transformation may be selected from *A. thaliana* plants of the Columbia ecotype.

10 Additionally disclosed are genetically modified (GM) plant cells and methods for generating them, wherein the plant cells have one or more nucleic acids introduced therein *via* methods of the present invention. In some embodiments, a plasmid comprising at least one gene of interest and a selectable marker may be introduced into a plant cell having a cell wall *via* a nanoparticle according to the present invention. In further embodiments,
15 stable transformants may be selected that have stably integrated at least one gene of interest and/or the selectable marker. In alternative embodiments, a plant cell now comprising at least one gene of interest may be propagated to produce other cells comprising a molecule of interest. In other embodiments, plant cells now comprising a molecule of interest may be a regenerable cell that may be used to regenerate a whole plant
20 including the molecule of interest.

Further disclosed are methods of creating regenerable plant cells comprising a molecule of interest for use in tissue culture. The tissue culture may be capable of regenerating plants having substantially the same genotype as the regenerable cells. The regenerable cells in such tissue cultures may be, for example, embryos; protoplasts;
25 meristematic cells; callus; pollen; leaves; anthers; roots; root tips; flowers; seeds; pods; or stems. Still further, some embodiments provide plants regenerated from the tissue cultures of the invention.

Further disclosed are methods for generating stabilized plant lines comprising a desired trait or nucleic acid molecule of interest, wherein the desired trait or nucleic acid
30 molecule of interest may be first introduced by uptake of a nanoparticle across a plant cell

wall. Methods of generating stabilized plant lines are well known to one of ordinary skill in the art, and may include techniques such as, but not limited to, selfing; backcrossing; hybrid production; crosses to populations; and the like. Thus, also disclosed are plants and plant cells comprising a desired trait or nucleic acid molecule of interest first introduced into the plant
5 cell (or its predecessors) by uptake of a nanoparticle across a cell wall. Plant cells comprising a desired trait or nucleic acid molecule of interest first introduced into the plant or cell (or its predecessors) by uptake of a nanoparticle across a cell wall can be used in crosses with other, different, plant cells to produce first generation (F₁) hybrid cells; seeds; and/or plants with desired characteristics.

10 In addition to the exemplary aspects and embodiments described above, further aspects and embodiments will become apparent in view of the following descriptions.

The invention as claimed relates to:

- a method of introducing a linear nucleic acid cassette into a plant cell having a cell wall, the method comprising: providing the plant cell having a cell wall; coating a
15 quantum dot nanoparticle with a biotinylated linear nucleic acid cassette, wherein the quantum dot nanoparticle comprises streptavidin that interacts with the biotinylated linear nucleic acid cassette; placing the plant cell having a cell wall and the coated quantum dot nanoparticle in contact with each other; allowing uptake of the quantum dot and the biotinylated linear nucleic acid cassette into the plant cell comprising a cell wall; and selecting cells that have
20 stably integrated the linear nucleic acid cassette sequence; and

- a method of introducing a linear nucleic acid cassette into plant material, the method comprising: providing plant material, wherein the plant material is selected from the group consisting of plant cells, plant tissues, and plants; providing a quantum dot nanoparticle; coating the quantum dot nanoparticle with a biotinylated linear nucleic acid
25 cassette, wherein the quantum dot nanoparticle comprises streptavidin that interacts with the biotinylated linear nucleic acid cassette; placing the plant material and the coated quantum dot nanoparticle in contact with each other; allowing uptake of the quantum dot nanoparticle and the biotinylated linear nucleic acid cassette into the plant material; and selecting plant material that has stably integrated the linear nucleic acid cassette sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 includes a diagram of unlinearized plasmid pDAB3831.

FIG. 2 includes the DNA sequence of plasmid pDAB3831. A DNA fragment from bp 7666-3870 was amplified using PCR and used for *Arabidopsis* transformation, which
5 generated stably integrated T₂ plants.

FIG. 3 includes a sequence alignment between the Phosphinothricin-N-acetyltransferase (PAT) DNA sequence from Dendrimer transformed *Arabidopsis* genome and the PAT sequence from the NCBI database.

FIG. 4 includes a sequence alignment between the Yellow Fluorescence
10 Protein (YFP) DNA sequence transformed *Arabidopsis* genome and the YFP sequence from the NCBI database.

SEQUENCE LISTING

SEQ ID NO: 1 shows a forward primer sequence used to amplify a 4.6 kbp complete expression cassette from plasmid pDAB3831:

15 /5Biosq/TGAAAGTGTACATCAACGAA.

SEQ ID NO: 2 shows a reverse primer sequence used to amplify a 4.6 kbp complete expression cassette from plasmid pDAB3831:

-7-

/5Biosq/ CCGCAACTATTTCAACAC.

SEQ ID NO:3 shows a forward primer sequence used to amplify the YFP gene:
TGTTCCACGGCAAGATCCCCTACG.

SEQ ID NO:4 shows a reverse primer sequence used to amplify the YFP gene:
5 TATTCATCTGGGTGTGATCGGCCA.

SEQ ID NO:5 shows a forward primer sequence used to amplify the PAT gene:
GGAGAGGAGACCAGTTGAGATTAG.

SEQ ID NO:6 shows a reverse primer sequence used to amplify the PAT gene:
AGATCTGGGTAACTGGCCTAACTG.

10

MODE(S) FOR CARRYING OUT THE INVENTION

I. Overview of several embodiments

Methods of the invention allowing non-invasive gene transfer may be very useful for generating genetically-modified plants with desirable traits. Non-invasive gene transfer may facilitate the specific targeting and editing of molecular sites within the cells for areas, such as incorporating desirable input, output, and agronomic traits in crop plants. Described methods may also be useful as a non-GMO option for transient transformation of plants, expanding technology for trait introgression and disease resistance to tree or vegetable crops, wherein the technology is currently limited.

20 A recent patent application (U.S.S.N. 60/978,059) demonstrates a non-invasive means of DNA delivery based on nanoparticles using a variety on nanoparticle-pay-loads, *inter alia*, to deliver circular plasmid DNA, and unequivocally demonstrates the stable integration of transgenes in T₁ seeds of *Arabidopsis* plants. The transgenic plants containing the circular plasmid DNA produced therein displayed desired herbicide
25 tolerance phenotypes and showed high levels of tolerance when sprayed with field levels of glufosinate ammonium at least 4 times concurrently. U.S.S.N. 60/978,059 demonstrated, *inter alia*, genetic transformation in *Arabidopsis* by positively charged gold nanoparticles using circular plasmid DNA. The present study describes, *inter alia*, the use of functionalized linear nucleic acid cassette molecules for stable genetic transformation of
30 plants.

-8-

U.S.S.N. 60/978,059 described, *inter alia*, positively charged nanoparticle-mediated plasmid DNA delivery. However, the demonstration of stable genomic integration of transgene using linear plasmid-based delivery has not been reported to date. This disclosure describes the use of nanoparticle-mediated functionalized linear

5 nucleic acid cassette molecules for stable genetic transformation in plants. Molecular analysis indicated the expression of PAT along with YFP in transgenic T₁ *Arabidopsis* plants transformed with a *pat* gene and a *yfp* gene by methods of the invention. The T₁ transgenic plants are fertile and produce seed. These seeds may be propagated, and a segregation analysis may be performed along with Molecular and protein analyses.

10 Disclosed are methods that allow the generation of simple DNA integration events in plants, and thereby streamline subsequent introgression efforts. The use of functionalized linear nucleic acid cassette molecules provides advantages in genetic transformation compared to, for example, plasmids. For example, functionalized linear nucleic acid cassette molecules may not comprise vector backbone sequences or selectable

15 marker genes.

II. Terms

In the description and tables which follow, a number of terms are used. In order to provide a clear and consistent understanding of the specification and claims, including the

20 scope to be given such terms, the following definitions are provided:

Backcrossing: As used herein the term, "backcrossing," may be a process in which a breeder repeatedly crosses hybrid progeny back to one of the parents, for example, a first generation hybrid F₁ with one of the parental genotypes of the F₁ hybrid.

Embryo: As used herein the term, "embryo," may refer to the small plant

25 contained within a mature seed.

Nanoparticle: As used herein the term, "nanoparticle," may refer to a microscopic particle with at least one nanoscale dimension, for example, less than 100 nm. Nanoparticles suitable for use in the present invention may have a size of 1 nm - 0.84 μm. One class of nanoparticles is "quantum dots" (QD). A quantum dot may have a median

30 diameter of 1 nm – 10 nm, for example, 2-4 nm. Other varieties of nanoparticle include,

-9-

without limitation: gold nanoparticles; gold-coated nanoparticles; porous nanoparticles; mesoporous nanoparticles; silica nanoparticles; polymer nanoparticles, such as dendrimers; tungsten nanoparticles; gelatin nanoparticles; nanoshells; nanocores; nanospheres; nanorods; magnetic nanoparticles; and combinations thereof.

5 Among available nanoparticles, luminescent semiconductor nanocrystals (QDs) provide many demonstrated applications in biological imaging and sensing. Their utility is derived from the combination of unique photo-physical characteristics and sizes comparable to that of a large protein. The hydrodynamic radius of hydrophilic CdSe-ZnS QDs varies from 5 nm (for nanocrystals cap exchanged with molecular ligands) to 20 nm
10 for nanocrystals encapsulated within block copolymers. A single QD can be conjugated to several biomolecules (*e.g.*, antibodies; peptides; and nucleic acid molecules) to provide multifunctional QD bioconjugates with enhanced avidity. In addition, their strong resistance to chemical and photo-degradation can potentially allow long-term fluorescent monitoring of specific biological processes. Nie and Emory (1997) *Science* 275:1102-6.
15 Multiple non-covalent conjugation schemes based on metal affinity self-assembly and biotin-avidin binding can be simultaneously applied within the same complex, without requiring further purification, to produce multifunctional QD bioconjugates that are stable even in intracellular environments. Yezhelyev et al. (2008) *J. Am. Chem. Soc.* 130(28):9006-12. By utilizing an average of 10 YFPs plus a nominal 50 cell-penetrating
20 peptides (CPPs) per QD, intracellular delivery of protein cargos with molecular weights of at least 300 kDa and a spatial extension of 150 angstroms can be achieved. *Id.* The delivered cargos for QD-b-PE conjugates have a much larger range of sizes and molecular weights; for instance, with an average of 2.5 Streptavidin-b-PE per conjugate, the delivered assemblies have a molecular weight that potentially exceeds 10^3 kDa, and overall
25 dimensions approaching 500 angstroms. Molecular weight and size can be increased substantially if conjugates with higher b-PE valencies are used.

Nucleic acid molecule: A polymeric form of nucleotides, which can include both sense and anti-sense strands of RNA, cDNA, genomic DNA, artificial chromosomes (ACEs), and synthetic forms and mixed polymers of the foregoing. A nucleotide refers to
30 a ribonucleotide, deoxyribonucleotide, or a modified form of either type of nucleotide. A

-10-

“nucleic acid molecule,” as used herein, is synonymous with “nucleic acid” and “polynucleotide.” A nucleic acid molecule is usually at least 10 bases in length, unless otherwise specified. The term includes single- and double-stranded forms of DNA. A nucleic acid molecule may include either or both naturally-occurring and modified nucleotides linked together by naturally-occurring and/or non-naturally occurring nucleotide linkages.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects transcription or expression of the coding sequence. When recombinantly produced, operably linked nucleic acid sequences may be contiguous, and, where necessary to join two protein-coding regions, in the same reading frame. However, nucleic acids need not be contiguous to be operably-linked.

PEGylated: As used herein the term, “PEGylated,” may refer to nanoparticles (e.g., gold nanoparticles; and quantum dots), wherein surfaces of the nanoparticles have been modified with polyethylene glycol (PEG) for improved biocompatibility. PEGylated nanoparticles may be further coated with various targeting ligands, for example, peptides and antibodies, for enhanced delivery efficiency to specific cells and tissues. PEG has been conjugated to nanoparticles with various drugs; liposomes; and polymeric micelles to, for example, prolong the blood circulation time of the coated nanoparticles by reducing the nonspecific adsorption of proteins *via* a steric stabilization effect.

Quantum dot: As used herein the term, “quantum dot,” (QD) (also sometimes known as nanocrystals) may refer to a semiconductor nanostructure that confines the motion of conduction band electrons, valence band holes, or excitons (bound pairs of conduction band electrons and valence band holes) in all three spatial directions. The confinement may be due, for example, to electrostatic potentials (generated by external electrodes, doping, strain, impurities, etc.); the presence of an interface between different semiconductor materials (e.g., in core-shell nanocrystal systems); the presence of the semiconductor surface (e.g., semiconductor nanocrystal); or combinations thereof. A quantum dot may have a discrete quantized energy spectrum. The corresponding wave

-11-

functions may be spatially localized within the quantum dot, but extend over many periods of the crystal lattice. A quantum dot contains a small finite number (for example, of the order of 1-100) of conduction band electrons; valence band holes; or excitons (*i.e.*, a finite number of elementary electric charges).

5 Quantum dots are a special class of semiconducting materials, which may be crystals composed of periodic groups of II-VI, III-V, or IV-VI materials. Their sizes may range, for example, from 2-10 nanometers (10-50 atoms) in diameter. In some embodiments, quantum dots may be made of Cadmium Selenide Zinc Sulfide Core Shell (CdSe/ZnS), and have a range of useful electrical and optical properties that diverge in
10 character from those of bulk material. Quantum dot nanoparticles have been investigated as an imaging agent *in vivo* and *in vitro*, because of their high quantum yield; high molar extinction coefficient; and high resistance to photobleaching.

 Resistant to Glyphosate: Resistance to a dosage of glyphosate refers to the ability of a plant to survive (*i.e.* the plant may be not killed) by that dosage of glyphosate. In
15 some cases, tolerant plants may temporarily yellow, or otherwise exhibit some glyphosate-induced injury (*e.g.*, excessive tillering and/or growth inhibition), but recover.

 Stabilized: As used herein the term, "stabilized," may refer to characteristics of a plant that are reproducibly passed from one generation to the next generation of inbred plants of the same variety.

20 Transgene: As used herein the term, "transgene," may refer to an exogenous nucleic acid sequence. In one example, a transgene is a gene sequence (*e.g.*, a herbicide-resistance gene); a gene encoding an industrially or pharmaceutically useful compound; or a gene encoding a desirable agricultural trait. In yet another example, the transgene is an antisense nucleic acid sequence, wherein expression of the antisense
25 nucleic acid sequence inhibits expression of a target nucleic acid sequence. A transgene may contain regulatory sequences operably linked to the transgene (*e.g.*, a promoter). In some embodiments, a functionalized linear nucleic acid cassette molecule of interest to be introduced by nanoparticle-mediated transformation comprises a transgene. However, in other embodiments, functionalized linear nucleic acid cassette molecule of interest
30 comprises an endogenous nucleic acid sequence, wherein additional genomic copies of the

-12-

endogenous nucleic acid sequence are desired; or a nucleic acid sequence that is in the antisense orientation with respect to a target nucleic acid molecule in the host organism.

Uptake: As used herein the term, "uptake," may refer to the translocation of a particle, such as a nanoparticle (for example, quantum dots; or gold nanoparticles), across a cell wall or a cellular membrane, wherein the translocation does not occur solely as a result of momentum imparted to the particle by something other than the cell into which the particle is being uptaken. Non-limiting examples of devices or methods which cause translocation of a particle across a cell wall or a cell membrane solely as a result of momentum imparted to the particle are biolistic, gene gun, microinjection, and/or impalefection technologies.

III. Nucleic Acid Molecule Delivery using Nanoparticles for Stable Transformation of Plant Cells

A. Overview

This invention describes, for example, new methods for plant transformation using nanoparticle-mediated transfer of functionalized linear nucleic acid cassette molecules for genetic transformation and the development of stable transgenic plants. Methods according to certain embodiments may offer not only rapid generation of a transgenic organism, but also several possibilities for desired genomic modifications when compared to other transformation methods. Embodiments of the invention have led to the first reported stably-transformed plant produced *via* nanoparticle-mediated linearized plasmid DNA delivery. Disclosed methods of genetic modification are a departure from traditional methods of genetic transformation of plants, are not dependent upon biolistic delivery, and may be very useful for generating transgenic crop plants.

Transgenic plants are typically produced by *Agrobacterium*-mediated or particle bombardment transformation. In addition to the gene of interest, transgenic plants often necessarily contain vector backbone sequences and selectable marker genes, for example, that confer resistance to antibiotics or herbicides. Since selectable marker genes and vector backbone sequences are both superfluous and undesirable in DNA transfer procedures, generation of vector-free transgenic plants is advantageous. The removal of

-13-

vector backbone sequences allowed by the methods of some embodiments may limit the amount of homologous recombination and the influence of recombinogenic elements on the integration process.

5 In embodiments of the present invention, direct creation of transgenic plants by non-invasive nanoparticle-mediated delivery of linear nucleic acid cassettes may now be achieved, for example, *via* the floral dip method. Such methods may provide a simpler way to carry out desired plant transformations than is otherwise available in the art. In some embodiments, methods may be used to create both vector-free and marker-free transgenic plants. In some embodiments, transformation methods are independent of
10 tissue culture, and therefore, may be more convenient and practical for sexually reproducing plants.

In some embodiments, methods of the invention may provide the ability to non-invasively transform non-*Agrobacterium* compatible plants, and/or their tissue culture suspension cells. Such methods may provide tremendous opportunities. Desirable input
15 and agronomic traits may require multiple gene delivery in the same transformation procedure. In some embodiments, methods of the invention allow delivery of multiple nucleic acid molecules, while eliminating the need to construct large plasmids containing all the genes of interest, which may be burdensome if at all possible.

20 B. Nucleic Acid Molecules

With the advent of molecular biological techniques that have allowed the isolation and characterization of genes that encode specific protein or RNA products (*e.g.*, interfering RNAs ("RNAi")), scientists in the field of plant biology developed a strong interest in engineering the genome of cells to contain and express foreign genes, or
25 additional or modified versions of native or endogenous genes (perhaps driven by different promoters) in order to, for example, alter the traits of a cell in a specific manner. Such foreign additional and/or modified genes are referred to herein collectively as "transgenes." Transgenes may, for example, encode a protein of interest, or be transcribed into RNAi. Over the last fifteen to twenty years, several methods for producing transgenic cells have
30 been developed and, in particular embodiments, the present invention relates to

-14-

transformed versions of cells and methods of producing them *via* introducing into a plant cell having a cell wall one or more functionalized linear nucleic acid cassette molecule(s) *via* uptake of a nanoparticle across a cell wall. In some embodiments, the transgene may be contained in a synthesized linear DNA cassette.

5 Cell transformation may involve a nucleic acid molecule that includes a gene under control of, or operatively linked to, a regulatory element (for example, a promoter, an enhancer, a termination sequence, or combinations thereof). Thus, a nucleic acid molecule may contain one or more such operably linked gene/regulatory element combinations.

In embodiments, a nucleic acid molecule of interest may be a functionalized linear
10 nucleic acid cassette molecule. Linear nucleic acid cassette molecules may be generated, for example, by digestion of a circular plasmid with at least one restriction endonuclease, such as to excise an expression cassette contained therein. Restriction endonucleases will cleave a plasmid at one or more recognition sites within the plasmid nucleotide sequence. Thus, plasmids may be designed to allow for the generation of one or more specific linear
15 nucleic acid cassette molecules by digestion with at least one particular restriction endonuclease. Alternatively, a given plasmid nucleotide sequence may be searched for recognition sites of one or more particular restriction endonuclease(s) that allow for generation of one or more specific linear nucleic acid cassette molecule(s). By selecting restriction sites that cleave at specific locations within a circular plasmid or linear nucleic
20 acid molecule, resulting linear nucleic acid cassette molecules may be generated that lack one or more sequences from the precursor nucleic acid molecule. For example, a linear nucleic acid cassette molecule may be generated that lacks extraneous nucleic acid sequences (*e.g.*, vector backbone; selection markers, such as bacterial selection markers; and unnecessary nucleic acid sequences that are homologous to genomic DNA of the
25 target cell). Alternatively, a linear nucleic acid cassette molecule may be synthesized that lacks extraneous nucleic acid sequences.

Linear nucleic acid cassette molecules may be synthesized using a continuous thermal cycling system. See International PCT Publication WO 2008/045288. Rather than using small tubes, continuous thermal cyclers use a constant or continuous stream of
30 fluid repetitively passed through different temperature zones to amplify DNA. PCR

-15-

reaction mixture is injected into a carrier fluid with which the PCR reaction mixture is immiscible, and the carrier fluid then passes through a plurality of temperature zones to facilitate DNA amplification within the PCR reaction mixture. The specific DNA sequence present in the sample is amplified as it passes through cyclically through the temperature zones. PCR product may be purified on a gel filtration column, followed by purification.

Nucleic acid molecules may be conjugated to nanoparticles using a variety of reagents with different functional groups. Various chemical reactions for nucleic acid molecule conjugation to nanoparticles are listed in Table 1.

Table 1.

Particle	Surface groups
620 nm PDDA functionalized QDs	N ⁺ (CH ₃) ₂ Cl ⁻
620 nm Amine functionalized QDs	Amino groups
620 nm Streptavidin functionalized QDs	Streptavidin
Mercapto acetic acid, HSCH ₂ COOH	Carboxyl and thiol
3-mercaptopropionimide hydrochloride (iminothiolane)	sulfhydryl

In embodiments wherein the linear nucleic acid cassette molecule of interest comprises one or more gene(s), the gene(s) may be a dominant or recessive allele. By way of example, the gene(s) may confer such traits as herbicide resistance, insect resistance, resistance for bacterial resistance, fungal resistance, viral disease resistance, male fertility, male sterility, enhanced nutritional quality, and industrial usage. Genes conferring these traits and other traits are known in the art, and any gene may be introduced into a plant cell comprising a cell wall according to methods of the invention.

Expression Vectors for Linearization and Uptake *via* Nanoparticles: Marker Genes

Expression vectors for linearization and uptake *via* nanoparticles may optionally include at least one genetic marker, for example, operably linked to a regulatory element that allows transformed cells containing the marker to be either recovered by negative

-16-

selection (*i.e.*, inhibiting growth of cells that do not contain the selectable marker gene) or by positive selection (*i.e.*, screening for the product encoded by the genetic marker). Many selectable marker genes for transformation are well known in the art, and include for example and without limitation: genes that code for enzymes that metabolically detoxify a selective chemical agent which may be an antibiotic or an herbicide; or genes that encode an altered target which may be insensitive to the inhibitor. Specific positive selection methods are also known in the art. However, in some embodiments, linearized nucleic acid cassette molecules do not comprise marker genes.

One selectable marker gene which may be suitable for plant transformation with certain nucleic acid molecules is the neomycin phosphotransferase II (*nptII*) gene, optionally under the control of plant regulatory signals, which confers resistance to kanamycin. See, e.g., Fraley *et al.* (1983) Proc. Natl. Acad. Sci. U.S.A. 80:4803. Another selectable marker gene which may be used is the hygromycin phosphotransferase gene, which confers resistance to the antibiotic hygromycin. See, e.g., Vanden Elzen *et al.* (1985) Plant Mol. Biol. 5:299.

Additional selectable marker genes which may be used in methods of the invention include those of bacterial origin, for example, those that confer resistance to antibiotics such as gentamycin acetyl transferase, streptomycin phosphotransferase, aminoglycoside-3'-adenyl transferase, and bleomycin. See Hayford *et al.* (1988) Plant Physiol. 86:1216; Jones *et al.* (1987) Mol. Gen. Genet. 210:86; Svab *et al.* (1990) Plant Mol. Biol. 14:197; and Hille *et al.* (1986) Plant Mol. Biol. 7:171. Other selectable marker genes may confer resistance to herbicides such as glyphosate; glufosinate; or bromoxynil. See Comai *et al.* (1985) Nature 317:741-744; Gordon-Kamm *et al.* (1990) Plant Cell 2:603-618; and Stalker *et al.* (1988) Science 242:419-423.

Other selectable marker genes which may be used in methods of the invention include those that are not of bacterial origin. These genes include, for example and without limitation, mouse dihydrofolate reductase; plant 5-enolpyruvylshikimate-3-phosphate synthase; and plant acetolactate synthase. See Eichholtz *et al.* (1987) Somatic Cell Mol. Genet. 13:67; Shah *et al.* (1986) Science 233:478; and Charest *et al.* (1990) Plant Cell Rep. 8:643.

-17-

Another class of marker genes suitable for plant transformation may require screening of presumptively transformed plant cells rather than direct genetic selection of transformed cells for resistance to a toxic substance, such as an antibiotic. These genes are particularly useful to quantify or visualize the spatial pattern of expression of a gene in specific tissues, and are frequently referred to as "reporter genes," because they can be fused to a gene or gene regulatory sequence for the investigation of gene expression. Commonly used genes for screening transformed cells include, without limitation, β -glucuronidase (GUS); β -galactosidase; luciferase; and chloramphenicol acetyltransferase. See Jefferson (1987) Plant Mol. Biol. Rep. 5:387; Teeri *et al.* (1989) EMBO J. 8:343; Koncz *et al.* (1987) Proc. Natl. Acad. Sci U.S.A. 84:131; and DeBlock *et al.* (1984) EMBO J. 3:1681. Recently, *in vivo* methods for visualizing GUS activity that do not require destruction of plant tissue have been made available. Molecular Probes publication 2908 (1993) Image Green™, p. 1-4; and Naleway *et al.* (1991) J. Cell Biol. 115:151a.

More recently, genes encoding Fluorescent Proteins (*e.g.*, GFP, EGFP, EBFP, ECFP, and YFP) have been utilized as markers for gene expression in prokaryotic and eukaryotic cells. See Chalfie *et al.* (1994) Science 263:802. Thus, fluorescent proteins and mutations of fluorescent proteins may be used as screenable markers in some embodiments.

20

Expression Vectors for Uptake *via* Nanoparticle: Promoters

Genes included in linear nucleic acid cassette molecules may optionally be driven by a nucleotide sequence comprising a regulatory element, for example, a promoter. Several types of promoters are now well known in the transformation arts, as are other regulatory elements that can be used alone or in combination with promoters.

25

A promoter is a region of DNA that may be upstream from the start of transcription, and may be involved in recognition and binding of RNA polymerase and/or other proteins to initiate transcription. A "plant promoter" may be a promoter capable of initiating transcription in plant cells. Examples of promoters under developmental control include promoters that preferentially initiate transcription in certain tissues, such as leaves;

30

-18-

roots; seeds; fibers; xylem vessels; tracheids; or sclerenchyma. Such promoters are referred to as "tissue-preferred." Promoters which initiate transcription only in certain tissues are referred to as "tissue-specific." A "cell type" specific promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An "inducible" promoter may be a promoter which may be under environmental control. Examples of environmental conditions that may affect transcription by inducible promoters include, without limitation, anaerobic conditions or the presence of light. Tissue-specific, tissue-preferred, cell type specific, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter which may be active under most environmental conditions.

1. Inducible Promoters

An inducible promoter may be operably linked to a gene for expression in a cell. Optionally, the inducible promoter may be operably linked to a nucleotide sequence encoding a signal sequence which may be operably linked to a gene for expression in a cell. With an inducible promoter, the rate of transcription increases in response to an inducing agent.

Any inducible promoter can be used in embodiments of the instant invention. See Ward *et al.* (1993) Plant Mol. Biol. 22:361-366. Exemplary inducible promoters include without limitation: those from the ACEI system that responds to copper (Mett *et al.* (1993) Proc. Natl. Acad. Sci. U.S.A. 90:4567-71); an *In2* gene from maize that responds to benzenesulfonamide herbicide safeners (Hershey *et al.* (1991) Mol. Gen. Genetics 227:229-237; and Gatz *et al.* (1994) Mol. Gen. Genetics 243:32-38); and Tet repressor from Tn10 (Gatz *et al.* (1991) Mol. Gen. Genetics 227:229-237). A particularly useful inducible promoter may be a promoter that responds to an inducing agent to which plants do not normally respond. Such an exemplary inducible promoter is the inducible promoter from a steroid hormone gene, the transcriptional activity of which may be induced by a glucocorticosteroid hormone. Schena *et al.* (1991) Proc. Natl. Acad. Sci. U.S.A. 88:0421.

-19-

2. Constitutive Promoters

A constitutive promoter may be operably linked to a gene for expression in a cell, or the constitutive promoter may be operably linked to a nucleotide sequence encoding a signal sequence which may be operably linked to a gene for expression in a cell.

5 Different constitutive promoters may be utilized in embodiments of the instant invention. Exemplary constitutive promoters include without limitation: promoters from plant viruses, such as the 35S promoter from CaMV (Odell *et al.* (1985) *Nature* 313:810-812); promoters from rice actin genes (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and
10 Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); maize H3 histone (Lepetit *et al.* (1992) *Mol. Gen. Genetics* 231:276-285 and Atanassova *et al.* (1992) *Plant Journal* 2 (3): 291-300); and the ALS promoter, Xba1/NcoI fragment 5' to the *Brassica napus* ALS3 structural gene (or a nucleotide sequence similarity to said
15 Xba1/NcoI fragment). See International PCT Publication WO 96/30530.

3. Tissue-specific or Tissue-preferred Promoters

A tissue-specific promoter may be operably linked to a gene for expression in a cell. Optionally, the tissue-specific promoter may be operably linked to a nucleotide sequence encoding a signal sequence which may be operably linked to a gene for
20 expression in a cell. Plants transformed with a gene of interest operably linked to a tissue-specific promoter may produce the protein product of the transgene exclusively, or preferentially, in a specific tissue.

Any tissue-specific or tissue-preferred promoter may be utilized in embodiments of the instant invention. Exemplary tissue-specific or tissue-preferred promoters include
25 without limitation: a root-preferred promoter, for example, a promoter from the phaseolin gene (Murai *et al.* (1983) *Science* 23:476-82 and Sengupta-Gopalan *et al.* (1985) *Proc. Natl. Acad. Sci. U.S.A.* 82:3320-4); a leaf-specific and light-induced promoter, for example, a promoter from cab or rubisco (Simpson *et al.* (1985) *EMBO J.* 4(11):2723-2729 and Timko *et al.* (1985) *Nature* 318:579-82); an anther-specific
30 promoter, for example, a promoter from LAT52 (Twell *et al.* (1989) *Mol. Gen. Genetics*

-20-

217:240-5); a pollen-specific promoter, for example, a promoter from Zm13 (Guerrero *et al.* (1993) Mol. Gen. Genetics 244:161-8); and a microspore-preferred promoter, for example, a promoter from apg (Twell *et al.* (1993) Sex. Plant Reprod. 6:217-24).

Transport of protein produced by transgenes to a subcellular compartment, such as the chloroplast; vacuole; peroxisome; glyoxysome; or mitochondrion, or for secretion into the apoplast, may be accomplished by means of operably linking the nucleotide sequence encoding a signal sequence to the 5' and/or 3' region of a gene encoding the protein of interest. Targeting sequences at the 5' and/or 3' end of the gene may determine, for example, during protein synthesis and processing, where the encoded protein may be ultimately compartmentalized. Alternatively, such subcellular compartment targeting proteins may be directly linked to a nanoparticle to direct the nanoparticle coated with the nucleic acid molecule of interest to the desired subcellular compartment.

The presence of a signal sequence may direct a polypeptide to either an intracellular organelle or subcellular compartment, or for secretion to the apoplast. Many signal sequences are known in the art. *See, e.g.,* Becker *et al.* (1992) Plant Mol. Biol. 20:49; Close, P. S., Master's Thesis, Iowa State University (1993), Knox *et al.* (1987) Plant Mol. Biol. 9:3-17; Lerner *et al.* (1989) Plant Physiol. 91:124-9; Fontes *et al.* (1991) Plant Cell 3:483-96; Matsuoka *et al.* (1991) Proc. Natl. Acad. Sci. U.S.A. 88:834; Gould *et al.* (1989) J. Cell. Biol. 108:1657; Creissen *et al.* (1991) Plant J. 2:129; Kalderon *et al.* (1984) Cell 39:499-509; Steifel *et al.* (1990) Plant Cell 2:785-93.

Foreign Protein Genes and Agronomic Genes

Transgenic plants according to embodiments of the present invention may produce a foreign protein in commercial quantities. Thus, techniques for the selection and propagation of transformed plants yield a plurality of transgenic plants which are harvested in a conventional manner. A foreign protein then may be extracted from a tissue of interest, or from total biomass. Protein extraction from plant biomass can be accomplished by known methods which are discussed, for example, in Heney and Orr (1981) Anal. Biochem. 114:92-6.

-21-

In some aspects of the invention, plant material provided for commercial production of foreign protein may be a plant, plant tissue, or plant cell. In some aspects, the biomass of interest may be plant seed. For the transgenic plants that show higher levels of expression, a genetic map can be generated, for example, *via* conventional RFLP, PCR and SSR analysis, which identifies the approximate chromosomal location of the integrated DNA molecule. For exemplary methodologies in this regard, see Glick and Thompson, *Methods in Plant Molecular Biology and Biotechnology* CRC Press, Boca Raton 269:284 (1993). Map information concerning chromosomal location may be useful, for example, for proprietary protection of a subject transgenic plant, or for biosafety evaluation. If unauthorized propagation may be undertaken and crosses made with other germplasm, the map of the integration region can be compared to similar maps for suspect plants to determine if the latter have a common parentage with the subject plant. Map comparisons may involve hybridizations, RFLP, PCR, SSR and sequencing, all of which are conventional techniques.

Likewise, agronomic genes may be expressed in transformed cells or their progeny. More particularly, plants can be genetically engineered *via* methods of the invention to express various phenotypes of agronomic interest. Exemplary genes that may be used in this regard include, but are not limited to, those categorized below.

1. Genes That Confer Resistance to Pests or Disease:

A) Plant disease resistance genes. Plant defenses are often activated by specific interaction between the product of a disease resistance gene (R) in the plant and the product of a corresponding avirulence (Avr) gene in the pathogen. A plant variety may be transformed with cloned resistance genes to engineer plants that are resistant to specific pathogen strains. *See, e.g., Jones et al. (1994) Science 266:789 (cloning of the tomato Cf-9 gene for resistance to Cladosporium fulvum); Martin et al. (1993) Science 262:1432 (tomato Pto gene for resistance to Pseudomonas syringae pv. tomato encodes a protein kinase); Mindrinos et al. (1994) Cell 78:1089 (RSP2 gene for resistance to Pseudomonas syringae).*

-22-

B) A gene conferring resistance to a pest, for example, soybean cyst nematode. *See, e.g.*, International PCT Publication WO 96/30517; and International PCT Publication WO 93/19181.

5 C) A *Bacillus thuringiensis* protein, a derivative thereof, or a synthetic polypeptide modeled thereon. *See, e.g.*, Geiser *et al.* (1986) Gene 48:109 (cloning and nucleotide sequence of a Bt δ -endotoxin gene). Moreover, DNA molecules encoding δ -endotoxin genes can be purchased from American Type Culture Collection (Manassas, VA), for example, under ATCC Accession Nos. 40098; 67136; 31995; and 31998.

10 D) A lectin. *See*, for example, Van Damme *et al.* (1994) Plant Molec. Biol. 24:25 (nucleotide sequences of several *Clivia miniata* mannose-binding lectin genes).

E) A vitamin-binding protein, for example, avidin. *See* International PCT Publication US93/06487 (use of avidin and avidin homologues as larvicides against insect pests).

15 F) An enzyme inhibitor, for example, a protease or proteinase inhibitor, or an amylase inhibitor. *See, e.g.*, Abe *et al.* (1987) J. Biol. Chem. 262:16793 (nucleotide sequence of rice cysteine proteinase inhibitor); Huub *et al.* (1993) Plant Molec. Biol. 21:985 (nucleotide sequence of cDNA encoding tobacco proteinase inhibitor I); Sumitani *et al.* (1993) Biosci. Biotech. Biochem. 57:1243 (nucleotide sequence of *Streptomyces nitrosporeus* alpha-amylase inhibitor) and U.S. Pat. No. 5,494,813.

20 G) An insect-specific hormone or pheromone, for example, an ecdysteroid or juvenile hormone, a variant thereof, a mimetic based thereon, or an antagonist or agonist thereof. *See, e.g.*, Hammock *et al.* (1990) Nature 344:458 (baculovirus expression of cloned juvenile hormone esterase, an inactivator of juvenile hormone).

25 H) An insect-specific peptide or neuropeptide which, upon expression, disrupts the physiology of the affected pest. *See, e.g.*, Regan (1994) J. Biol. Chem. 269:9 (expression cloning yields DNA coding for insect diuretic hormone receptor); and Pratt *et al.* (1989) Biochem. Biophys. Res. Comm. 163:1243 (an allostatin may be identified in *Diploptera puntata*). *See also* U.S. Pat. No. 5,266,317 (genes encoding insect-specific, paralytic neurotoxins).

-23-

I) An insect-specific venom produced in nature by a snake, a wasp, or any other organism. *See, e.g., Pang et al. (1992) Gene 116:165 (heterologous expression in plants of a gene coding for a scorpion insectotoxic peptide).*

5 J) An enzyme responsible for a hyperaccumulation of a monoterpene, a sesquiterpene, a steroid, hydroxamic acid, a phenylpropanoid derivative or another non-protein molecule with insecticidal activity.

K) An enzyme involved in the modification, including the post-translational modification, of a biologically active molecule, for example, a glycolytic enzyme; a proteolytic enzyme; a lipolytic enzyme; a nuclease; a cyclase; a transaminase; an esterase; 10 a hydrolase; a phosphatase; a kinase; a phosphorylase; a polymerase; an elastase; a chitinase; or a glucanase, whether natural or synthetic. *See International PCT Publication WO 93/02197 (nucleotide sequence of a callase gene). DNA molecules which contain chitinase-encoding sequences can be obtained, for example, from the ATCC, under Accession Nos. 39637 and 67152. See also Kramer et al. (1993) Insect Biochem. Molec. Biol. 23:691 (nucleotide sequence of a cDNA encoding tobacco hornworm chitinase); and Kawalleck et al. (1993) Plant Molec. Biol. 21:673 (nucleotide sequence of the parsley ubi4-2 polyubiquitin gene).*

L) A molecule that stimulates signal transduction. *See, e.g., Botella et al. (1994) Plant Molec. Biol. 24:757 (nucleotide sequences for mung bean calmodulin cDNA clones); and Griess et al. (1994) Plant Physiol. 104:1467 (nucleotide sequence of a maize calmodulin cDNA clone).*

M) A hydrophobic moment peptide. *See, e.g., International PCT Publication WO 95/16776 (peptide derivatives of Tachyplesin which inhibit fungal plant pathogens); and International PCT Publication WO 95/18855 (synthetic antimicrobial peptides that confer 25 disease resistance).*

N) A membrane permease, a channel former, or a channel blocker. *See, e.g., Jaynes et al. (1993) Plant Sci 89:43 (heterologous expression of a cecropin- β lytic peptide analog to render transgenic tobacco plants resistant to *Pseudomonas solanacearum*).*

O) A viral-invasive protein or a complex toxin derived therefrom. For example, 30 the accumulation of viral coat proteins in transformed plant cells imparts resistance to viral

-24-

infection and/or disease development effected by the virus from which the coat protein gene may be derived, as well as by related viruses. *See Beachy et al.* (1990) *Ann. rev. Phytopathol.* 28:451. Coat protein-mediated resistance has been conferred upon transformed plants against alfalfa mosaic virus, cucumber mosaic virus, tobacco streak virus, potato virus X, potato virus Y, tobacco etch virus, tobacco rattle virus and tobacco mosaic virus. *Id.*

P) An insect-specific antibody or an immunotoxin derived therefrom. Thus, an antibody targeted to a critical metabolic function in the insect gut may inactivate an affected enzyme, killing the insect. Cf. Taylor *et al.*, Abstract #497, Seventh Int'l Symposium on Molecular Plant-Microbe Interactions (Edinburgh, Scotland) (1994) (enzymatic inactivation in transgenic tobacco via production of single-chain antibody fragments).

Q) A virus-specific antibody. *See, e.g.*, Tavladoraki *et al.* (1993) *Nature* 366:469 (transgenic plants expressing recombinant antibody genes are protected from virus attack).

R) A developmental-arrestive protein produced in nature by a pathogen or a parasite. For example, fungal endo α -1,4-D-polygalacturonases facilitate fungal colonization and plant nutrient release by solubilizing plant cell wall homo- α -1,4-D-galacturonase. *See Lamb et al.* (1992) *Bio/Technology* 10:1436. *See also Toubart et al.* (1992) *Plant J.* 2:367 (cloning and characterization of a gene which encodes a bean endopolygalacturonase-inhibiting protein).

S) A developmental-arrestive protein produced in nature by a plant. *See, e.g.*, Logemann *et al.* (1992) *Bio/Technology* 10:305 (transgenic plants expressing the barley ribosome-inactivating gene have an increased resistance to fungal disease).

2. Genes That Confer Resistance to an Herbicide:

A) An herbicide that inhibits the growing point or meristem, for example, an imidazolinone or a sulfonylurea. Exemplary genes in this category code for mutant ALS and AHAS enzyme as described, for example, by Lee *et al.* (1988) *EMBO J.* 7:1241, and Miki *et al.* (1990) *Theor. Appl. Genet.* 80:449, respectively.

B) Glyphosate resistance conferred by, for example, mutant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) genes (*via* the introduction of

-25-

recombinant nucleic acids and/or various forms of *in vivo* mutagenesis of native EPSPs genes); aroA genes and glyphosate acetyl transferase (GAT) genes, respectively); other phosphono compounds, such as glufosinate phosphinothricin acetyl transferase (PAT) genes from *Streptomyces* species, including *Streptomyces hygroscopicus* and *Streptomyces viridichromogenes*); and pyridinoxy or phenoxy proprionic acids and cyclohexones (ACCase inhibitor-encoding genes). See, e.g., U.S. Pat. No. 4,940,835; and U.S. Pat. 6,248,876 (nucleotide sequences of forms of EPSPs which can confer glyphosate resistance to a plant). A DNA molecule encoding a mutant aroA gene can be obtained under ATCC accession number 39256. See also U.S. Pat. No. 4,769,061 (nucleotide sequence of a mutant aroA gene). European patent application No. 0 333 033 and U.S. Pat. No. 4,975,374 disclose nucleotide sequences of glutamine synthetase genes, which may confer resistance to herbicides such as L-phosphinothricin. Nucleotide sequences of exemplary PAT genes are provided in European application No. 0 242 246, and DeGreef *et al.* (1989) Bio/Technology 7:61 (production of transgenic plants that express chimeric bar genes coding for PAT activity). Exemplary of genes conferring resistance to phenoxy proprionic acids and cyclohexones, such as sethoxydim and haloxyfop, include the Acc1-S1, Acc1-S2 and Acc1-S3 genes described by Marshall *et al.* (1992) Theor. Appl. Genet. 83:435. GAT genes capable of conferring glyphosate resistance are described, for example, in WO 2005012515. Genes conferring resistance to 2,4-D, phenoxyproprionic acid and pyridyloxy auxin herbicides are described, for example, in WO 2005107437.

C) An herbicide that inhibits photosynthesis, such as a triazine (psbA and gs+ genes) or a benzonitrile (nitrilase gene). See, e.g., Przibila *et al.* (1991) Plant Cell 3:169 (transformation of *Chlamydomonas* with plasmids encoding mutant psbA genes). Nucleotide sequences for nitrilase genes are disclosed in U.S. Pat. No. 4,810,648, and DNA molecules containing these genes are available under ATCC Accession Nos. 53435; 67441; and 67442. See also Hayes *et al.* (1992) Biochem. J. 285:173 (cloning and expression of DNA coding for a glutathione S-transferase).

-26-

3. Genes That Confer or Contribute to a Value-Added Trait, such as:

A) Modified fatty acid metabolism, for example, by transforming a plant with an antisense gene of stearyl-ACP desaturase to increase stearic acid content of the plant. *See Knultzon et al. (1992) Proc. Natl. Acad. Sci. U.S.A. 89:2624.*

5 B) Decreased phytate content. Introduction of a phytase-encoding gene may enhance breakdown of phytate, adding more free phosphate to the transformed plant. *See, e.g., Van Hartingsveldt et al. (1993) Gene 127:87 (nucleotide sequence of an *Aspergillus niger* phytase gene).* A gene may be introduced to reduce phytate content. In maize for example, this may be accomplished by cloning and then reintroducing DNA associated
10 with the single allele which may be responsible for maize mutants characterized by low levels of phytic acid. *See Raboy et al. (1990) Maydica 35:383.*

C) Modified carbohydrate composition effected, for example, by transforming plants with a gene coding for an enzyme that alters the branching pattern of starch. *See, e.g., Shiroza et al. (1988) J. Bacteol. 170:810 (nucleotide sequence of *Streptococcus*
15 mutant fructosyltransferase gene); Steinmetz et al. (1985) Mol. Gen. Genet. 20:220 (levansucrase gene); Pen et al. (1992) Bio/Technology 10:292 (α -amylase); Elliot et al. (1993) Plant Molec. Biol. 21:515 (nucleotide sequences of tomato invertase genes); Sogaard et al. (1993) J. Biol. Chem. 268:22480 (barley α -amylase gene); and Fisher et al. (1993) Plant Physiol. 102:1045 (maize endosperm starch branching enzyme II).*

20

C. Nanoparticles

According to some embodiments of the invention, methods are provided of introducing a functionalized linear nucleic acid cassette molecule of interest into a plant cell comprising a cell wall. In some embodiments, the method may comprise placing a
25 nanoparticle coated with a functionalized linear nucleic acid cassette molecule of interest in contact with the cell, and allowing uptake of the nanoparticle across the cell wall. In particular embodiments, the nanoparticle may be reversibly or irreversibly contain, be coated with, or otherwise be bound to and/or carry a functionalized linear nucleic acid cassette molecule of interest. In these and further embodiments, the nanoparticle may be
30 functionalized with a group that reacts with a group on the functionalized linear nucleic

-27-

acid cassette molecule of interest to produce a nanoparticle conjugated to the functionalized linear nucleic acid cassette molecule of interest. In certain embodiments, a functionalized linear nucleic acid cassette molecule of interest may be introduced to the nanoparticles before contact with a plant cell having a cell wall, or concurrently with the introduction of the nanoparticle to a plant cell having a cell wall. Examples of nanoparticles that can be used in embodiments of the present invention include without limitation quantum dots; other semiconductor nanoparticles; positively-charged nanoparticles; gold nanoparticles; gold coated nanoparticles; porous nanoparticles; mesoporous nanoparticles; silica nanoparticles; polymer nanoparticles such as dendrimers; tungsten nanoparticles; gelatin nanoparticles; nanoshells; nanocores; nanospheres; nanorods; and magnetic nanoparticles.

In particular embodiments of the invention, the surface of the nanoparticle may be functionalized, which may, for example, allow for targeted uptake or allow for reversible or irreversible binding of other substances to the surface of the nanoparticle. By way of non-limiting example, the surface of a nanoparticle (e.g., quantum dots) might be functionalized with a self-assembled monolayer of, for example, alkanethiolates, which can be further functionalized or derivatized. In a further non-limiting example, the surface of a nanoparticle may be derivatized with linkers which themselves may be further functionalized or derivatized, for example, streptavidin. In one embodiment, a nanoparticle may be PEGylated. In other embodiments, the nanoparticle may comprise, or may be multifunctionalized with, one or more of a core (active or inactive); a steric coat (active or inert); a cleavable linkage; and/or a targeting molecule or ligand.

In some embodiments, the nanoparticle may be a streptavidin-QD conjugate. The streptavidin-QD conjugate is made from a nanometer-scale crystal of a semiconductor material (CdSe), which is coated with an additional semiconductor shell (ZnS) to improve the optical properties of the material. It has a narrow, symmetric emission spectrum with the emission maximum near 605 nm. The core-shell material is further coated with a polymer shell that allows the materials to be conjugated to biological molecules and to retain their optical properties. This polymer shell is directly coupled to streptavidin. The streptavidin-QD conjugate is the size of a large macromolecule or protein (~15–20 nm).

-28-

The surface has been prepared to have a low nonspecific signal when incubated with samples in a variety of aqueous buffers. Quantum dots may be coupled to streptavidin directly through an active ester coupling reaction. This yields a material with streptavidin covalently attached on the surface (typically 5–10 streptavidins/quantum dot conjugate),
5 which results in streptavidin-quantum dot conjugates with high specific biological activity.

According to embodiments of the present invention, a plant cell having a cell wall may be any plant cell comprising an intact and whole cell wall. Examples of cells having a cell wall include without limitation: algal; tobacco; carrot; maize; canola; rapeseed; cotton; palm; peanut; soybean; sugarcane; *Oryza sp.*; *Arabidopsis sp.*; and *Ricinus sp.*
10 Embodiments of the invention may include cells comprising a cell wall from any tissue or wherever they are found, including without limitation: in embryos; meristematic cells; callus; pollen, including haploid and double haploid microspores; leaves; anthers; roots; root tips; flowers; seeds; pods; stems; and tissue culture.

In particular embodiments of the invention, a functionalized linear nucleic acid
15 cassette molecule of interest may be any functionalized linear nucleic acid cassette molecule that can be delivered to a plant cell having a cell wall according to the present invention. Functionalized linear nucleic acid cassette molecules of interest may comprise nucleic acid sequences of, without limitation: DNA; RNA; RNAi molecules; genes; plasmids; cosmid; YACs; and BACs. Functionalized linear nucleic acid molecules of
20 interest may be introduced to a plant cell having a cell wall concurrently with, for example and without limitation: polypeptides; enzymes; hormones; glyco-peptides; sugars; fats; signaling peptides; antibodies; vitamins; messengers; second messengers; amino acids; cAMP; drugs; herbicides; fungicides; antibiotics; and/or combinations thereof.

Nanoparticles such as quantum dots may be functionalized with PEG using the
25 protocol of Dubertret *et al.* (2002) Science 298:1759, or by a protocol modified therefrom according to the discretion of the skilled artisan. For example, TOPO (tri-octyl phosphine oxide)-coated CdSe/ZnS quantum dots may be suspended with PEG-PE in chloroform, followed by evaporation of the solvent and solubilization of the resulting PEGylated quantum dots with water.

-29-

In aspects of the invention, the nanoparticle may be uptaken into various parts of cells. Examples of locations that a nanoparticle may be uptaken into include without limitation: the cytosol; the nucleus; tonoplasts; plastids; etioplasts; chromoplasts; leucoplasts; elaioplasts; proteinoplasts; amyloplast; chloroplasts; and the lumen of a double membrane. In other embodiments, nanoparticle uptake into a plant cell comprising a cell wall may occur *via* the symplastic or apoplastic pathway.

D. Stably Transformed Plant Cells

A stably-transformed plant cell according to the invention may be any plant cell capable of being transformed with a functionalized linear nucleic acid cassette molecule of interest by nanoparticle-mediated transformation. Accordingly, the plant cell may be isolated from or cultured from a dicot or monocot. The plant cell may also be present in plant tissue or a whole plant. Non-limiting examples of stably transformed plant cells from dicotyledonous plants according to the invention include: alfalfa; beans; broccoli; cabbage; carrot; cauliflower; celery; Chinese cabbage; cotton; cucumber; eggplant; lettuce; melon; pea; pepper; peanut; potato; pumpkin; radish; rapeseed; spinach; soybean; squash; sugarbeet; sunflower; tobacco; tomato; and watermelon. Non-limiting examples of stably transformed plant cells from monocotyledonous plants according to the invention include corn; onion; rice; sorghum; wheat; rye; millet; sugarcane; oat; triticale; switchgrass; and turfgrass.

The herbicide, glufosinate ammonium (GLA), may be sprayed at a field level concentration for screening transgenic plants that express a glufosinate resistance gene. *Arabidopsis* T₁ seedlings produced using methods of the invention have shown herbicide resistance against five consecutive applications of a field level dosage of glufosinate, for example, on alternate days beginning 7 days after germination. The genomic DNA from these transgenic plants were analyzed for the presence of *pat* and *yfp* by PCR, and the results have shown *pat* and *yfp* target DNA sequences. Sequencing of the PCR products results have revealed the correct sequences of *pat* and *yfp* transgenes in T₁ *Arabidopsis* produced using methods of the invention.

Transgenic plants according to the invention may be regenerated from stably transformed plant cells produced by methods of the invention. Such plants may be used or cultivated in any manner, wherein presence of the nucleic acid molecules of interest is desirable. Accordingly, transgenic plants may be engineered to, *inter alia*, have one or
 5 more desired traits, by being transformed with functionalized linear nucleic acid cassette molecules *via* nanoparticle-mediated transformation, and cropped and cultivated by any method known to those of skill in the art.

The following examples are provided to illustrate certain particular features and/or embodiments. The examples should not be construed to limit the invention to the
 10 particular features or embodiments exemplified.

EXAMPLES

Example 1: Preparation of nanoparticles for plant cell transformation

Preparation of DNA; plasmid DNA

15 pDAB3831 plasmid DNA, Figure 1, was isolated and prepared for Linear-DNA / Streptavidin Coated Quantum Dot -- mediated plant transformation. This plasmid contains the PAT selectable marker gene driven by the Arabidopsis Ubiquitin 10 promoter (*AtUbi10*) and the *Philadium* Yellow Fluorescence Protein gene (*PhiYFP*) driven by the Cassava Vein Mosaic Virus promoter (*CsVMV*). Transformation experiments were tested
 20 using linearized DNA.

To linearize pDAB3831, a PCR reaction was completed. pDAB3831 was PCR amplified using a Continuous Thermal Cycling system. International PCT Publication WO 2008/045288. A sample was prepared containing: 12% MgCl₂ (25mM), 0.33% Taq DNA polymerase (5 units/μL), 2.0% dNTP's (deoxyadenosine triphosphate (dATP),
 25 deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP), 8.0% template (2μg/mL), 61.66% Pluronic* F108 solution (1.5% solution), 4% forward primer, 4% reverse primer, and 8% reaction buffer (10X concentration). The adjacent sectors of the system were set at the temperature of 95°C, 59°C, and 72°C for dissociation, annealing and extension purposes, respectively.
 30 The PCR reaction mixture was pumped through the system using a pressurized vessel at

*Trademark

-31-

20 PSI. After the reaction mixture was fed to the temperature control body, mineral oil was used to push the sample through the entire length of tubing. The flow rate of the reaction mixture was controlled with a flow valve to 0.25 mL/min. The specific DNA sequence present in the sample was amplified as it passed cyclically through the temperature zones. After the thirtieth cycle, the contents were collected. PCR product was purified on a gel filtration column followed by ethanol precipitation. A sample of the purified product was analyzed on an Agilent Bioanalyzer™, as well as Agarose gel electrophoresis, to confirm the size and the concentration of the PCR product.

Template used for the PCR described above was DAS plasmid pDAB3831. Forward primer SEQ ID NO:1 and reverse primer SEQ ID NO:2 were synthesized to amplify the 4.6 kbp complete expression cassette (*i.e.*, the linearized DNA) containing both genes and their promoters. In addition, to facilitate the conjugation of the linear dsDNA to the surface of nanoparticles, a biotin molecule was chemically linked to the phosphate group of the primers using Biotin-TEG-CE-phosphoramidite. This phosphoramidite has an extended 15-atom mixed polarity spacer arm based on a triethylene glycol linker. The benefits of an extended spacer arm separating the biotin function from the rest of an oligo is to reduce any possible steric hindrance effects during binding to the streptavidin molecule. When the forward primer was labeled, the biotin is at the beginning of the DNA. When the reverse primer was labeled, the biotin is at the end of the DNA fragment. The biotinylated (both orientations) DNA fragment can therefore be attached to streptavidin coated nanoparticles. Using the biotinylated oligos and the continuous thermal cycling system, approximately 20 mg of the linear DNA fragment was produced.

25 Complexation of the Linear-DNA/nanoparticles

Streptavidin Coated Quantum Dots were obtained from Evident Technology (Troy, NY). 1 mL of Streptavidin Coated Quantum Dots (4 nmol) were incubated with 0.5 mg biotinylated linearized plasmid DNA at room temperature for 30 minutes to form the Linear-DNA/QD complex.

Example 2: Transformation of *Arabidopsis* floral budsPlant material for *in planta* transformation

Synchronized germination of the seed is important to ensure the uniformity of floral development in the T₀ plants. *A. thaliana* cv. Columbia seed was suspended in 0.1% (w/v) agar solution and incubated at 4°C for 48 hours to complete stratification. 60 mg of seed was weighed and transferred to a 15 mL tube. 13 mL of 0.1% (w/v) agar solution was added and was vortexed until seed was evenly dispersed. This makes a seed solution concentration of 4.6 mg seed/1 mL of 0.1% (w/v) agar solution (or about 230 seeds/mL). 6 tubes (72 mL solution) were prepared to sow 4 flats that contain 18 (3 1/2 inch) pots in each tray. The seed solution was incubated at 4°C for 48 hours to complete stratification. Each pot was sown individually at 1.0 mL of stratified seed solution per pot. When all the pots were sown, propagation domes were placed on the trays to keep the soil moist. The domes were removed 5 days after the sow date. Seeds were germinated and plants were grown in a Conviron® (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 $\mu\text{mol}/\text{m}^2\text{sec}$ under constant temperature (22°C) and humidity (40-50%). Plants were watered 10 to 14 days after sowing the plants with Hoagland's solution and subsequently with DI water to keep the soil moist but not wet. After 4 weeks post-sow date, the flowers were cut back to produce a more even growth of secondary flowers. In the 5th week post-sowing, the plants were prepared for the transformation process.

In planta transformation

Linear-DNA / QD – mediated transformation of *A. thaliana* cv. Columbia was completed using a modified protocol from Clough and Bent. Clough and Bent (1998) Plant J. 16:735-43. A 20 mL suspension was made with the linear DNA / QD complex solution at a concentration of 0.5 mg of Linear-DNA and 4 nM of PQD and used for treatments of the *Arabidopsis* plants (mostly immature flower clusters with some fertilized siliques). Before dipping plants, Silwet* L-77 to a concentration of 0.05% (v/v) (250 μL /500 mL) - 0.005% was added to the Linear-DNA / PQD solution and mixed well.

*Trademark

-33-

Above-ground parts of plant were dipped in Linear-DNA / PQD solution for 30 seconds, with gentle agitation. Treated plants were placed on their sides for 30 minutes in shade at 22-24°C. The plants were transferred to the Conviron under conditions as described above and allowed to grow to maturity and to collect seeds.

5 Selection trays (10.5" x 21" x 1" trays) were used to screen bulk harvest seed from T₀ plants, approximately 10,000 seeds on each tray. Two controls were used to ensure selection spraying was done correctly; Col-0 negative transformant control and Columbia Col-0 wild type spiked with homozygous seed for PAT (phosphinothricin acetyl transferase) selectable marker as a positive transformant control. To achieve
10 synchronization, seeds were stratified in a 0.1% (w/v) agar solution for 48 hours prior to sowing. To provide 10,000 seeds per selection tray, 200 mg of seeds were added to a 0.1% (w/v) agar solution and vortexed until the seeds were evenly suspended. The stratified seeds were then sowed on selection trays filled with Sunshine mix LP5 and sub-irrigated with Hoagland's solution. For the selection spray to be effective, it is
15 important that the 40 mL of suspended seed is sown evenly onto the selection tray. After sowing, propagation domes were placed on each selection tray and plants were grown for selection. Propagation domes were removed approximately 5 days post-sowing.

Example 3: Analysis of transformed *Arabidopsis*

20 Selection of Transformed Plants

Freshly harvested T₁ seed was allowed to dry for 7 days at room temperature. T₁ seed was sown in 26.5 x 51-cm germination trays, each receiving a 200 mg aliquot of stratified T₁ seed (~10,000 seed) that had previously been suspended in 40 mL of 0.1% (w/v) agarose solution and stored at 4°C for 2 days to complete dormancy requirements
25 and ensure synchronous seed germination.

Sunshine Mix LP5 was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 mL aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes for 4-5 days. Domes were removed 1 day prior to initial transformant
30 selection using glufosinate postemergence spray.

Seven days after planting (DAP) T₁ plants (cotyledon and 2-4-leaf stage, respectively) were sprayed five times consecutively within five days with a 0.2% (w/v) solution of Liberty herbicide (200 g ae/L glufosinate, Bayer Crop Sciences, Kansas City, MO) at a spray volume of 10 mL/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ae/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix^{*} 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22°C growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22±5°C, 50±30% RH, 14 h light:10 dark, minimum 500 µE/m²s⁻¹ natural + supplemental light).

Molecular analysis and evidence for the genomic integration of transgenes

Genomic DNA from *A. thaliana* transgenic plants was extracted from leaf material of 6-week-old plants using Plant DNAzolTM (Invitrogen) according to the manufacturer's instructions. PCR primers were designed for detection of the YFP and PAT transgenes. The YFP primers are presented as SEQ ID NO:3 and SEQ ID NO:4. The PAT primers are presented as SEQ ID NO:5 and SEQ ID NO:6.

gDNA PCR Amplification of Transgenes

PCR amplification reactions for PAT and YFP were completed using the TaKaRa ExTaqTM kit (Takara, Otsu, Shiga, Japan). Gene products were amplified in a total reaction volume of 50 µL. The PCR reaction contained 100 ng genomic DNA template, 1X ExTaq reaction buffer, 0.2 mM dNTP, 10 pMol of each primer, and 0.025 units/µL ExTaq. The following PCR conditions were used: 1 cycle at 96°C for 5min, and 31 cycles of the following conditions 94°C for 15 sec., 65°C for 30 sec, 72 °C for 1 min and a final extension of 72°C for 7 min. PCR amplification product was analyzed by 0.8% TAE agarose gel electrophoresis and visualized by ethidium bromide staining. The DNA fragments were purified from the agarose gel using the QIAEXTM II gel purification kit (Qiagen, Valencia, CA).

*Trademark

-35-

The PCR fragments were sequenced using the PAT forward primer (SEQ ID NO:5) and YFP forward primer (SEQ ID NO:3) using advanced Sanger sequencing technology (MWG Biotech, Huntsville, AL). The sequence data was analyzed using Sequencher software.

5 The sequencing results of the PAT and YFP PCR amplicons matched the expected nucleotide sequence for these genes. These results clearly indicate that the PAT and YFP sequences from pDAB3831 were stably integrated into the gDNA of *Arabidopsis* using the nanoparticle and linear-DNA cassette transformation protocol.

10 The present results indicates that the PAT and YFP sequences delivered through positively charged nanoparticle-mediated linearized DNA delivery in Example 1 and thus providing an evidence of stable genomic integration of transgenes in the genomic DNA of *Arabidopsis* plants.

15 **Example 4: Nanoparticle-mediated delivery of functionalized linear nucleic acid cassette molecules to cultured plant cells**

Single cell plant material is prepared.

For example, both BY2 cells and NT1 cells are used. BY2 cells are a non-green, fast growing tobacco cell line. NT1 cells are photoautotrophic cells isolated from tobacco.

20 Three to four days prior to transformation, a one-week-old suspension culture is subcultured to fresh medium by transfer of 2 ml of NT1 or BY2 culture into 40 ml NT1B or LSBY2 media containing 50 nM DAS-PMTI-1 (a microtubule inhibitor) and 0.5- 0.1% (v/v) DMSO in a 250-mL flask. Single cells are collected either at four days or seven days after the microtubule inhibitor treatment. The BY2 single cells used are processed through

25 a Beckman Flow cytometer to count the viable cells. The cells are examined using a Differential Interference Contrast (DIC) microscope attached to a confocal imaging system to determine that single cells comprise large numbers of plastids (amyloplasts) distributed throughout the cytoplasm of the cell. Cells are sub-cultured once in every 14 days by transferring 1 mL of suspension at 3.0OD⁶⁰⁰. Cultured cells are used as target cells for

30 transformation.

Nanoparticle preparation and treatment of cells

Plasmid DNA is isolated and prepared for Linear-DNA / Quantum Dot (QD) -- mediated plant transformation. The plasmid contains the PAT selectable marker gene driven by the *Arabidopsis* Ubiquitin 10 promoter (*AtUbi10*) and the *Philadium* Yellow Fluorescence Protein gene (*PhiYFP*) driven by the Cassava Vein Mosaic Virus promoter (*CsVMV*). An *Escherichia coli* strain containing the plasmid is inoculated and grown to turbidity in Luria-Bertani broth containing ampicillin at 37°C. DNA is isolated using the Qiagen* Plasmid Midi-Prep kit (Qiagen, Valencia, CA).

To linearize the plasmid DNA, a PCR reaction is completed using a Continuous Thermal Cycling system. International PCT Publication WO 2008/045288. A sample is prepared containing: 12% MgCl₂ (25mM), 0.33% Taq DNA polymerase (5 units/μL), 2.0% dNTP's (deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP), 8.0% template (2μg/mL), 61.66% Pluronic F108 solution (1.5% solution), 4% forward primer, 4% reverse primer, and 8% reaction buffer (10X concentration). The adjacent sectors of the system are set at the temperature of 95°C, 59°C, and 72°C for dissociation, annealing and extension purposes, respectively. The PCR reaction mixture is pumped through the system using a pressurized vessel at 20 PSI. After the reaction mixture is fed to the temperature control body, mineral oil is used to push the sample through the entire length of tubing. The flow rate of the reaction mixture is controlled with a flow valve to 0.25 mL/min. The specific DNA sequence present in the sample is amplified as it passes cyclically through the temperature zones. After the thirtieth cycle, the contents are collected. PCR product is purified on a gel filtration column followed by ethanol precipitation. A sample of the purified product is analyzed on an Agilent Bioanalyzer™, as well as Agarose gel electrophoresis, to confirm the size and the concentration of the PCR product.

A forward primer and reverse primer are synthesized to amplify the complete expression cassette (*i.e.*, the linearized DNA) containing both genes and their promoters. In addition, to facilitate the conjugation of the linear dsDNA to the surface of nanoparticles, a biotin molecule is chemically linked to the phosphate group of the primers

*Trademark

-37-

using Biotin-TEG-CE-phosphoramidite. The biotinylated (both orientations) DNA fragment can therefore be attached to streptavidin coated nanoparticles. Using the biotinylated oligos and the continuous thermal cycling system, the linear DNA fragment is produced in milligram quantities.

5

Complexation of the Linear-DNA/nanoparticles

Streptavidin Coated Quantum Dots are obtained from Evident Technology (Troy, NY). 1 mL of Streptavidin Coated Quantum Dots (4 nmol) are incubated with 0.5 mg biotinylated linearized plasmid DNA at room temperature for 30 minutes to form the

10 Linear-DNA/QD complex.

A concentration of 1-3 $\mu\text{L/mL}$ linear DNA-conjugated nanoparticles are added to 500 μL of cells in a 24-well micro titer plate and rotated on a shaker gently for 20 minutes in the dark. The nanoparticles are transported across the cell walls.

15 Example 5: Multifunctionalized Nanoparticle-mediated *in planta* transformation of *Arabidopsis*

In planta transformation for *Arabidopsis* can be performed using a modified from Clough and Bent, 1998. Concentration of DNA on the multifunctionalized nanoparticle

20 along with the molecules of homing protein transduction domain (PTDs) and NLS units are optimized to achieve increased transformation efficiency.

Plant material: Healthy *Arabidopsis* plants are grown under long days in pots in soil until flowering. First bolts are clipped to encourage proliferation of many secondary bolts. Plants are ready roughly 4-6 days after clipping. *Arabidopsis thaliana* Columbia

25 (Col-0) ecotype is selected as the background (T_0 plant) for floral *in planta* transformation. Synchronized germination of the seed is important to ensure the uniformity of floral development in the T_0 plants. Wild Type seed is suspended in 0.1% agar solution and is incubated at 4°C for 48 hours to complete stratification. 60mg of seed is weighed on weigh paper and transferred to a 15 mL tube. 13 mL of 0.1% agar solution is added and

30 vortexed until seed is evenly dispersed. This makes a concentration of 4.6 mg seed/ 1 mL solution (or about 230 seeds / mL). 6 tubes (72 mL solution) are prepared to sow 4 flats

that contain 18 (3 1/2 inch) pots in each tray and 2 total pots are sowed. The solution is incubated at 4°C for 48 hours to complete stratification. Each pot is sown individually at 1.0 mL of stratified seed solution per pot. When all the pots are sown, propagation domes are placed on the trays to keep the soil moist. The domes are removed five days after the
 5 sow date. Seeds are germinated and plants are grown in a Conviron® (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150 $\mu\text{mol}/\text{m}^2\text{sec}$ under constant temperature (22°C) and humidity (40-50%). Plants are watered 10 to 14 days after sowing the plants with Hoagland's solution and
 10 subsequently with DI water to keep the soil moist but not wet. After 4 weeks post-sow date, the flowers are cut back to produce a more even growth of secondary flowers. In the 5th week post-sowing, the plants are prepared for the transformation process.

Nanoconjugate preparation for floral treatments: Nanoparticles of 2-120 nm size ranges are chosen for treatments and are multifunctionalized with a linear DNA cassette
 15 and the homing peptide units according to Derufus *et. al.* (2007). Quantum dots with emission maxima of 655 or 705 nm and modified with streptavidin and amino groups are obtained. QD concentrations are measured by optical absorbance at 595 nm, using extinction coefficients provided by the supplier. Cross-linkers used are sulfo-LC-SPDP (sulfosuccinimidyl 6-(3'-[2-pyridyldithio]-propionamido)hexanoate) (Pierce) and
 20 sulfo-SMCC (sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) (Sigma). Amino-modified streptavidin-QDs are conjugated to biotin-conjugated linear DNA cassettes and homing peptides using cross-linkers. QDs are resuspended in 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2, using Amicon* Ultra-4 (100 kDa cutoff) filters. Cross-linker (1000-fold excess) is added to QDs and allowed to react for
 25 1 h. Samples are filtered on a NAP-5 gravity column (to remove excess cross-linker) into similar buffer supplemented with 10 mM EDTA. Peptides are typically used from lyophilized powder. Peptide and linear DNA cassettes are added to filtered QDs and allowed to react overnight at 4°C. Using three Amicon filters, product is filtered twice with Dulbecco's phosphate-buffered saline (PBS), twice with a high salt buffer (1.0 M
 30 sodium chloride, 100 mM sodium citrate, pH 7.2), and twice again with PBS. High salt

*Trademark

washes are required to remove electrostatically bound DNA and peptide, which is not removed with PBS washes alone. Sulfo-SMCC has an *N*-hydroxysuccinimide(NHS) ester at one end, which reacts with amino-modified QDs to form an amide bond. Sulfo-LC-SPDP also contains an amine-reactive *N*-hydroxysuccinimide (NHS) ester
 5 which reacts rapidly with any primary amine-containing molecule thereby forming a stable amide bond.

In planta transformation and screening T₁ resistant plants: A final volume of 250-500 mL suspension is made with the nanoparticle, homing peptide and linear DNA cassette (NHD) conjugate solution and then the *Arabidopsis* plants (mostly immature
 10 flower clusters with some fertilized siliques) are used for treatments. Before dipping plants, Silwet L-77 to a concentration of 0.05% (250 ul/500 ml) - 0.005% is added to the NHpD conjugate solution and mixed well. Above-ground parts of plant are dipped in NHpD conjugate solution for 2 to 30 seconds, with gentle agitation. Treated plants are kept under a dome or cover for 16 to 24 hours at 22-24°C. The plants are transferred to the
 15 Conviron and allowed to grow to maturity and to collect seeds. Selection trays (10.5"x21"x1" trays) are used to screen bulk harvest seed from T₀ plants, approximately 10,000 seeds on each tray. Two controls are used to ensure selection spraying is done correctly, Col-0 negative transformant control and Columbia Col-0 wild type spiked with homozygous seed for PAT (Phosphinothricin acetyl transferase) selectable marker as a
 20 positive transformant control. To achieve synchronization, seeds are stratified in a 0.1% (w/v) agar solution for 48 hours prior to sowing. To provide 10,000 seeds per selection tray, 200 mg of seeds are added to a 0.1% agar solution and vortexed until the seeds are evenly suspended. The stratified seeds are then sowed on selection trays filled with Sunshine mix^{*} LP5 and sub-irrigated with Hoagland's solution. For the selection spray to
 25 be effective, it is important that the 40 mL of suspended seed is sown evenly onto the selection tray. After sowing, propagation domes are placed on each selection tray, the seeds are grown for selection using the conditions mentioned earlier. Propagation domes are removed approximately 5 days post-sowing. Seedlings are sprayed 5 days post-sowing and again 10 days post-sowing spray seedlings with a 0.2% (v/v) solution (20µl/10ml
 30 dH₂O) of glufosinate ammonium (LIBERTY® Herbicide from Bayer CropSciences) in a

*Trademark

-40-

spray volume of 10 mL/tray (703L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280g /ha glufosinate per application. The amount of Liberty® to prepare is calculated as follows: (703L/ha spray volume = 280 GPA). (280 g ai/ha) x (1 ha/703L) x (1 L/200 g ai glufosinate) = 0.20% solution (or 20 µL / 10 mL). 10 mL of
5 the solution is pipetted into a 20 mL scintillation vial for each tray to be sprayed. The spray is delivered using a horizontal and vertical application pattern. Four to seven days after the second spray herbicide resistant plants are identified.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 55118-9 Seq 14-12-12 vl.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

SEQUENCE TABLE

<110> Dow Agrosiences LLC
 Yau, Kerrm Y.
 Samuel, Jayakumar P
 Burroughs, Frank G.
 Samboju, Narasimha C.
 Webb, Steven R.

<120> PRODUCTION OF FUNCTIONALIZED LINEAR DNA CASSETTE AND QUANTUM DOT/NANOPARTICLE MEDIATED DELIVERY IN PLANTS

<130> 2971 10045.1PC (68330-WO-PCT)

<150> 61/362,222
 <151> 2010-07-07

<160> 6

<170> PatentIn version 3.5

<210> 1
 <211> 20
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Forward primer for plasmid pDAB3831

<400> 1
 tgaaagtgtgta catcaacgaa

<210> 2
 <211> 18
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Reverse primer for plasmid pDAB3831

40b

<400> 2
ccgcaactat ttcaacac

18

<210> 3
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for YFP gene

<400> 3
tggtccacgg caagatcccc tacg

24

<210> 4
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for YFP gene

<400> 4
tattcatctg ggtgtgatcg gccca

24

<210> 5
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Forward primer for PAT gene

<400> 5
ggagaggaga ccagttgaga ttag

24

<210> 6
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Reverse primer for PAT gene

<400> 6
agatctgggt aactggccta actg

24

CLAIMS:

1. A method of introducing a linear nucleic acid cassette into a plant cell having a cell wall, the method comprising:
providing the plant cell having a cell wall;
coating a quantum dot nanoparticle with a biotinylated linear nucleic acid cassette, wherein the quantum dot nanoparticle comprises streptavidin that interacts with the biotinylated linear nucleic acid cassette;
placing the plant cell having a cell wall and the coated quantum dot nanoparticle in contact with each other;
allowing uptake of the quantum dot and the biotinylated linear nucleic acid cassette into the plant cell comprising a cell wall; and
selecting cells that have stably integrated the linear nucleic acid cassette sequence.
2. The method according to claim 1, further comprising allowing uptake of the coated quantum dot nanoparticle into a compartment of the plant cell comprising a cell wall.
3. The method according to claim 2, further comprising coating the coated quantum dot nanoparticle with a subcellular compartment targeting protein.
4. The method according to claim 3, wherein the compartment is selected from the group consisting of cytosol, nucleus, tonoplasts, plastid, etioplast, chromoplast, leucoplast, elaioplast, proteinoplast, amyloplast, chloroplast, and the lumen of the double membrane.
5. The method according to claim 1, wherein the plant cell having a cell wall is a plant cell from a commercial crop species.
6. The method according to claim 5, wherein the plant cell is selected from the group consisting of tobacco, carrot, maize, canola, rapeseed, cotton, palm, peanut, soybean, *Oryza sp.*, *Arabidopsis sp.*, *Ricinus sp.*, and sugarcane cells.

7. The method according to claim 5, wherein the plant cell is from a tissue selected from the group consisting of embryo, meristematic, callus, pollen, leaves, anthers, roots, root tips, flowers, seeds, pods and stems.

8. The method according to claim 1, wherein the plant cell having a cell wall is a cultured cell.

9. The method according to claim 1, wherein the linear nucleic acid cassette comprises a nucleic acid sequence selected from the group consisting of DNA, RNA, RNAi molecules, and genes.

10. The method according to claim 9, wherein the linear nucleic acid cassette comprises a gene.

11. The method according to claim 10, wherein the gene is a foreign protein gene, an agronomic gene, or a marker gene.

12. The method according to claim 1, wherein the linear nucleic acid cassette is obtained from PCR amplification of a nucleic acid sequence.

13. The method according to claim 12, wherein the nucleic acid sequence is obtained from a nucleic acid molecule selected from the group consisting of plasmids, cosmids, artificial chromosomes, yeast artificial chromosomes, and bacterial artificial chromosomes.

14. The method according to claim 1, wherein the selected cells are regenerable cells.

15. The method according to claim 14, further comprising regenerating a plant from the regenerable cells.

16. A method of introducing a linear nucleic acid cassette into plant material, the method comprising:
providing plant material, wherein the plant material is selected from the group consisting of
plant cells, plant tissues, and plants;
providing a quantum dot nanoparticle;
coating the quantum dot nanoparticle with a biotinylated linear nucleic acid cassette, wherein
the quantum dot nanoparticle comprises streptavidin that interacts with the
biotinylated linear nucleic acid cassette;
placing the plant material and the coated quantum dot nanoparticle in contact with each other;
allowing uptake of the quantum dot nanoparticle and the biotinylated linear nucleic acid
cassette into the plant material; and
selecting plant material that has stably integrated the linear nucleic acid cassette sequence.

17. The method of claim 16, wherein the plant material is plant tissue selected from the group consisting of embryo, meristematic tissue, callus, pollen, leaves, anthers, roots, root tips, flowers, seeds, pods and stems.

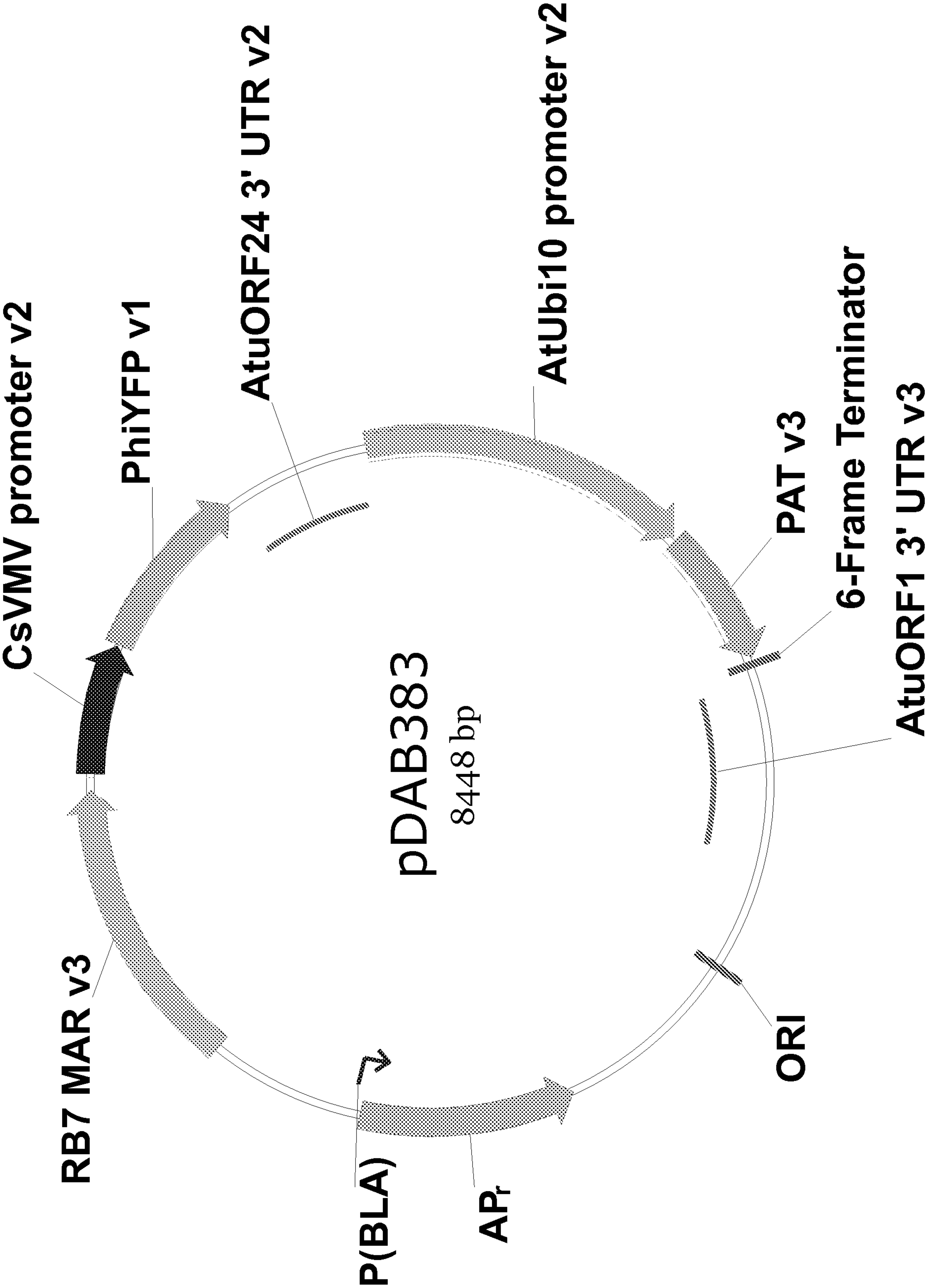


Fig. 1

1	GGCCGCTAAA	CCCAGAAAGGT	AATTATCCAA	GATGTAGCAT	CAAGAAATCCA	ATGTTTACGG	GAAAAACTAT	GGAAGTATTA	TGTAAGCTCA	GCAAGAAAGCA
	CCGGCGATT	GGGTCTTCCA	TTAATAGGT	CTACATCGTA	GTTCTTAGGT	TACAAATGCC	CTTTTGTGATA	CCTTCATAAT	ACATTCGAGT	CGTTCCTCGT
101	GATCAATATG	CGGCACATAT	GCAACCTATG	TTCAAAAATG	AAGAAATGTAC	AGATACAAGA	TCCTATACTG	CCAGAAATACG	AAGAAGAATA	CGTAGAAAATT
	CTAGTTATAC	GCCGTGTATA	CGTTGGATAC	AAGTTTTTAC	TTCTTACATG	TCTATGTTCT	AGGATATGAC	GGTCCTTATG	TTCCTCTTAT	GCATCTTTAA
201	GAAAAAGAAG	AACCAGGCGA	AGAAAAGAAT	CTTGAAAGACG	TAAGCACTGA	CGACAACAAT	GAAAAAGAGA	AGATAAGGTC	GGTGATTGTG	AAAGAGACAT
	CTTTTTCTTC	TTGGTCCGCT	TCTTTTCTTA	GAACTTCTGC	ATTCTGTACT	GCTGTTGTFA	CTTTTCTTCT	TCTATTCCAG	CCACTAACAC	TTTCTCTGTA
301	AGAGGACACA	TGTAAGGTGG	AAAATGTAAG	GGCGGAAAAGT	AACCTTATCA	CAAAAGGAATC	TTATCCCCCA	CTACTTATCC	TTTTATATTT	TTCCGTGTCA
	TCTCCTGTGT	ACATTCCACC	TTTTACATTC	CCGCCTTTCA	TTGGAATAGT	GTTCCTTAG	AATAGGGGGT	GATGAATAGG	AAAATATAAA	AAGGCACAGT
401	TTTTTTGCCCT	TGAGTTTTTCC	TATATAAGGA	ACCAAGTTTCG	GCATTTGTGA	AAACAAGAAA	AAATTTGGTG	TAAGCTATTT	TCITTGAAGT	ACTGAGGATA
	AAAAACGGGA	ACTCAAAAGG	ATATATTCTT	TGGTTCAAGC	CGTAAACACT	TTTGTTCCTT	TTTAAACCAC	ATTCGATAAA	AGAAACTTCA	TGACTCCTAT
501	CAACTTCAGA	GAAATTTGTA	AGTTTGTAGA	TCTCCATGGG	CTCCAGCGGC	GCCCTGCTGT	TCCACGGCAA	GATCCCCCTAC	GTGGTGGAGA	TGGAGGGCAA
	GTTGAAGTCT	CTTTAAACAT	TCAAACATCT	AGAGGTACCC	GAGTCCGCCG	CGGGACGACA	AGTGCCCGTT	CTAGGGGATG	CACCACCTCT	ACCTCCCCGT
601	TGTGGATGGC	CACACCTTCA	GCATCCGCGG	CAAGGGCTAC	GGCGATGCCA	GGTGGATGCC	GGTGGATGCC	CAGTTCATCT	GCACCACCGG	CGATGTGCCC
	ACACCTACCG	GTGTGGAAGT	CGTAGGCGCC	GTTCCCGATG	CCGCTACGGT	CGCACCCGTT	CCACCCTACGG	GTCAAAGTAGA	CGTGGTGGCC	GCTACACGGG
701	GTGCCCCTGGA	GCACCCTGGT	GACCACCCTG	ACCTACGGCG	CCCAGTGCTT	CGCCAAAGTAC	GGCCCCGAGC	TGAAGGATTT	CTACAAGAGC	TGCATGCCCG
	CACGGGACCT	CGTGGGACCA	CTGGTGGGAC	TGGATGCCGC	GGGTACGAA	GCGGTTCATG	CCGGGCTCG	ACTTCCTAAA	GATGTTCTCG	ACGTACGGGC
801	ATGGCTACGT	GCAGGAGCGC	ACCATCACCT	TCGAGGGCGA	TGGCAATTTC	AAGACCCCGC	CCGAGGTGAC	CTTCGAGAAAT	GGCAGCGTGT	ACAATCGCGT
	TACCGATGCA	CGTCTCGCG	TGGTAGTGA	AGCTCCCGCT	ACCGTTAAAG	TTCTGGGCGC	GGCTCCACTG	GAAGCTCTTA	CCGTCCGACA	TGTTAGCGCA
901	GAAAGCTGAAT	GGCCAGGGCT	TCAAGAAGGA	TGGCCACGTG	CTGGGCAAGA	ATCTGGAGTT	CAATTTCAAC	CCCCACTGCC	TGTACATCTG	GGCGATCAG
	CTTCGACTTA	CCGGTCCCGA	AGTTCTTCTT	ACCGGTGCAC	GACCCGTTCT	TAGACCTCAA	GTAAAGTGG	GGGTGACGG	ACATGTAGAC	CCCGCTAGTC
1001	GCCAATCACG	GCCTGAAGAG	CGCCTTCAAG	ATCTGCCACG	AGATCACCGG	CAGCAAGGGC	GATTTCATCG	TGGCCGATCA	CACCCAGATG	AATACCCCA
	CGGTTAGTGC	CGGACTTCTC	GCGGAAGTTC	TAGACGGTGC	TCTAGTGGCC	GTCGTTCCCG	CTAAAGTAGC	ACCGCTAGT	GTGGGTCTAC	TTATGGGGGT
1101	TCGGCGGCGG	CCCCGTGCAC	GTGCCCGAGT	ACCACCACAT	GAGCTACCAC	GTGAAGCTGA	GCAAGGATGT	GACCGATCAC	CGCGATAATA	TGAGCCTGAA
	AGCCGCCGCC	GGGGCACGTG	CACGGGCTCA	TGGTGGTGTA	CTCGATGGTG	CAC TTCGACT	CGTTCCTACA	CTGGCTAGTG	GCGCTATTAT	ACTCGGACTT

Fig- 2

1201	GGAGACCGTG	CGCGCCGTGG	ATTGCCGCAA	GACCTACCTG	TGAGAGCTCG	CATGCGGTCA	CCAAACCTTG	GACTCCCATG	TTGGCAAAGG	CAACCAAACA
	CCTCTGGCAC	CGCGGCACC	TAACGGCGTT	CTGGATGGAC	ACTCTCGAGC	GTACGCCAGT	GGTTTGGAAC	CTGAGGGTAC	AACCGTTTCC	GTTGGTTTGT
1301	AACAAATGAAT	GATCCGCTCC	TGCATATGGG	GCGGTTTGAG	TATTTCAACT	GCCATTTGGG	CTGAAATTGAA	GACATGCTCC	TGTCAGAAAT	TCCGTGATCT
	TTGTTTACTTA	CTAGGCGAGG	ACGTATACCC	CGCCAAACTC	ATAAAGTTGA	CGGTAAACCC	GACTTAACTT	CTGTACGAGG	ACAGTCTTTA	AGGCACTAGA
1401	TACTCAATAT	TCAGTAATCT	CGGCCAATAT	CCTAAATGTG	CGTGGCTTTA	TCTGTCTTTG	TATTGTTTCA	TCAATTCAATG	TAAAGTTTGC	TTTTCATATG
	ATGAGTTATA	AGTCATTAGA	GCCGGTTATA	GGATTTACAC	GCACCGAAAT	AGACAGAAAC	ATAACAAAAGT	AGTTAAGTAC	ATTGCAAACG	AAAAGTATAC
1501	AATTTTCAAA	TAAATTATCG	CGATAGTACT	ACGAAATATT	CGTATCGCTG	ATCTTCTCAA	TCACAAATGAT	GCGTAGTGAC	CCGACAAAATA	ATTTAAGCGT
	TTAAAAAGTTT	ATTTAATAGC	GCTATCATGA	TGCTTATAAA	GCATAGCGAC	TAGAAGAGTT	AGTGTTACTA	CGCATCACTG	GGCTGTTTAT	TAAATTGCGA
1601	CCTTAATACC	AATCCCTAAAA	TAATTGAGGC	AAATAAAAATT	TTTTTGTAAT	TTTTATGATA	GCAGATCGAT	TCTCCAGCAA	GCCTGCAACA	AAATATTGTG
	GGAAATTATGG	TTAGGATTTT	ATTAACTCCG	TTTATTTTAA	AAAAACATTA	AAAATACTAT	CGTCTAGCTA	AGAGTTCGTT	CGGACGTTGT	TTTATAAACAC
1701	TATTTCTAAA	TAGATTTTGA	TATTAAAAATC	CCGAGAAAAGC	AAAAATTGCAT	TTAACAAAAAC	AGTAAATTTAG	TACATTAATA	AAAAATTATGC	TCGGCCGGCC
	ATAAAGATTT	ATCTAAAAC	ATAATTTTAG	GGCTCTTTTCG	TTTTTAACGTA	AATTGTTTTG	TCATTAATATC	ATGTAATTAT	TTTTTAATACG	AGCCGGCCCG
1801	GCGGCCGCTT	AATTAAAATT	AAATGTTTAA	ACCCGCCCTG	CAGGTCAACG	GATCAGGATA	TTCTTGTTTA	AGATGTTGAA	CTCTATGGAG	GTTTGTATGA
	CGCCGGCGAA	TTAAATTTAAA	TTTACAAAAT	TGGGGCGGAC	GTCCAGTTGC	CTAGTCCCTAT	AAGAACAAAT	TCTACAACTT	GAGATACCTC	CAAAACATACT
1901	ACTGATGATC	TAGGACCGGA	TAAGTTCCCT	TCCTCATAGC	GAACTTATTC	AAAGAAATGTT	TTGTGTATCA	TTCTTGTTAC	ATTGTTATTA	ATGAAAAAAT
	TGACTACTAG	ATCCTGGCCT	ATTCAAAGGA	AGAAGTATCG	CTTGAATAAG	TTTCTTTACAA	AACACATAGT	AAGAACCAATG	TAAACAATAAT	TACTTTTTTA
2001	ATTATTGGTC	ATTGGA	CTGA	CTGA	CTGA	CTGA	CTGA	CTGA	CTGA	CTGA
	TAAATAACCAG	TAACCTGACT	TGTGCTCACA	ATTTATACCT	GGTCCGGGGT	TTATTTCTAGG	TAACTATATA	CTTAAATTTAT	TGTTCTTAT	TAGCTCAGTG
2101	CAAACCACTT	GCCTTTTTTA	ACGAGACTTG	TTCAACCAACT	TGATACAAAA	GTCATTATCC	TATGCAAAATC	AAATAATCATA	CAAAAAATATC	CAATAACACT
	GTTTGGTGAA	CGGAAAAAAT	TGCTCTGAAC	AAGTGGTTGA	ACTATGTTTT	CAGTAATAGG	ATACGTTTAG	TTATTAGTAT	GTTTTTTATAG	GTTATTGTGA
2201	AAAAAATTAA	AAGAAATGGA	TAAATTCACA	ATATGTTATA	CGATAAAGAA	GTTACTTTTC	CAAGAAATTC	ACTGATTTTA	TAAAGCCCACT	TGCATTAGAT
	TTTTTTTAAAT	TTCTTTACCT	ATTAAAAGTG	TATACAAATAT	GCTATTTCTT	CAATGAAAAAG	GTTCTTTAAG	TGACTAAAAAT	ATTCCGGGTGA	ACGTAATCTA

Fig. 2 cont.

2301	EcoRI										TTCAATTATT
	AAATGGCAAA	AAAAAACAAA	AAGGAAAAAG	AAATAAGCAC	GAAGAAATTCT	AGAAAAATACG	AAATACGCTT	CAATGCAGTG	GGACCCACGG		
2401	TTTACCGTTT	TTTTTTTGTT	TTCCCTTTCT	TTATTTCTGT	CTTCTTAAGA	TCTTTTATGC	TTTATGCGAA	GTACGTCAC	CCTGGGTGCC	AAGTTAATAA	
	GCCAAATTTT	AGCTCCACCG	TATATTTTAA	AAATAAAAACG	ATAATGCTAA	AAAAATATAA	ATCGTAACGA	TCGTTAAATC	TCAACGGCTG	GATCTTATGA	
2501	CGGTTAAAAG	TCGAGGTGGC	ATATAAATTT	TTTATTTTGC	TATTACGATT	TTTTTATATT	TAGCATTGCT	AGCAATTTAG	AGTTGCCGAC	CTAGAATACT	
	CGACCGTTAG	AAATTGTTGT	TGTCGACGAG	TCAGTAATAA	ACGGCGTCAA	AGTGGTTGCA	GCCGGCACAC	ACGAGTCGTG	TTTATCAACT	CAAAGCACAA	
2601	GCTGGCAATC	TTTAACACCA	ACAGCTGCTC	AGTCATTATT	TGCCGCAGTT	TCACCAACGT	CGGCCGTGTG	TGCTCAGCAC	AAATAGTTGA	GTTTCGTGTT	
	ATACTTTTCC	TCAACCTAAA	AATAAGGCAA	TTAGCCAAAA	ACAACTTTGC	GTGTAAACAA	CGCTCAATAC	ACGTGTCATT	TTATTATTAG	CTATTGCTTC	
2701	TATGAAAAGG	AGTTGGATTT	TTATTCCGTT	AATCGGTTTT	TGTTGAAACG	CACATTTGTT	GCGAGTTATG	TGCACAGTAA	AATAATAATC	GATAACGAAG	
	HindIII										
2801	ACCGCCCTTAG	CTTTCTCGTG	ACCTAGTCGT	CCTCGTCTTT	TCTTCTTCTT	CTTCTATAAA	ACAATACCCA	AAGCTTCTTC	TTCAACAATTC	AGATTTCAAT	
	TGGCGGAATC	GAAAGAGCAC	TGGATCAGCA	GGAGCAGAAA	AGAAAGAAAG	GAAGATATTT	TGTTATGGGT	TTCGAAGAAG	AAGTGTTAAG	TCTAAAAGTTA	
2901	TTCTCAAAAT	CTTAAAAACT	TTCTCTCAAT	TCCTCTTACC	GTGATCAAGG	TAAATTTCTG	TGTTCTTTAT	TCCTCCTAAA	TCCTCGATTT	TGTTTTCTGTT	
	AAGAGTTTAA	GAATTTTGA	AAGAGAGTTA	AGAGAGATGG	CAC TAGTTCC	ATTTAAAGAC	ACAAGGAATA	AGAGAGTTTT	AGAAAGCTAAA	ACAAAAAGCAA	
3001	CGATCCCAAT	TTCGTATATG	TTCTTTTGGT	TAGATTCTGT	TAATCTTAGA	TCGAAGACGA	TTTTCTGGGT	TTGATCGTTA	GATATCATCT	TAAATTCCTGA	
	GCTAGGGTTA	AAGCATATAC	AAGAAACCAA	ATCTAAGACA	ATTAGAAATC	AGCTTCTGCT	AAAAGACCCA	AACTAGCAAT	CTATAGTAGA	ATTAAGAGCT	
3101	TTAGGGTTTC	ATAAATATCA	TCCGATTTGT	TCAAATAAAT	TGAGTTTTGT	CGAATAATTA	CTCTTCGATT	TGTGATTTCT	ATCTAGATCT	GGTGTTAGTT	
	AATCCCAAAG	TATTTATAGT	AGGCTAAACA	AGTTTATTAA	ACTCAAAACA	GCTTATTAAT	GAGAAAGCTAA	ACACTAAAGA	TAGATCTAGA	CCACAATCAA	
3201	BamHI NcoI										
	TCTAGTTTGT	GCGATCGAAT	TTGTGATTA	ATCTGAGTTT	TTCTGATTAA	CAGGTAAGGA	TCCAACCATG	GCTTCTCCGG	AGAGGAGACC	AGTTGAGATT	
3301	AGATCAAACA	CGCTAGCTTA	AACAGCTAAT	TAGACTCAAA	AAGACTAATT	GTCCATTCCCT	AGGTTGGTAC	CGAAGAGGCC	TCTCCTCTGG	TCAACTCTAA	
	AGGCCAGCTA	CAGCAGCTGA	TATGGCCGCG	GTTTGTGATA	TCGTTAAACCA	TTACATTGAG	ACGTCTACAG	TGAACTTTAG	GACAGAGCCA	CAAACACCCAC	
3401	TCCGGTCGAT	GTCGTCGACT	ATACCGGCGC	CAAACACTAT	AGCAATTGGT	AATGTAACTC	TGCAGATGTC	ACTTGAAATC	CTGTCTCGGT	GTTTGTGGTG	
	AAGAGTGGAT	TGATGATCTA	GAGAGGTTGC	AAGATAGATA	CCCTTGGTTG	GTTGCTGAGG	TTGAGGGTGT	TGTGGCTGGT	ATTGCTTACG	CTGGGCCCTG	
	TTCTCACCTA	ACTACTAGAT	CTCTCCAACG	TTCTATCTAT	GGGAACCAAC	CAACGACTCC	AACTCCCACA	ACACCGACCA	TAAACGAATGC	GACCCGGGAC	
	BamHI										
	GAAGGCTAGG	AACGCTTACG	ATTGGACAGT	TGAGAGTACT	GTTTACGTGT	CACATAGGCA	TCAAAGGTTG	GGCCTAGGAT	CCACATTGTA	CACACATTTG	
	CTTCCGATCC	TTGCGAATGC	TAACCTGTCA	ACTCTCATGA	CAAAATGCACA	GTGTATCCGT	AGTTTCCAAC	CCGGATCCCTA	GGTGTAAACAT	GTGTGTAAAC	

Fig. 2 cont.

3501	CTTAAGTCTA	TGGAGGCGCA	AGGTTTTAAG	TCTGTGGTTG	CTGTTATAGG	CCTTCCAAAC	GATCCATCTG	TTAGGTTGCA	TGAGGCTTTG	GGATACACAG
	GAAATTCAGAT	ACCTCCGCGT	TCCAAAATTC	AGACACCAAC	GACAATATCC	GGAAGGTTTG	CTAGGTAGAC	AATCCAACGT	ACTCCGAAAC	CCTATGTGTC
	SmaI XmaI AvaI									
3601	CCCGGGGTAC	ATTGCGCGCA	GCTGGATACA	AGCATGGTGG	ATGGCATGAT	GTTGGTTTTT	GGCAAAAGGA	TTTTGAGTTG	CCAGCTCCTC	CAAGGCCAGT
	GGGCCCCATG	TAACGCGCGT	CGACCTATGT	TCGTACCACC	TACCGTACTA	CAACCAAAA	CCGTTTCCCT	AAAACTCAAC	GGTCGAGGAG	GTTCCGGTCA
	BamHI									
3701	TAGGCCAGTT	ACCCAGATCT	GAGGTACCCT	GAGCTTGAGC	TTATGAGCTT	ATGAGCTTAG	AGCTCGGATC	CAC TAGTAAC	GGCCGCCAGT	GTGCTGGAAT
	ATCCGGTCAA	TGGGTCTAGA	CTCCATGGGA	CTCGAACTCG	AATACTCGAA	TACTCGAATC	TCGAGCCCTAG	GTGATCATTG	CCGCGCGTCA	CACGACCCTTA
	SmaI XmaI AvaI									
3801	TCGCCCTTGA	CTAGATAGGC	GCCCAGATCG	GCGGCAATAG	CTTCTTAGCG	CCATCCCGGG	TTGATCCTAT	CTGTGTTGAA	ATAGTTGCGG	TGGGCAAGGC
	AGCGGGAAC	GATCTATCCG	CGGGTCTAGC	CGCCGTTATC	GAAGAATCGC	GGTAGGGCCC	AACTAGGATA	GACACAACTT	TATCAACGCC	ACCCGTTCCG
3901	TCTCTTTTCA	AAAGACAGGC	GGCCAAAGGA	ACCCAAAGGTG	AGGTGGGCTA	TGGCTCTCAG	TTCCCTTGTTG	AAGCGCTTGG	TCTAAGGTGC	AGAGGTGTTA
	AGAGAAAGTC	TTTCTGTCCG	CCGGTTTCCT	TGGGTTCCAC	TCCACCCGAT	ACCGAGAGTC	AAGGAACACC	TTCGCGAACC	AGATTCCACG	TCTCCACAAT
4001	GCGGGATGAA	GCAAAAGTGT	CCGATTGTAA	CAAGATATGT	TGATCCTACG	TAAGGATATT	AAAGTATGTA	TTCATCACTA	ATATAATCAG	TGTATTCCAA
	CGCCCTACTT	CGTTTTCACA	GGCTAACATT	GTTCATACA	ACTAGGATGC	ATTCCATATA	TTTCATACAT	AAGTAGTGAT	TATATTAGTC	ACATAAGGTT
4101	TATGTACTAC	GATTTCCAAT	GTCCTTTATTG	TCGCCGTATG	TAATCGGCGT	CACAAAATAA	TCCCCGGTGA	CTTTCCTTTA	ATCCAGGATG	AAATAATATG
	ATACATGATG	CTAAAGGTTA	CAGAAATAAC	AGCGGCATAC	ATTAGCCGCA	GTGTTTATT	AGGGCCACT	GAAAGAAAAT	TAGGTCCTAC	TTTATTATAC
4201	TTATTATAAT	TTTTGCGATT	TGGTCCGTTA	TAGGAATTGA	AGTGTGCTTG	CGGTCGCCAC	CACTCCCAT	TCATAATTTT	ACATGTATTT	GAAAAATAAA
	AATAATATTA	AAAACGCTAA	ACCAGGCAAT	ATCCTTAACT	TCACACGAAC	GCCAGCGGTG	GTGAGGGTAA	AGTATTAAAA	TGTACATAAA	CTTTTATTAT
4301	AAATTATGGT	ATTCAATTTA	AACACGTATA	CTTGTAAGA	ATGATATCTT	GAAAGAAATA	TAGTTTAAAT	ATTTATTGAT	AAAAATACAA	GTCAGGTATT
	TTAAATACCA	TAAGTTAAAT	TTGTGCATAT	GAACATTTCT	TACTATAGAA	CTTTCCTTAT	ATCAAAATTA	TAAATAACTA	TTTTATTGTT	CAGTCCATAA
4401	ATAGTCCAAG	CAAAAACATA	AATTATTTGA	TGCAAGTTTA	AATTCAGAAA	TATTTCAATA	ACTGATTATA	TCAGCTGGTA	CATTGCCGTA	GATGAAAGAC
	TATCAGGTTT	GTTTTTTGTAT	TTAAATAAACT	ACGTTCAAAT	TTAAGTCTTT	ATAAAGTTAT	TGACTAATAT	AGTCGACCAT	GTAACGGCAT	CTACTTTCTG

Fig. 2 cont.

4501	TGAGTGC	GAT	ATTATG	TGGTGT	AATACATAGC	GGCCGGGGTTT	CTAGTCACCG	GTGTAGCTTG	GCGTAATCAT	GGTCATAGCT	GTTTCCTGTG	TGAAATTGTT
	ACTCACGCTA	TAATACCA	CA	TTATGTATCG	TTATGTATCG	CCGGCCCCAAA	GATCAGTGGC	CACATCGAAC	CGCATTAGTA	CCAGTATCGA	CAAAAGGACAC	ACTTTAACAA
4601	ATCCGCTCAC	AATTCCACAC	AACATACGAG	CCGGAAGCAT	AAAGTGTA	AA	AAAGTGTA	GCCTGGGGTG	CCTAATGAGT	GAGCTAACTC	ACATTAAATTG	CGTTGCGCTC
	TAGGCGAGTG	TTAAGGTGTG	TTGTATGCTC	GGCCTTCGTA	TTTCACATTT	TTTCACATTT	CGGACCCCCAC	CGGACCCCCAC	GGATTACTCA	CTCGATTGAG	TGTAATTAAC	GCAACGCGAG
4701	ACTGCCCGCT	TTCCAGTCCG	GAAACCTGTC	GTGCCAGCTG	CATTAATGAA	TCGGCCCAACG	CGCGGGGAGA	GGCGGTTTGC	GTAATTGCGG	GTATTGGGCG	GTAATTGGGCG	CTCTTCCGCT
	TGACGGGCGA	AAGGTCAGCC	CTTTGGACAG	CACGGTCGAC	GTAATTACTT	AGCCGGTTGC	GCGCCCCCTCT	CGGCCAAACG	CATAACCCCG	CATAACCCCG	CATAACCCCG	GAGAAAGCGA
4801	GGGCACGCTG	CGCACGCTGC	GCACGCTTCC	TCGCTCACTG	ACTCGCTGCG	CTCGGTGCGT	CGGCTGCGGC	GAGCGGTATC	AGCTCACTCA	AAGCGGTAA	AAGCGGTAA	AAGCGGTAA
	CGCGTGCGAC	GGGTGCGACG	CGTGCGAAGG	AGCGAGTGAC	TGAGCGACGC	GAGCCAGCAA	GCCGACGCG	GAGCCAGCAA	GCCGACGCG	CTCGCCATAG	TCGAGTGAGT	TTCCGCCATT
4901	TACGGTTATC	CACAGAAATCA	GGGATAACG	CAGGAAAGAA	CATGTGAGCA	AAAGGCCAGC	AAAAGGCCAG	GAACCGTAAA	AAGGCCGCGT	AAGGCCGCGT	AAGGCCGCGT	TGCTGGCGTT
	ATGCCAATAG	GTGTCTTAGT	CCCTATTG	GTCCTTTCTT	GTACACTCGT	TTTCCGTCG	TTTCCGTCG	TTTCCGTCG	TTTCCGTCG	CTTGCAATTT	TTCCGGCGCA	ACGACCGCAA
5001	TTTTCCATAG	CTCCGCCCCC	CTGACGAGCA	TCACAAAAAT	CGACGCTCAA	GTCAGAGGTG	GCGAAAACCCG	ACAGGACTAT	AAAGATACCA	AAAGATACCA	AAAGATACCA	GGCGTTTCCC
	AAAGGTATCC	GAGGCGGGG	GACTGCTCGT	AGTGTTT	GCTGCGAGTT	CAGTCTCCAC	CGCTTTGGG	TGTCCTGATA	TTTCTATGGT	TTTCTATGGT	TTTCTATGGT	CCGCAAGGG
5101	CCTGGAAGCT	CCCTCGTGCG	CTCTCCTGTT	CCGACCCCTGC	CGCTTACCGG	ATACCTGTCC	GCCTTTCTCC	CTTCGGGAAG	CGTGGCGCTT	CGTGGCGCTT	CGTGGCGCTT	TCTCATAGCT
	GGACCTTCGA	GGGAGCACGC	GAGAGGACAA	GGCTGGGACG	GCGAATGGCC	TATGGACAGG	GCGAAAAGAGG	GAAGCCCTTC	GCACCGCGAA	GCACCGCGAA	GCACCGCGAA	AGAGTATCGA
5201	CACGCTGTAG	GTATCTCAGT	TCGGTGTAGG	TCGTTTGGCTC	CAAGCTGGGC	TGTGTGCACG	AACCCCCCGT	TCAGCCCGAC	CGCTGCGCCT	CGCTGCGCCT	CGCTGCGCCT	TATCCGGTAA
	GTGCGACATC	CATAGAGTCA	AGCCACATCC	AGCAAGCGAG	GTTCGACCCG	ACACACGTGC	TTGGGGGGCA	AGTCGGGCTG	GCGACGCGGA	GCGACGCGGA	GCGACGCGGA	ATAGGCCATT
5301	CTATCGTCTT	GAGTCCAACC	CGGTAAGACA	CGACTTATCG	CCACTGGCAG	CAGCCACTGG	TAAACAGGATT	AGCAGAGCGA	GGTATGTAGG	GGTATGTAGG	GGTATGTAGG	CGGTGCTACA
	GATAGCAGAA	CTCAGGTTGG	GCCATTCTGT	GCTGAATAGC	GGTGACCGTC	GTCGGTGACC	ATTGTCCCTAA	TCGTCTCGCT	CCATACATCC	CCATACATCC	CCATACATCC	GCCACGATGT
5401	GAGTTCCTGA	AGTGGTGGCC	TAACTACGGC	TACACTAGAA	GGACAGTATT	TGGTATCTGC	GCTCTGCTGA	AGCCAGTTAC	CTTCGGAAAA	CTTCGGAAAA	CTTCGGAAAA	AGAGTTGGTA
	CTCAAGAACT	TCACCACCGG	ATTGATGCCG	ATGTGATCTT	CCTGTCTATA	ACCATAGACG	CGAGACGACT	TCGGTCAATG	GAAAGCCTTT	GAAAGCCTTT	GAAAGCCTTT	TCTCAACCAT
5501	GCTCTTGATC	CGGCAAAACA	ACCACCGCTG	GTAGCGGTGG	TTTTTTTGT	TGCAAGCAGC	AGATTACGCG	CAGAAAAAAA	GGATCTCAAG	GGATCTCAAG	GGATCTCAAG	AAGATCCCTT
	CGAGAACTAG	GCCGTTTGT	TGGTGGCGAC	CATCGCCACC	AAAAAAACA	ACGTTCTGTC	TCCTAATGCGC	GTCTTTTTT	CCTAGAGTTC	CCTAGAGTTC	CCTAGAGTTC	TTCTAGGAAA
5601	GATCTTTTCT	ACGGGGTCTG	ACGCTCAGTG	GAACGAAAAAC	TCACGTTAAG	GGATTTTGGT	CATGAGATTA	TCAAAAAAGGA	TCTTCACCTA	TCTTCACCTA	TCTTCACCTA	GATCCTTTTA
	CTAGAAAAAG	TGCCCCAGAC	TGCGAGTCAC	CTTGCTTTTG	AGTGCAATTC	CCTAAAACCA	GTA	GTACTCTAAT	AGTTTTCCT	AGAAAGTGAT	AGAAAGTGAT	CTAGGAAAAT
5701	AATTAAAAAT	GAAGTTT	TAA	ATCAATCTAA	AGTATATATG	GTCTGACAGT	TACCAATGCT	TAATCAGTGA	GGCACCTATC	GGCACCTATC	GGCACCTATC	TCAGCGATCT
	TTAATTTT	CTTCAAAAT	TT	TAGTTAGATT	TCATATATAC	CAGACTGTCA	ATGTTACGA	ATTAGTCACT	CCGTGGATAG	CCGTGGATAG	CCGTGGATAG	AGTCGCTAGA

Fig. 2 cont.

5801	GTCTATTTCG	TTCATCCATA	GTTGCCCTGAC	TCCCCGTCGT	GTAGATAACT	ACGATACGGG	AGGGCTTACC	ATCTGGCCCC	AGTGCTGCAA	TGATACCGCG
	CAGATAAAGC	AAGTAGGTAT	CAACGGACTG	AGGGGCAGCA	CATCTATTGA	TGCTATGCC	TCCCGAATGG	TAGACCGGG	TCACGACGTT	ACTATGGCGC
5901	AGACCCACGC	TCACCGGCTC	CAGATTTATC	AGCAATAAAC	CAGCCAGCCG	GAAGGGCCGA	GCGCAGAAGT	GGTCCTGCAA	CTTTATCCGC	CTCCATCCAG
	TCTGGGTGCG	AGTGCCCGAG	GTCTAAATAG	TCGTTATTTG	GTGCGTCGGC	CTTCCCGGCT	CGCGTCTTCA	CCAGGACGTT	GAAATAGCG	GAGGTAGGTC
6001	TCTATTAAAT	GTTGCCGGGA	AGCTAGAGTA	AGTAGTTCGC	CAGTTAATAG	TTTGCGCAAC	GTTGTTGCCA	TTGCTACAGG	CATCGTGGTG	TCACGCTCGT
	AGATAAATTAA	CAACGGCCCT	TCGATCTCAT	TCATCAAGCG	GTCAATTATC	AAACGCGTIG	CAACAAACGGT	AACGATGTCC	GTAGCACCCAC	AGTGCAGGCA
6101	CGTTTGGTAT	GGCTTCAATC	AGCTCCGGTT	CCCAACGATC	AAGCGAGTT	ACATGATCCC	CCATGTTGTG	CAAAAAAGCG	GTTAGCTCCT	TCGGTCCCTCC
	GCAAAACCATA	CCGAAGTAAG	TCGAGGCCAA	GGTTTGCTAG	TTCCGCTCAA	TGTACTAGGG	GGTACAACAC	GTTTTTTTCG	CAATCGAGGA	AGCCAGGAGG
6201	GATCGTTGTC	AGAAGTAAGT	TGGCCGCAGT	GTTATCACTC	ATGGTTATGG	CAGCACIGCA	TAAATTCTCTT	ACTGTCATGC	CATCCGTAAG	ATGCTTTTCT
	CTAGCAACAG	TCTTTCATTCA	ACCGGCGTCA	CAATAGTGAG	TACCAATACC	GTCGTGACGT	ATTAAGAGAA	TGACAGTACG	GTAGGCATTC	TACGAAAAAGA
6301	GTGACTGGTG	AGTACTCAAC	CAAGTCATTC	TGAGAAATAGT	GTATGCGGGC	ACCGAGTTGC	TCTTGCCCCG	CGTCAATACG	GGATAATACC	GCGCCACATA
	CACTGACCAC	TCATGAGTTG	GTTCAGTAAG	ACTCTTATCA	CATACGCCGC	TGGCTCAACG	AGAACGGGCC	GCAGTTATGC	CCTATTATGG	CGCGGTGTAT
6401	GCAGAACTTT	AAAAGTGCTC	ATCATTTGAA	AACGTTCTTC	GGGGCGAAAA	CTCTCAAGGA	TCTTACCGCT	GTTGAGATCC	AGTTCGATGT	AACCCACTCG
	CGTCTTGAAA	TTTTCACGAG	TAGTAACCTT	TTGCAAGAAG	CCCCGCTTTT	GAGAGTTICCT	AGAAATGGCGA	CAACTCTAGG	TCAAGCTACA	TTGGGTGAGC
6501	ApaLI									
	TGCACCCCAAC	TGATCTTCAG	CATCTTTTAC	TTTCACCAGC	GTTTCTGGGT	GAGCAAAAAC	AGGAAGGCAA	AATGCCGCAA	AAAAGGGAAT	AAGGGCGACA
	ACGTGGGTG	ACTAGAAGTC	GTAGAAAAATG	AAAGTGGTCG	CAAAAGACCCA	CTCGTTTTTG	TCCCTCCGTT	TTACGGCGTT	TTTTCCCTTA	TTCCCGCTGT
6601	CGGAAATGTT	GAATACTCAT	ACTCTTCCTT	TTTCAATATT	ATTGAAGCAT	TTATCAGGGT	TATTGTCTCA	TGAGCGGATA	CATATTTGAA	TGTATTTAGA
	GCCTTTTACAA	CTTATGAGTA	TGAGAAAGGAA	AAAGTTATAA	TAACTTCGTA	AATAGTCCCA	ATAACAGAGT	ACTCGCCTAT	GTATAAACTT	ACATAAAATCT
6701	AAAATAAACA	AATAGGGGTT	CCGCGCACAT	TTCCCCGAAA	AGTGCCACCT	GACGTCTAAG	AAACCATTAT	TATCATGACA	TTAACCTATA	AAAAATAGCG
	TTTTTATTGT	TTATCCCCAA	GGCGCGTGTA	AAGGGGCTTT	TCACGGTGGA	CTGCAGATTC	TTTGGTAATA	ATAGTACTGT	AATTGGATAT	TTTTATCCGC
6801	TATCACGAGG	CCCTTTTCGTC	TCGCGCGTTT	CGGTGATGAC	GGTGAAAACC	TCTGACACAT	GCAGCTCCCG	GAGACGGTCA	CAGCTTGTCT	GTAAGCGGAT
	ATAGTGCTCC	GGGAAAGCAG	AGCGCGCAAA	GCCACTACTG	CCACTTTTGG	AGACTGTGTA	CGTCGAGGGC	CTCTGCCAGT	GTCGAACAGA	CATTCCGCTA
6901	GCCGGGAGCA	GACAAGCCCG	TCAGGGCGCG	TCAGCGGGTG	TTGGCGGGTG	TCGCGGCTGG	CTTAACTATG	CGGCATCAGA	GCAGATTGTA	CTGAGAGTGC
	CGGCCCTCGT	CTGTTTCGGC	AGTCCCGCGC	AGTCGCCCCAC	AACCGCCAC	AGCCCCGACC	GAATTGATAC	GCCGTAGTCT	CGTCTAACAT	GACTCTCACC
	ApaLI									

Fig. 2 cont.

ApaLI	
7001	ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCCGCCATT CAGGCTGCGC AACGTGTGGG AAGGGCGATC TGGTATACGC CACACTTTAT GCGGTGTCTA CGCATTCCTC TTTTATGGCG TAGTCCGCGG TAAGCGGTAA GTCCGACGCG TTGACAAACC TTCCCGCTAG
7101	GGTGCGGGCC TCTTCGCTAT TACGCCAGCT GCGGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGTA ACGCCAGGT TTTCCCAAGT ACGACGTTGT CCACGCCCGG AGAAGCGATA ATGCGGTCGA CCGCTTTCCC CCTACACGAC GTTCCGCTAA TTCAAACCCAT TCGGTCCCA AAGGTCAG TGCTGCAACA
7201	AAAACGACGG CCAGTGAATT ACACCGGTGT GATCATGGGC CGCGATTAAA AATCTCAATT ATATTTGGTC TAATTTAGTT TGGTATTGAG TAAACAAAT TTTTTGCTGCC GGTCACCTAA GTGGCCACA CTAGTACCCG GCGCTAATTT TTAGAGTTAA TATAAACCCAG ATTAAATCAA ACCATAACTC ATTTTGTTTA
7301	TCGAACCAAA CCAAAATATA AATATATAGT TTTTATATAT ATGCCCTTAA GACTTTTAT AGAATTTTCT TTAAAAAATA TCTAGAAATA TTTGCGACTC AGCTTGGTTT GGTTTTATAT TTATATATCA AAAATATATA TACGGAAATT CTGAAAAATA TCTTAAAAAG AATTTTTTAT AGATCTTTAT AACCGCTGAG
7401	TTCTGGCATG TAATATTTTG TTAATATAGA AGTGCTCCAT TTTTATTAACT TTTTAAATAAT TGGTTGTACG ATCACTTTCT TATCAAGTGT TACTAAAAATG AAGACCGTAC ATTATAAAGC AATTATACT TCACGAGGTA AAAATAATTG AAAATTAATTG ACCAACATGC TAGTGAAAGA ATAGTTCACA ATGATTTTAC
7501	CGTCAATCTC TTTGTTCTTC CATATTCATA TGTCAAAACC TATCAAAAATT TTTTATATAT CTTTTCGAAT TTGAAAGTGAA ATTTTCGATAA TTTAAAAATTA GCAGTTAGAG AAACAAGAAG GTATAAGTAT ACAGTTTTGG ATAGTTTAA GAAATATATAG AATAAGCTTA AACTTCACCT TAAAGCTATT AAATTTTAAT
7601	AATAGAACAT ATCATTAATT AGGTATCATA TGTCAAAACC TACTTAATTA TACTTAATTA ATATTTTAAAT TTAACCTTGA AAGTGTCAT AAGCGAAAA TTATCTTGTA TAGTAATAAA TCCATAGTAT AACTAAAAAT ATGAATTAAT ATGAACTAACC GATTTAAACC AATTGAAACT TTCACATGTA GTTGCTTTT AATCAGTTTG
7701	GACTAAAAA AATAAATATC ATGTGTTATT AAGAAAAATC TCCTATAAGA ATATTTTAAAT AGATCATATG TTTGTAAAAA AAATTAATTT TTACTAACAC CTGATTTTAT TTATTTATAG TACACAATAA TTCTTTTAAG AGGATATTCT TATAAAATTA TCTAGTATAC TATAAATTTT AAACATTTT TTTAATTAATA AATGATTGTG
7801	ATATATTTAC TTATCAAAAA TTTGACAAAAG TAAGATTAAA ATAATATTCA TCTAACAAAA AAAAAACCAG AAAATGCTGA AAACCCGGCA AAACCGAAAC TATATAAATG AATAGTTTTT AAACGTGTTT AATCTAATTT TATTATAAGT TATTATAAGT AGATTGTTTT AGATTGTTT TTTTACGACT TTTGGCCGT TTTGGCTTGG
7901	AATCCAAACC GATATAGTTG GTTTGGTTTG ATTTTGATAT AAACCGAACC AACTCGGTCC ATTTGCACCC CTAATCATAA TAGCTTTAAT ATTTCAAGAT TTAGGTTTGG CTATATCAAC CAAACCAAC TAAACTATA TTTGGCTTGG TTGAGCCAGG TAAACGTGGG GATTAGTATT ATCGAAATTA TAAAGTTCTA
8001	ATTATTAAGT TAACGTTGTC AATATCCTGG AAATTTTGCA AAATGAATCA AGCCTATATG GCTGTAATAT GAATTTAAAA GCAGCTCGAT GTGGTGTAA TAAATAATTCA ATTGCAACAG TTATAGGACC TTTAAAACGT TTTACTTAGT TCGGATATAC CGACATTATA CTTAAATTTT CGTCGAGCTA CACCACCAT
8101	TATGTAATTT ACTTGATTCT AAAAAAATAT CCCAAGTATT AATAATTTCT GCTAGGAAGA AGGTTAGCTA CGATTTACAG CAAAGCCAGA ATACAATGAA ATACATTAAA TGAACTAAGA TTTTTTTATA GGGTTCATAA TTATTAAAGA CGATCCTTCT TCCAAATCGAT GCTAAATGTC GTTTCGGTCT TATGTTACTT

Fig. 2 cont.

8201

CCATAAAGTG ATTGAAGCTC GAAATATACG AAGGAACAAA TATTTTAAAA AAAATACGCA ATGACTTTGGA ACAAAGAGAAA GTGATATATT TTTTGTTCCTT
GGTATTTCAC TAACTTCGAG CTTTATATGC TTCCCTTGTTT ATAAAAATTT TTTTATGCGT TACTGAACCT TGTTTTCTTT CACTATATAA AAAACAAGAA

8301

AAACAAGCAT CCCCTCTAAA GAATGGCAGT TTTCCCTTTGC ATGTAACTAT TATGCTCCCT TCGTTACAAA AATTTTGAC TACTATTGGG AACTTCTTCT
TTTGTTTCGTA GGGGAGATTT CTTACCGTCA AAAGGAAACG TACATTGATA ATACGAGGA AGCAATGTTT TTAAAAACCTG ATGATAACCC TTGAAGAAGA

SmaI
XmaI
AvaI

8401

GAAAAATAGTG GCCACCGCTT AATTAAAGCG CGCCATGCCC GGGCAAGC
CTTTTATCAC CGGTGGCGAA TTAATTCCGC GCGGTACGGG CCCGTTTCG

10 / 11

```

GCAGCCNGANATGGCCGCGGTTNGTGATATCGTTAACCATTACATTGAGACGCTCTACAG
|||||  || ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
GCAGC-TGATATGGCCGCGGTTTGTGATATCGTTAACCATTACATTGAGACG-TCTACAG

TGAACTTTAGGACAGAGCCACAAACACCACAAGAGTGGATTGATGATCTAGAGAGGTTGC
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
TGAACTTTAGGACAGAGCCACAAACACCACAAGAGTGGATTGATGATCTAGAGAGGTTGC

AAGATAGATACCCTTGGTTGGTTGCTGAGGTTGAGGGTGTTGTGGCTGGTATTGCTTACG
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
AAGATAGATACCCTTGGTTGGTTGCTGAGGTTGAGGGTGTTGTGGCTGGTATTGCTTACG

CTGGGCCCTGGAAGGCTAGGAACGCTTACGATTGGACAGTTGAGAGTACTGTTTACGTGT
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
CTGGGCCCTGGAAGGCTAGGAACGCTTACGATTGGACAGTTGAGAGTACTGTTTACGTGT

CACATAGGCATCAAAGGTTGGGCCTAGGATCTACATTGTACACACATTTGCTTAAGTCTA
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
CACATAGGCATCAAAGGTTGGGCCTAGGATCCACATTGTACACACATTTGCTTAAGTCTA

TGGAGGCGCAAGGTTTTTAAGTCTGTGGTTGCTGTTATAGGCCTTCCAAACGATCCATCTG
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
TGGAGGCGCAAGGTTTTTAAGTCTGTGGTTGCTGTTATAGGCCTTCCAAACGATCCATCTG

TTAGGTTGCATGAGGCTTTGGGATACACAGCCCGGGGTACATTGCGCGCAGCTGGATACA
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
TTAGGTTGCATGAGGCTTTGGGATACACAGCCCGGGGTACATTGCGCGCAGCTGGATACA

AGCATGGTGGATGGCATGATGTTGGTTTTTGGCAAAGGGATTTTGAGTTGCCAGCTCCTC
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
AGCATGGTGGATGGCATGATGTTGGTTTTTGGCAAAGGGATTTTGAGTTGCCAGCTCCTC

CAAGGCCAGTTA
|||||
CAAGGCCAGTTA

```

PAT original sequence from Gen bank (NCBI) is the top sequence

PAT from PCR product from Genomic DAN of Superfect mediated transgenic *Arabidopsis* is the bottom sequence

Fig. 3

11 / 11

```
GCCAGCGTGGGCAAGGTGGATGCCCAG-TTCATCTGCACCACCGGCGATGTGCCCGTGCC
||||| | || | ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
GCCAGAGNGGNCCNGGTGGATGCCCAGGTTCATCTGCACCACCGGCGATGTGCCCGTGCC

CTGGAGCACCC-TGG-TGACCACCCTGACCTACGGCGCCCAGTGCTTCGCCAAGTACGGC
||||| | || | ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
CTGGAGCNCCCGTGGATGACCACCCTGANCTACGGCGCCCAGTGCTTCGCCAAGTACGGC

CCCGAGCTGAAGGATTTCTACAAGAGCTGCATGCCCGATGGCTACGTGCAGGAGCGCACC
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
CCCGANCTGAAGGATTTCTACAATAGCTGCATGCCCGATGGCTACGTGCAGGANCGCACC

ATCACCTTCGAGGGCGATGGCAATTTCAAGACCCGCGCCGAGGTGACCTTCGAGAATGGC
||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| ||||| |||||
ATCACCTTCGAGGGCGATGGCAATTTCAAGANCCGCGCCGAGGTGACCTTCGAGAATGGC

AGCGTGTACAATCGCGTGAAGCTGAATGGCCAGGGCTTCAAGAAGGATGG
| |||| | || | ||||| ||||| | |||| | || | |||
NGNNTGTANAATCNNGTNNAGCTGAATGGCCANGNCTTCNANAANGNTGG
```

Phi-YFP original sequence is the top sequence

YFP from PCR product from Genomic DAN of Superfect mediated transgenic *Arabidopsis* is the bottom sequence

Fig. 4

