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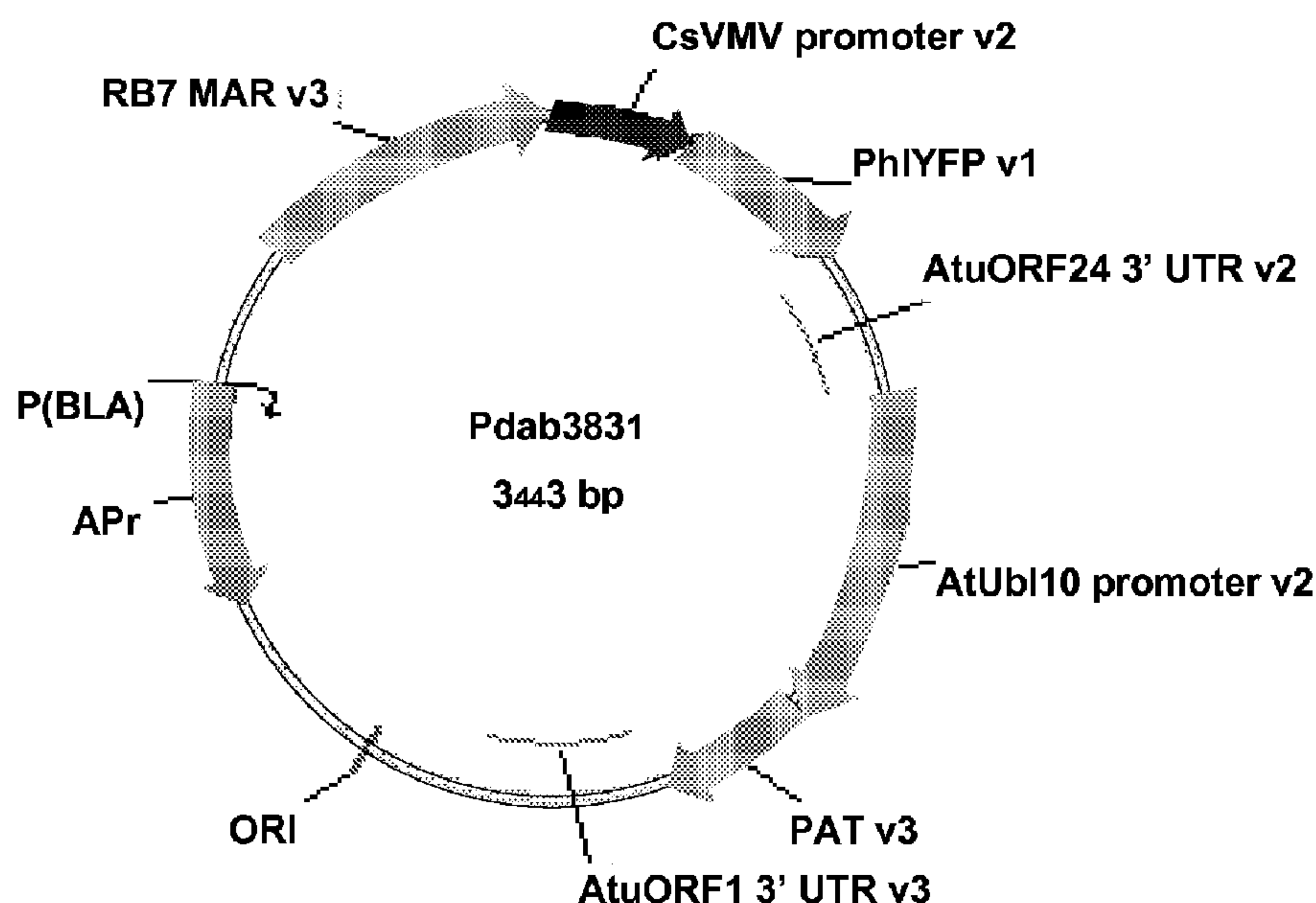
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(54) Titre : UTILISATION D'UNE NANOTECHNOLOGIE DENDRIMERE POUR LA DELIVRANCE DE BIOMOLECULES  
DANS DES CELLULES VEGETALES

(54) Title: USE OF DENDRIMER NANOTECHNOLOGY FOR DELIVERY OF BIOMOLECULES INTO PLANT CELLS



**Figure 1:** Map of plasmid pDAB3831.

(57) **Abrégé/Abstract:**

Provided are methods for introducing a molecule of interest into a plant cell having a cell wall by using dendrimers, and optionally one or more CPPs. Methods are provided for genetically or otherwise modifying plants and for treating or preventing disease in plant cells comprising a cell wall.

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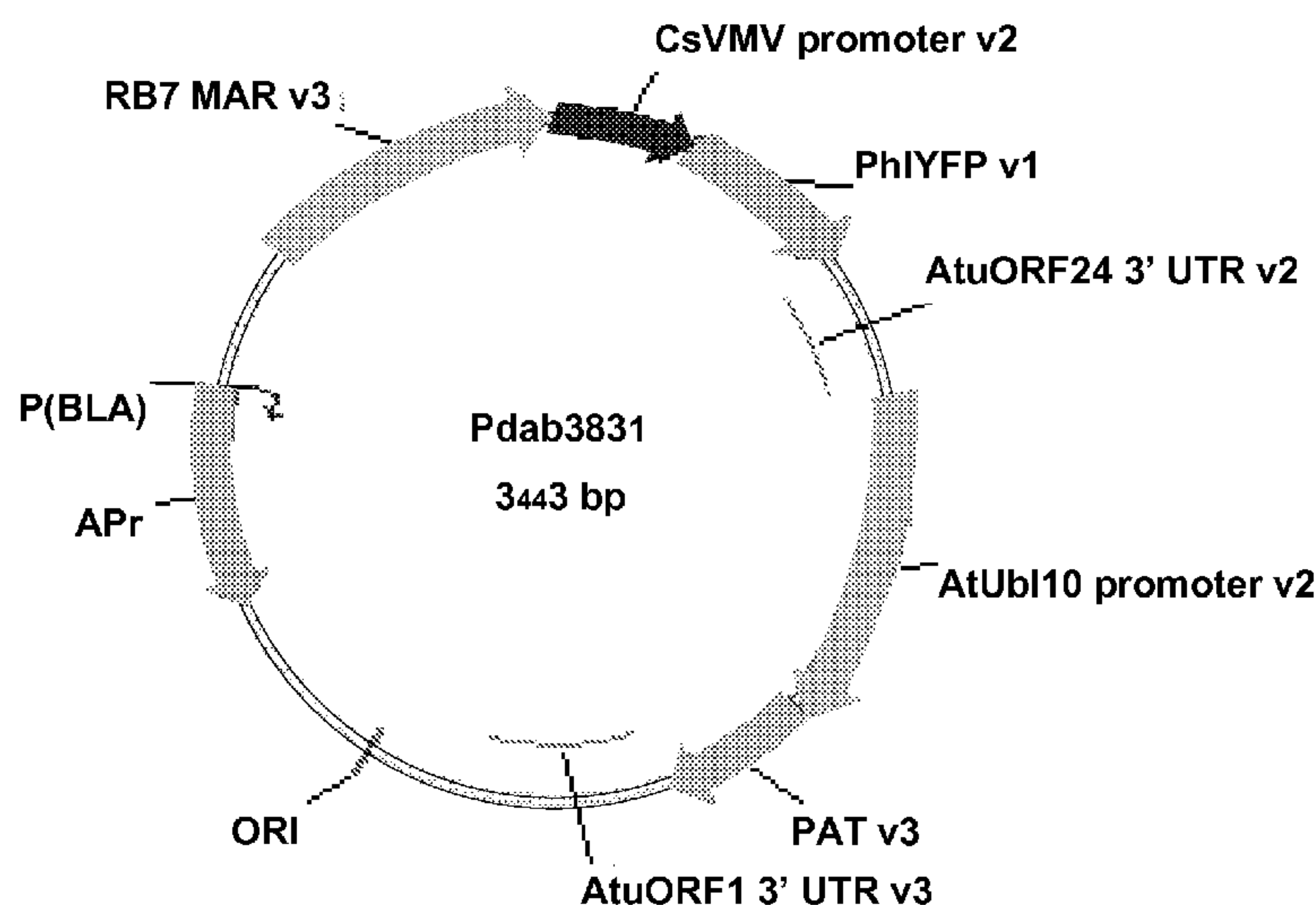


Figure 1: Map of plasmid pDAB3831.

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## USE OF DENDRIMER NANOTECHNOLOGY FOR DELIVERY OF BIOMOLECULES INTO PLANT CELLS

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### PRIORITY CLAIM

This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/252,607, filed October 16, 2009, for "USE OF DENDRIMER NANOTECHNOLOGY FOR DELIVERY OF BIOMOLECULES INTO PLANT CELLS."

10

### BACKGROUND

Nanoparticles have unique properties that have been exploited for use in the delivery of DNA to cells. Metal Nanoparticles, such as gold (Au) Nanoparticles have been used for DNA delivery because of their low cytotoxicity and ease of functionalization with various ligands of biological significance. In addition to metal Nanoparticles, semi-conductor Nanoparticles (e.g., quantum dots) ("QD") 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 the ligand attached to the QD surface (see, e.g., F. Patolsky et al., J. Am. Chem. Soc. 125, 13918 (2003)).

Nanoparticles have been used to deliver plasmid DNA to a variety of animal cells. It has been found that when DNA coated Nanoparticles are incubated with cells not having a cell wall, the cells take up the Nanoparticles and begin expressing any genes encoded on the DNA. However, the contemporary plant gene delivery is challenging due to the presence of plant cell walls, which leads to the common reliance on invasive delivery means for genetic transformation of plants. Where nanoparticle-mediated delivery to cells normally having a cell wall is desired, the cell's wall is stripped before the addition of the particles to protoplasts of plant (see, F. Torney et al., Nature Nanotechnol. 2, (2007)). In plant cells, the cell wall stands as a barrier for the delivery of exogenously applied molecules. Many invasive methods, like gene gun (biolistics), microinjection, electroporation, and Agrobacterium, have been employed to achieve gene and small molecule delivery into these walled plant cells, but delivery of proteins has only been achieved by microinjection. Delivery of small molecules and proteins in the presence of a plant cell wall remains unexplored

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and would be advantageous in order to develop enabling technologies to be deployed in intact plant cell/ tissue or organ for *in vitro* and *in vivo* manipulations.

Cell penetrating peptides (CPPs) are a novel and fast growing class of short peptides that are known to play an important role in translocation of a wide range of cargo complexes including proteins and DNA across the bio-membranes in mammalian and human cell lines. J. Schwartz and S. Zhang (2000), Peptide-Mediated Cellular Delivery, Curr. Opin. Mol. Ther. 2:162–167; Ü Langel (2002), Preface *in*: Cell Penetrating Peptides; Processes and Applications, Ü. Langel, Editor, CRC Press, Boca Raton; E. Vives and B. Lebleu (2002), The Tat-Derived Cell-Penetrating Peptide *in*: Cell-Penetrating Peptides; Processes and Applications, U. Langel, Editor, CRC Press, Boca Raton: pp. 3–22. While CPPs have been shown to facilitate cargo delivery in mammalian cells, the use of CPP in plant cells for transfection studies has been limited by a number of factors. A major obstacle to adapting this technology to plants is that, unlike animal cells, plant cells present a dual barrier system (cell wall and plasma membrane) for the internalization of CPPs and their cargos. Therefore, CPPs must overcome these two barriers for efficient translocation. CPPs have been used in plant cells but typically rely on use of permeabilization agents and techniques with the use of CPPs to effectuate delivery of cargo delivery to the plant cells. The HIV-1 TAT protein transduction domain (PTD) is one of the most well studied translocating peptides. Recent reports have shown the potential of TAT-PTD and its oligomers for plasmid delivery by forming a complex with the negatively charged DNA in mammalian cells. I. Ignatovich, E. Dizhe, A. Pavlotskaya, B. Akifiev, S. Burov, S. Orlov, and A. Perevozchikov (2003), Complexes of Plasmid DNA with Basic Domain 47-57 of the HIV-1 Tat Protein Are Transferred to Mammalian Cells by Endocytosis-mediated Pathways, J. Biol. Chem. 278:42625-42636; C. Rudolph, C. Plank, J. Lausier, U. Schillinger, R.H. Müller, and J. Rosenecker (2003), Oligomers of the Arginine-Rich Motif of the HIV-1 TAT Protein are Capable of Transferring Plasmid DNA into Cells, J. Biol. Chem. 278:11411–11418; Z. Siprashvili, F. Scholl, S. Oliver, A. Adams, C. Contag, P. Wender, and P. Khavari (2003), Gene Transfer via Reversible Plasmid Condensation with Cysteine-Flanked, Internally Spaced Arginine-Rich Peptides, Hum. Gene. Ther. 14 (13):1225-33; I. Hellgren, J. Gorman,



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and C. Sylvén (2004), Factors Controlling the Efficiency of Tat-mediated Plasmid DNA Transfer, J. Drug. Target. 12 (1):39-47.

Dendrimers are “cascade molecules” with unique core-shell macromolecular architecture. Dendrimers were first created in the laboratory in 1979 by Donald Tomalia (D.A. Tomalia et al., Preprints of the 1<sup>st</sup> SPSJ Int’l Polymer conference, Society of Polymer Science, Japan, Kyoto, 1984, p. 65; *see also* U. S. Patent No. 6,316,694). Dendrimers have been used to deliver DNA and other bio-molecules into animal cells. However, the presence of plant cell walls has presented challenges to gene delivery in plants. Additionally, the stable genomic integration of transgenes using dendrimer-based delivery has not been reported or demonstrated in plants. Thus, there still remains a need for a method of stable incorporation of genes and other molecules of interest in plants through use of dendrimer-based delivery.

#### DISCLOSURE

The following embodiments are described in conjunction with systems, tools and methods which are meant to be exemplary and illustrative, and not limiting in scope.

The present invention relates to methods using dendrimers, and optionally one or more CPPs, to non-invasively deliver molecular substances into cells having a cell wall for stable incorporation of the molecular substances therein.

One embodiment of the invention includes a method of introducing a molecule of interest into a plant cell having a cell wall to effect stable transformation of a plant and seeds. The method includes providing the plant cell having a cell wall and interacting a dendrimer, and optionally one or more CPPs, with a molecule of interest to form an activated dendrimer structure. The cell and the activated dendrimer structure are placed in contact with each other, under conditions permitting the uptake of the same into the cell having the cell wall.

Another embodiment of the invention includes a method of stably expressing a gene. The method includes providing a plant cell having a cell wall and interacting a dendrimer, and optionally one or more CPPs, with a gene to form an activated dendrimer structure. The plant cell having a cell wall and the activated dendrimer structure are placed in contact with each other, and the dendrimer and the gene are

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placed under conditions permitting the uptake of the same into the plant cell having the cell wall. The gene in the progeny of a plant having the plant cell is then expressed.

Yet another embodiment of the invention includes a method for transferring a molecular substance into a plant cell. The method includes interacting a dendrimer, and optionally one or more CPPs, with a plasmid DNA to form an activated dendrimer structure. The activated dendrimer structure is placed in contact with an intact wall-bearing plant cell under conditions permitting the uptake of the dendrimer and a gene from the plasmid DNA into the plant cell.

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

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows Plasmid pDAB3831.

FIG. 2 shows Plasmid pDAB7331.

FIG. 3 is an ethidium bromide stained gel image showing PAT PCR amplification from genomic DNA of *Arabidopsis* transgenic plant: 100 DNA ladder (Promega Inc) 1; *Arabidopsis* transgenic line from SUPERFECT™ with pDAB3831 treatment 2; Plasmid DNA pDAB3831 as a positive control; Asterisks indicate the PAT PCR amplicon.

FIG. 4 is a gel image showing YFP PCR amplification from genomic DNA of *Arabidopsis* transgenic plant: 100 DNA ladder (Promega Inc) 1; *Arabidopsis* transgenic line from SUPERFECT™ with pDAB3831 treatment 2; Plasmid DNA pDAB3831 as a positive control; Asterisks indicate the YFP PCR amplicon.

FIG. 5 shows PAT protein expression levels from leaf tissues of dendrimer-mediated (SUPERFECT™) transformed *Arabidopsis* plants; using a commercial ELISA kit, PAT protein was detected in the dendrimer-mediated transgenic plants and compared to plants that were transformed via *Agrobacterium tumefaciens*.



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## MODE(S) FOR CARRYING OUT THE INVENTION

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

5           Backcrossing. 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  $F_1$  with one of the parental genotypes of the  $F_1$  hybrid.

Embryo. The embryo may be the small plant contained within a mature seed.

10           Dendrimer. Dendrimers are three dimensional, hyperbranched, monodisperse nanometric macromolecules obtained by a reiterative sequence of reactions. Dendrimers are routinely synthesized as tunable “nanostructures” that may be designed and regulated as a function of their size, shape, surface chemistry and interior void space. Dendrimers can be obtained with structural control approaching that of traditional biomacromolecules, such as DNNPNA or proteins and are distinguished by  
15           their precise nanoscale scaffolding and nanocontainer properties. Dendrimers are microscopic particles with at least one nanoscale dimension, usually less than 100 nm. Dendrimers suitable for use in the present invention may have a size of 1 nm - 0.4  $\mu$ m.

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  
20           glyphosate. In 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. Stabilized refers to characteristics of a plant that are reproducibly passed from one generation to the next generation of inbred plants of the same variety.

25           Uptake. Uptake refers to the translocation of a particle, such as a dendrimer, 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  
30           solely as a result of momentum imparted to the particle are biolistic, gene gun, microinjection, and/or impalefection technologies.



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In a particular embodiment, the invention relates to the application of dendrimer as an option for nano-engineering to develop a payload in order to fashion materials for applications in small molecule, biomolecule delivery, gene delivery, imaging, and various biotechnological diagnostics and sensing functions. Dendrimer architecture provides a number of distinctive properties which differentiate them from other polymers and nanoparticles, such as the gradual stepwise method of synthesis, which can provide a well defined size and structure with a comparatively low polydispersity index. Additionally, dendrimer chemistry can be adaptable and, thus, facilitate synthesis of a broad range of molecules with different functionality. Use of dendrimers according to particular methods of the present invention facilitates biomolecules and gene delivery through use of a high density of terminal groups.

In other embodiments of the invention, multiple attachment sites or filling of an “added” or “guest” molecule may be engineered on the dendrimers at various and/or multiple sites. This property can be employed, for example, in specific targeting and editing of molecular sites within cells for areas such as biomimetics, targeted deliveries, for non-genetically modified organism options, and transient transformation options in a variety of tree or vegetable crops for trait and disease resistance options. Embodiments of the invention can also be employed to develop suitable bio-sensors. In addition, artificial chromosomes (ACES) may be employed with the methods of the invention as an alternative to current eukaryotic vectors for precise targeting and homologous recombination options.

According to embodiments of the invention, there may be provided a method of introducing a molecule of interest into a plant cell comprising a cell wall, the method comprising placing a dendrimer containing a molecule of interest in contact with the plant cell and allowing uptake of the dendrimer across the plant cell wall. In particular aspects of invention, the dendrimer may be any dendrimer and may reversibly or irreversibly contain, may interact with, or otherwise be bound to and/or carry a molecule of interest. In certain embodiments, a molecule of interest may be introduced to the dendrimer before contact with a plant cell having a cell wall or concurrently with the introduction of the dendrimer to a plant cell having a cell wall.

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



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having a cell wall include, but are not limited to, algal, tobacco, carrot, maize, canola, rapeseed, cotton, palm, peanut, soybean, sugarcane, *Oryza sp.*, *Arabidopsis sp.*, and *Ricinus sp.*, preferably tobacco, carrots maize, cotton, canola, soybean and sugarcane; more preferably tobacco and carrots. Embodiments of the invention may include cells  
5 comprising a cell wall from any tissue or wherever they are found, including but not limited to, in embryos, meristematic cells, callus, pollen, leaves, anthers, roots, root tips, flowers, seeds, pods, stems, and tissue culture.

In embodiments of the invention, a molecule of interest may be any molecule that can be delivered to a plant cell according to the present invention. Molecules of  
10 interest, or components of molecules of interest, may comprise, but are not limited to, nucleic acids, DNA, RNA, RNAi molecules, genes, plasmids, cosmids, YACs, BACs, Plant Artificial Chromosomes, Plant Mini-chromosomes, Plant Engineered Trait Loci DNA; polypeptides, enzymes, hormones, glyco-peptides, sugars, fats, signaling peptides, antibodies, vitamins, messengers, second messengers, amino acids, cAMP,  
15 drugs, herbicides, fungicides, antibiotics, and/or combinations thereof.

Embodiments of the invention include methods for the prevention or treatment of disease. Non-limiting example embodiments include the delivery of fungicides, antibiotics, and/or other drugs to cells in need thereof using methods of the present invention.

20 In aspects of the invention, the dendrimer may be uptaken into various parts of cells. Examples of locations that a dendrimer may be uptaken into include, but are not limited to, cytosol, nucleus, tonoplasts, plastids, etioplasts, chromoplasts, leucoplasts, elaioplasts, proteinoplasts, amyloplasts, chloroplasts, and the lumen of a double membrane. In other embodiments of the invention, dendrimer uptake into a cell  
25 comprising a cell wall may occur via the symplastic or apoplastic pathway.

Additional embodiments of the invention include genetically modified 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 one example of an embodiment, a plasmid comprising a gene of interest and a selectable marker  
30 may be introduced into a plant cell having a cell wall via a dendrimer according to the present invention. In further embodiments, stable transformants may be selected that have stably integrated the gene of interest and/or the selectable marker. In



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alternative embodiments, a plant cell now comprising the 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 including the molecule of interest.

5 In another aspect, the present invention provides methods of creating regenerable plant cells comprising a molecule of interest for use in tissue culture. The tissue culture will preferably be capable of regenerating plants having substantially the same genotype as the regenerable cells. The regenerable cells in such tissue cultures can be embryos, protoplasts, meristematic cells, callus, pollen, leaves, anthers, roots,  
10 root tips, flowers, seeds, pods or stems. Still further, an embodiment of the invention provides plants regenerated from the tissue cultures of the invention.

Alternatively, the present invention provides a method of introducing a desired trait into a plant cell having a cell wall, wherein the method comprises: placing a dendrimer and a molecule of interest capable of providing the desired trait to the plant  
15 cell in contact with the cell and allowing uptake of the dendrimer across the cell wall. Examples of desired traits include, but are not limited to, traits selected from male sterility, herbicide resistance, insect resistance, and resistance to bacterial disease, fungal disease, and/or viral disease.

Further aspects of the invention provide for the methods of generating of  
20 stabilized plant lines comprising a desired trait or molecule of interest, wherein the desired trait or molecule of interest may be first introduced by uptake of a dendrimer 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, backcrosses, hybrid production, crosses to populations, and the like. All plants  
25 and plant cells comprising a desired trait or molecule of interest first introduced into the plant cell (or its predecessors) by uptake of a dendrimer across a cell wall are within the scope of this invention. Advantageously, the plant cells comprising a desired trait or molecule of interest first introduced into the plant or cell (or its predecessors) by uptake of a dendrimer across a cell wall can be used in crosses with other, different, plant cells  
30 to produce first generation (F<sub>1</sub>) hybrid cells, seeds, and/or plants with superior characteristics.



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In embodiments wherein the 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) will 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.

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., 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 additional or modified versions of native or endogenous genes (perhaps driven by different promoters) in order to alter the traits of a cell in a specific manner. Such foreign additional and/or modified genes are referred to herein collectively as “transgenes.” Over the last fifteen to twenty years, several methods for producing transgenic cells have been developed and, in particular embodiments, the present invention relates to transformed versions of cells and methods of producing them via introducing into a cell having a cell wall a transgene via uptake of a dendrimer across a cell wall. In embodiments of the invention, the transgene may be contained in an expression vector.

Cell transformation may involve the construction of an expression vector which will function in a particular cell. Such a vector may comprise DNA that includes a gene under control of, or operatively linked to, a regulatory element (for example, a promoter). The expression vector may contain one or more such operably linked gene/regulatory element combinations. The vector(s) may be in the form of a plasmid and can be used alone or in combination with other plasmids to provide transformed cells using transformation methods as described herein to incorporate transgene(s) into the genetic material of a plant cell comprising a cell wall.

In particular embodiments of the invention, a multipurpose STARBURST® PAMAM (polyamidoamine) dendrimer prototype exhibits properties suitable to be used as: (i) targeted, diagnostic MRI (magnetic resonance imaging) INIR (near-IR) contrast agents, (ii) and/or for controlled delivery of cancer therapies. Among them, a lead candidate is (core: 1,4-diaminobutane; G (generation) [ PAMAM(CO<sub>2</sub>Na)<sub>64</sub>J. This dendritic nanostructure (i.e. ~5.0 nm diameter) was selected on the basis of a very favorable biocompatibility profile (The Nanotechnology Characterization Laboratory



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(NCL), an affiliate of the National Cancer institute (NCI), has completed extensive *in vitro* studies on the lead compound and have found it to be very benign and highly biocompatible, the expectation being that it will exhibit desirable mammalian kidney excretion properties and demonstrated targeting features. Dendrimers used with the methods of the invention represent a class of polymers characterized by their well-defined structure, with a high degree of molecular uniformity and low polydispersity. In addition, these dendrimers have been shown to be capable of bypassing efflux transporters.

Use of dendrimers according to methods of the present invention has produced stably transformed plants and demonstrated the expression of the stably transformed herbicide gene with the phenotype where high herbicide tolerance was rendered into the transgenic T1 plant. This plant was shown to be fertile as it produced T2 seeds.

In a particular embodiment, SUPERFECT™ Transfection Reagent was used. This reagent is a polycation having a defined shape and diameter, available as Qiagen's SUPERFECT™ reagent (Qiagen Catalog #301307) as a solution of specifically designed activated dendrimers. Dendrimers are spherical polyamidoamine molecules with branches radiating from a central core and terminating at charged amino groups. Chemical activation promotes efficient uptake of DNA by eukaryotic cells. While not being limited to a particular theory, this reagent is thought to assemble DNA into compact structures, thereby optimizing the entry of DNA into cells. To stabilize the SUPERFECT™-DNA complexes during their transport to the nucleus, the SUPERFECT™ reagent is designed to buffer the lysosome after fusion with the endosome, leading to pH inhibition of lysosomal nucleases.

## 25 Expression Vectors for Uptake via Dendrimer: Marker Genes

Expression vectors may include at least one genetic marker, operably linked to a regulatory element (a promoter, for example) that allows transformed cells containing the marker to be either recovered by negative 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 transformation arts and include, for example, genes that code for enzymes that metabolically detoxify a selective chemical agent



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which may be an antibiotic or an herbicide, or genes that encode an altered target which may be insensitive to the inhibitor. A few positive selection methods are also known in the art.

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

10 Additional selectable marker genes of bacterial origin that confer resistance to antibiotics include gentamycin acetyl transferase, streptomycin phosphotransferase, aminoglycoside-3'-adenyl transferase, and the bleomycin resistance determinant. See Hayford et al., Plant Physiol. 86:1216 (1988); Jones et al., Mol. Gen. Genet., 210:86 (1987); Svab et al., Plant Mol. Biol. 14:197 (1990); Hille et al., Plant Mol. Biol. 7:171  
15 (1986). Other selectable marker genes confer resistance to herbicides such as glyphosate, glufosinate or bromoxynil. See Comai et al., Nature 317:741-744 (1985); Gordon-Kamm et al., Plant Cell 2:603-618 (1990); and Stalker et al., Science 242:419-423 (1988).

Other selectable marker genes suitable for plant transformation are not of  
20 bacterial origin. These genes include, for example, mouse dihydrofolate reductase, plant 5-enolpyruvylshikimate-3-phosphate synthase and plant acetolactate synthase. See Eichholtz et al., Somatic Cell Mol. Genet. 13:67 (1987); Shah et al., Science 233:478 (1986); Charest et al., Plant Cell Rep. 8:643 (1990).

Another class of marker genes suitable for plant transformation requires  
25 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  
30 expression. Commonly used genes for screening transformed cells include  $\beta$ -glucuronidase (GUS),  $\beta$ -galactosidase, luciferase and chloramphenicol acetyltransferase. See Jefferson, R. A., Plant Mol. Biol. Rep. 5:387 (1987); Teeri et al.,



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EMBO J. 8:343 (1989); Koncz et al., Proc. Natl. Acad. Sci. U.S.A. 84:131 (1987); DeBlock et al., EMBO J. 3:1681 (1984).

Recently, *in vivo* methods for visualizing GUS activity that do not require destruction of plant tissue have been made available. Molecular Probes publication  
5 2908, Imagene Green<sup>TM</sup>, p. 1-4(1993); and Naleway et al., J. Cell Biol. 115:151a (1991). However, these *in vivo* methods for visualizing GUS activity have not proven useful for recovery of transformed cells because of low sensitivity, high fluorescent backgrounds, and limitations associated with the use of luciferase genes as selectable markers.

10 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., Science 263:802 (1994). Fluorescent proteins and mutations of fluorescent proteins may be used as screenable markers.

#### 15 Expression Vectors for Uptake via Dendrimer: Promoters

Genes included in expression vectors must 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.

20 As used herein, "promoter" includes reference to a region of DNA that may be upstream from the start of transcription and that may be involved in recognition and binding of RNA polymerase and 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  
25 preferentially initiate transcription in certain tissues, such as leaves, 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  
30 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 effect transcription by inducible promoters include anaerobic conditions or the presence of

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light. Tissue-specific, tissue-preferred, cell type specific, and inducible promoters constitute the class of “non-constitutive” promoters. A “constitutive” promoter may be a promoter which may be active under most environmental conditions.

5 A. 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  
10 response to an inducing agent.

Any inducible promoter can be used in the instant invention. See Ward et al., Plant Mol. Biol. 22:361-366 (1993). Exemplary inducible promoters include, but are not limited to: those from the ACEI system that responds to copper (Mett et al., PNAS 90:4567-4571 (1993)); In2 gene from maize that responds to benzenesulfonamide  
15 herbicide safeners (Hershey et al., Mol. Gen. Genetics 227:229-237 (1991); and Gatz et al., Mol. Gen. Genetics 243:32-38 (1994)); and Tet repressor from Tn10 (Gatz et al., Mol. Gen. Genetics 227:229-237 (1991)). A particularly useful inducible promoter may be a promoter that responds to an inducing agent to which plants do not normally respond. An exemplary inducible promoter may be the inducible promoter from a  
20 steroid hormone gene, the transcriptional activity of which may be induced by a glucocorticosteroid hormone. Schena et al., Proc. Natl. Acad. Sci. U.S.A. 88:0421 (1991).

B. Constitutive Promoters

25 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.

Different constitutive promoters can be utilized in the instant invention.  
30 Exemplary constitutive promoters include, but are not limited to: promoters from plant viruses, such as the 35S promoter from CaMV (Odell et al., Nature 313:810-812 (1985)); promoters from rice actin genes (McElroy et al., Plant Cell 2:163-171 (1990));



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ubiquitin (Christensen et al., Plant Mol. Biol. 12:619-632 (1989); and Christensen et al., Plant Mol. Biol. 18:675-689 (1992)); pEMU (Last et al., Theor. Appl. Genet. 81:581-588 (1991)); MAS (Velten et al., EMBO J. 3:2723-2730 (1984)); and maize H3 histone (Lepetit et al., Mol. Gen. Genetics 231:276-285 (1992); and Atanassova et al., Plant Journal 2 (3):291-300 (1992)). The ALS promoter, Xba1/NcoI fragment 5' to the Brassica napus ALS3 structural gene (or a nucleotide sequence similarity to said Xba1/NcoI fragment), represents a particularly useful constitutive promoter. See PCT application WO 96/30530.

10 C. 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 expression in a cell. Plants transformed with a gene of interest operably linked to a tissue-specific promoter can produce the protein product of the transgene exclusively, or preferentially, in a specific tissue.

Any tissue-specific or tissue-preferred promoter can be utilized in the instant invention. Exemplary tissue-specific or tissue-preferred promoters include, but are not limited to, a root-preferred promoter--such as that from the phaseolin gene (Murai et al., Science 23:476-482 (1983); and Sengupta-Gopalan et al., Proc. Natl. Acad. Sci. U.S.A. 82:3320-3324 (1985)); a leaf-specific and light-induced promoter such as that from cab or rubisco (Simpson et al., EMBO J. 4(11):2723-2729 (1985); and Timko et al., Nature 318:579-582 (1985)); an anther-specific promoter such as that from LAT52 (Twell et al., Mol. Gen. Genetics 217:240-245 (1989)); a pollen-specific promoter such as that from Zm13 (Guerrero et al., Mol. Gen. Genetics 244:161-168 (1993)) or a microspore-preferred promoter such as that from apg (Twell et al., Sex. Plant Reprod. 6:217-224 (1993)).

Transport of protein produced by transgenes to a subcellular compartment, such as the chloroplast, vacuole, peroxisome, glyoxysome, cell wall or mitochondrion or for secretion into the apoplast, can 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



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structural gene may determine, during protein synthesis and processing, where the encoded protein may be ultimately compartmentalized. Alternatively such subcellular compartment targeting proteins can be directly linked to a dendrimer to direct the dendrimer coated with the molecule of interest to the desired subcellular compartment.

5           The presence of a signal sequence directs 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., *Plant Mol. Biol.* 20:49 (1992); P.S. Close, Master's Thesis, Iowa State University (1993); C. Knox et al., "Structure and Organization of Two Divergent Alpha-Amylase Genes from Barley," *Plant Mol.*  
10 *Biol.* 9:3-17 (1987); Lerner et al., *Plant Physiol.* 91:124-129 (1989); Fontes et al., *Plant Cell* 3:483-496 (1991); Matsuoka et al., *Proc. Natl. Acad. Sci.* 88:834 (1991); Gould et al., *J. Cell. Biol.* 108:1657 (1989); Creissen et al., *Plant J.* 2:129 (1991); Kalderon, et al., A short amino acid sequence able to specify nuclear location, *Cell* 39:499-509 (1984); Steifel, et al., Expression of a maize cell wall hydroxyproline-rich glycoprotein  
15 gene in early leaf and root vascular differentiation, *Plant Cell* 2:785-793 (1990).

#### Foreign Protein Genes and Agronomic Genes

With transgenic plants according to the present invention, a foreign protein can be produced in commercial quantities. Thus, techniques for the selection and  
20 propagation of transformed plants, which are well understood in the art, yield a plurality of transgenic plants which are harvested in a conventional manner, and a foreign protein then can 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, by Heney and Orr, *Anal. Biochem.* 114:92-6 (1981).

25           In aspects of the invention, the transgenic plant provided for commercial production of foreign protein may be a cell or a plant. In other aspects, the biomass of interest may be seed. For the relatively small number of transgenic plants that show higher levels of expression, a genetic map can be generated primarily via conventional RFLP, PCR and SSR analysis, which identifies the approximate chromosomal location  
30 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



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location may be useful for proprietary protection of a subject transgenic plant. 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  
5 comparisons would involve hybridizations, RFLP, PCR, SSR and sequencing, all of which are conventional techniques.

Likewise, agronomic genes can be expressed in transformed cells or their progeny. More particularly, plants can be genetically engineered via the methods of the invention to express various phenotypes of agronomic interest. Exemplary genes  
10 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 and That Encode:

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

B) A gene conferring resistance to a pest, such as soybean cyst nematode. See e.g., PCT Application WO 96/30517; PCT Application WO 93/19181.

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

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

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E) A vitamin-binding protein, such as avidin. See PCT application US93/06487. The application teaches the use of avidin and avidin homologues as larvicides against insect pests.

F) An enzyme inhibitor, for example, a protease or proteinase inhibitor or an amylase inhibitor. See, e.g., Abe et al., J. Biol. Chem. 262:16793 (1987) (nucleotide sequence of rice cysteine proteinase inhibitor); Huub et al., Plant Molec. Biol. 21:985 (1993) (nucleotide sequence of cDNA encoding tobacco proteinase inhibitor I); Sumitani et al., Biosci. Biotech. Biochem. 57:1243 (1993) (nucleotide sequence of *Streptomyces nitrosporeus*  $\alpha$ -amylase inhibitor) and U.S. Pat. No. 5,494,813 (Hepher and Atkinson, issued Feb. 27, 1996).

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

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

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

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, a hydrolase, a phosphatase, a kinase, a phosphorylase, a polymerase, an



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elastase, a chitinase and a glucanase, whether natural or synthetic. See PCT application WO 93/02197 in the name of Scott et al., which discloses the 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  
5 also Kramer et al., Insect Biochem. Molec. Biol. 23:691 (1993), who teach the nucleotide sequence of a cDNA encoding tobacco hornworm chitinase; and Kawalleck et al., Plant Molec. Biol. 21:673 (1993), who provide the nucleotide sequence of the parsley ubi4-2 polyubiquitin gene.

L) A molecule that stimulates signal transduction. For example, see the  
10 disclosure by Botella et al., Plant Molec. Biol. 24:757 (1994), of nucleotide sequences for mung bean calmodulin cDNA clones, and Griess et al., Plant Physiol. 104:1467 (1994), who provide the nucleotide sequence of a maize calmodulin cDNA clone.

M) A hydrophobic moment peptide. See PCT application WO 95/16776 (disclosure of peptide derivatives of Tachyplesin which inhibit fungal plant pathogens)  
15 and PCT application WO 95/18855 (teaches synthetic antimicrobial peptides that confer disease resistance).

N) A membrane permease, a channel former or a channel blocker. For example, see the disclosure of Jaynes et al., Plant Sci 89:43 (1993), of heterologous expression of a cecropin- $\beta$  lytic peptide analog to render transgenic tobacco plants  
20 resistant to *Pseudomonas solanacearum*.

O) A viral-invasive protein or a complex toxin derived therefrom. For example, the accumulation of viral coat proteins in transformed plant cells imparts resistance to viral 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  
25 et al., Ann. rev. Phytopathol. 28:451 (1990). 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  
30 antibody targeted to a critical metabolic function in the insect gut would 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)



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(enzymatic inactivation in transgenic tobacco via production of single-chain antibody fragments).

Q) A virus-specific antibody. See, for example, Tavladoraki et al., Nature 366:469 (1993), who show that transgenic plants expressing recombinant antibody  
5 genes are protected from virus attack.

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

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

## 2. Genes That Confer Resistance to an Herbicide:

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

B) Glyphosate (resistance conferred by, e.g., mutant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPs) genes (via the introduction of recombinant nucleic acids and/or various forms of *in vivo* mutagenesis of native EPSPs  
25 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, for example, U.S. Pat. No.  
30 4,940,835 to Shah, et al. and U.S. Pat. 6,248,876 to Barry et. al., which disclose 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



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accession number 39256, and the nucleotide sequence of the mutant gene may be disclosed in U.S. Pat. No. 4,769,061 to Comai. European patent application No. 0 333 033 to Kumada et al., and U.S. Pat. No. 4,975,374 to Goodman et al., disclose nucleotide sequences of glutamine synthetase genes which confer resistance to herbicides such as L-phosphinothricin. The nucleotide sequence of a PAT gene may be provided in European application No. 0 242 246 to Leemans et al., DeGreef et al., Bio/Technology 7:61 (1989), describe the production of transgenic plants that express chimeric bar genes coding for PAT activity. Exemplary of genes conferring resistance to phenoxy propionic acids and cyclohexones, such as sethoxydim and haloxyfop include the Acc1-S1, Acc1-S2 and Acc1-S3 genes described by Marshall et al., Theor. Appl. Genet. 83:435 (1992). GAT genes capable of conferring glyphosate resistance are described in WO 2005012515 to Castle et al. Genes conferring resistance to 2,4-D, phenoxypropionic acid and pyridyloxy auxin herbicides are described in WO 2005107437 assigned to Dow AgroSciences LLC.

C) An herbicide that inhibits photosynthesis, such as a triazine (psbA and gs<sup>+</sup> genes) or a benzonitrile (nitrilase gene). Przibila et al., Plant Cell 3:169 (1991), describe the transformation of Chlamydomonas with plasmids encoding mutant psbA genes. Nucleotide sequences for nitrilase genes are disclosed in U.S. Pat. No. 4,810,648 to Stalker, and DNA molecules containing these genes are available under ATCC Accession Nos. 53435, 67441, and 67442. Cloning and expression of DNA coding for a glutathione S-transferase may be described by Hayes et al., Biochem. J. 285:173 (1992).

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., Proc. Natl. Acad. Sci. U.S.A. 89:2624 (1992).

B) Decreased phytate content--1) Introduction of a phytase-encoding gene would enhance breakdown of phytate, adding more free phosphate to the transformed plant. For example, see Van Hartingsveldt et al., Gene 127:87 (1993), for a disclosure of the nucleotide sequence of an Aspergillus niger phytase gene. 2) A gene could be introduced that reduced phytate content. In maize for example, this could be

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accomplished by cloning and then reintroducing DNA associated with the single allele which may be responsible for maize mutants characterized by low levels of phytic acid. See Raboy et al., *Maydica* 35:383 (1990).

5 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 Shiroza et al., *J. Bacteol.* 170:810 (1988) (nucleotide sequence of *Streptococcus* mutants fructosyltransferase gene); Steinmetz et al., *Mol. Gen. Genet.* 20:220 (1985) (nucleotide sequence of *Bacillus subtilis* may be levansucrase gene); Pen et al., *Bio/Technology* 10:292 (1992) (production of transgenic plants that express *Bacillus* 10 licheniform may be  $\alpha$ -amylase); Elliot et al., *Plant Molec. Biol.* 21:515 (1993) (nucleotide sequences of tomato invertase genes); Sogaard et al., *J. Biol. Chem.* 268:22480 (1993) (site-directed mutagenes may be of barley  $\alpha$ -amylase gene); and Fisher et al., *Plant Physiol.* 102:1045 (1993) (maize endosperm starch branching enzyme II).

15

## EXAMPLES

The present invention is further described in the following examples, which are offered by way of illustration and are not intended to limit the invention in any manner.

20

### EXAMPLE 1

#### PREPARATION OF DENDRIMER / DNA COMPLEX AND TREATMENT OF CELLS

##### 1.1 Preparation of plasmid

pDAB3831 plasmid DNA (FIG. 1) (SEQ. ID. NOS. 1 and 2) was isolated and 25 prepared for dendrimer-mediated plant transformation. Transformation experiments were tested using both circularized DNA and linearized DNA.

To linearize pDAB3831, a PCR reaction was completed. pDAB3831 was PCR amplified using a Continuous Thermal Cycling system, which has been described previously in, for example, WO 2008045288. Rather than using small tubes, 30 continuous thermal cyclers use a constant or continuous stream of fluid repetitively passed through different temperature zones to amplify DNA. PCR reaction mixture was injected into a carrier fluid with which the PCR reaction mixture is immiscible.



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The carrier fluid was then passed through a plurality of temperature zones to facilitate DNA amplification within the PCR reaction mixture. A sample was prepared containing: 12%  $\text{MgCl}_2$  (25 mM), 0.33% Taq DNA polymerase (5 units/ $\mu\text{l}$ ), 2.0% dNTP's (deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), 5 deoxyguanosine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP), 8.0% template (2  $\mu\text{g/ml}$ ), 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. The PCR reaction 10 mixture was pumped through the system using a pressurized vessel at 13.79 N/cm<sup>2</sup>. 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 15 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, 20 which 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*). Forward primer SEQ ID NO:3 and reverse primer SEQ ID NO:4 were synthesized to amplify the 4.6 kbp complete expression cassette (i.e. the linearized DNA) containing both genes and their 25 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. This extended spacer arm can separate the biotin function from the rest of an oligo to 30 advantageously reduce any possible steric hindrance effects during binding to the streptavidin molecule. When the forward primer was labeled, the biotin was at the beginning of the DNA. When the reverse primer was labeled, the biotin was at the end

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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.

5

## 1.2 Preparation of the DNA/Dendrimer Complex

The dendrimers used for these experiments were spherical cationic polyamidoamine (PAMAM) cascade polymers that consist of primary amines on the surface and tertiary amines in the interior. The dendrimers are partially degraded by  
10 heat treatment in solvolytic solvents, thereby resulting in less sterical constraint and greater flexibility. The dendrimer's highly positive charge facilitates electrostatic interactions with DNA, and the flexible structure allows the dendrimer to compact when bound to DNA and swell when released from the DNA. The transfection or transformation efficiency is increased as a result of the positive charge and the flexible  
15 structural property of the dendrimer.

Dendrimers were obtained from Qiagen (Germantown, MD), which are marketed as SUPERFECT™ Transfection Reagent (Cat # 301305). The plasmid DNA was mixed with 0.6 ml of SUPERFECT™ reagent and incubated for 30 minutes at 24°C to form a DNA/Dendrimer complex. Varying concentrations of circularized  
20 plasmid DNA (0.1 mg and 0.5 mg) were used to form the DNA/Dendrimer complex. In addition, the linearized DNA described above in Example 1.1 was used to form a DNA/Dendrimer complex. Concentrations of 0.1 mg and 0.5 mg of linear DNA were used. After formation of the DNA/Dendrimer complex, a 10 ml volume solution containing 5% sucrose and .02-.04% Silwet-L77 was added to the DNA/Dendrimer  
25 reaction.

## EXAMPLE 2

### DNA/Dendrimer Complex Delivery and Stable Transformation of *ARABIDOPSIS THALIANA*

#### 30 2.1 Plant material for *in planta* transformation:

Synchronized germination of the seed is important to ensure the uniformity of floral development in the T0 plants. *Arabidopsis thaliana* cv. Columbia seed was



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suspended in 0.1% 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% agar solution was added and was vortexed until seed was evenly dispersed. This created a concentration of 4.6 mg seed/ 1 ml solution (or about 230 seeds / ml). Six  
5 tubes (72 ml solution) were prepared to sow 4 flats that contained 18 (3 1/2 inch) pots in each tray. The 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  
10 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  
15 the soil moist but not wet. After four weeks post-sow date, the flowers were cut back to produce a more even growth of secondary flowers. In the fifth week post-sowing, the plants were prepared for the transformation process.

## 2.2 *In planta* transformation and screening T1 resistant plants:

20 Dendrimer-mediated transformation of *Arabidopsis thaliana* cv. Columbia was completed using a modified protocol from Clough and Bent. (S.J. Clough and A.F. Bent, 1998, Plant J 16:735-43). A 10 ml suspension was made with the DNA /Dendrimer solution and used for treatments of the Arabidopsis plants (mostly immature flower clusters with some fertilized siliques). Both circular DNA/  
25 Dendrimer complexes and linear DNA/Dendrimer complexes were used independent of one another. Before dipping plants, Silwet L-77 to a concentration of 0.05% (250  $\mu\text{l}/500\text{ ml}$ ) - 0.005% was added to the DNA/Dendrimer solution and mixed well. Above-ground parts of plant were dipped in DNA/Dendrimer solution for 2-30 seconds, with gentle agitation. Treated plants were kept under a plastic dome cover for  
30 16-24 hours at 22-24°C. The plants were transferred to the CONVIRONS® and allowed to grow to maturity and to grow seeds. Selection trays (10.5"x21"x1" trays) were used to screen bulk harvest seed from T<sub>0</sub> plants, approximately 10,000 seeds on



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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 synchronization, seeds were stratified in a 0.1% 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% 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. To increase effectiveness of the selection spray, 40 ml of suspended seed was 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 five days post-sowing.

Additionally, a control experiment was completed wherein a solution containing only DNA, without dendrimers, was used to transform *Arabidopsis thaliana*. The previously described protocol was used for transformation of naked DNA. Both linear and circular forms of DNA were used independent of one another.

An additional control transformation for *Arabidopsis thaliana* using *Agrobacterium* was completed. This transformation was used as a benchmark to determine the efficiency of the dendrimer-mediated transformation. The plasmid, pDAB7331 (FIG. 2), was transformed into *Agrobacterium* using a modified protocol from Hanahan (1983), this strain was used for the *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* cv. Columbia.

*Arabidopsis* was transformed using the floral dip method described by Clough and Bent. A selected *Agrobacterium* colony was used to inoculate one or more 100 ml pre-cultures of YEP broth containing spectinomycin (100 mg/L) and kanamycin (50 mg/L). The culture(s) was incubated overnight at 28°C with constant agitation at 225 rpm. The cells were pelleted at approximately 5000 xg for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 400 ml dunking media containing: 5% (w/v) sucrose, 10 µg/L 6-benzylaminopurine, and 0.04% Silwet L-77. Approximately one month old plants were dipped into the media for 5-10 minutes with gentle agitation. The plants were laid down on their sides and covered (transparent or opaque) for 2-3 hours, and then



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placed upright. The plants were grown at 22°C, with a 16-hour light/8-hour dark photoperiod. Approximately four weeks after dipping, the seeds were harvested.

### 2.3 Selection of transformed plants

- 5           Freshly harvested T<sub>1</sub> seed were allowed to dry for seven days at room temperature. T<sub>1</sub> seed were sown in 26.5 x 51-cm germination trays, each receiving a 200 mg aliquots of stratified T<sub>1</sub> seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4°C for two days to complete dormancy requirements and ensure synchronous seed germination.
- 10           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 one day prior to initial transformant selection using glufosinate post-emergence spray.
- 15           Seven days after planting (DAP), T<sub>1</sub> plants (cotyledon and 2-4-lf stage, respectively) were sprayed five times within five days with a 0.2% 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
- 20           growing) were identified 4-7 days after the final spraying and transplanted individually into three-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 directly to a greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22±5°C, 50±30% RH, 14 h light:10 dark, minimum
- 25           500 µE/m<sup>2</sup>s<sup>-1</sup> natural + supplemental light).

## EXAMPLE 3

### MOLECULAR ANALYSIS OF THE GENOMIC INTEGRATION OF TRANSGENES

#### 30           IN THE T<sub>1</sub> PROGENY OF *ARABIDOPSIS THALIANA* CV *COLUMBIA*

#### 3.1           gDNA PCR amplification of transgenes

Genomic DNA from *Arabidopsis* transgenic plants was extracted from total leaf material of six-week-old plants using a Plant DNAzol kit according to the

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manufacturer's instructions. PCR primers were designed for detection of the YFP and PAT transgenes. The YFP primers are represented as SEQ ID NO:5 and SEQ ID NO:6. The PAT primers are represented as SEQ ID NO:7 and SEQ ID NO:8.

5 PCR amplification reactions for PAT and YFP were completed using the TaKaRa EXTAQ™ 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 .025 units/µL ExTaq. The following PCR conditions were used: 1 cycle at 96°C for five minutes and 31 cycles of the following conditions 94°C for 15 seconds, 65°C for 30  
10 seconds, 72°C for one minute, and a final extension of 72°C for 7 minutes. PCR amplification product was analyzed by 0.8% TAE agarose gel electrophoresis and visualized by ethidium bromide staining. FIG. 5 and FIG. 6 show the amplification products that were obtained from these reactions.

The PCR fragments were sequenced using the PAT forward primer (SEQ ID  
15 NO:7) and YFP forward primer (SEQ ID NO:5) using advanced Sanger sequencing technology (MWG Biotech, Huntsville, AL). The sequence data was analyzed using Sequencher software.

The sequencing results of the PAT and YFP PCR amplicons matched the expected nucleotide sequence for these genes. These results indicate that the PAT and  
20 YFP sequences from pDAB3831 were stably integrated into the gDNA of *Arabidopsis* using the SUPERFECT™ Transfection Reagent.

### 3.2 ELISA screening of PAT

Protein was extracted from six-week old transgenic *Arabidopsis* plant leaves  
25 for detection of expressed PAT protein via ELISA (enzyme linked immuno-sorbent assay). A microfuge tube containing the leaf samples was chilled in liquid nitrogen. The leaf material was ground to a powder using a disposable homogenizer. After equilibrating on ice for 5 minutes, 200 µl of extraction buffer (PBST; 20 mM phosphate buffered saline containing .05% (v/v) TWEEN® 20) was added. The  
30 contents were mixed with a vortex and centrifuged at 4°C for 10 minutes at 13,000 xg. Supernatant was extracted from the cell debris and stored on ice until further analysis.



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The ELISA was performed using a modified protocol for the QUALIPLATE™ Kit for LIBERTYLINK® PAT/*pat*- (Envirologix, Portland, ME). The ELISA plate and other reagents were equilibrated at room temperature. 50 µl of Enzyme Conjugate was added to each well of the plate. Another 50 µl of Extraction Buffer was added to each well of the plate. Serial dilutions of the purified transgenic *Arabidopsis* protein were added to the wells. Concentrations of 10, 5, 2.5, and 1.25 ng/ml were used. Additional standards and plant extracts were added to wells as controls. The plate was shaken at 200 rpm and incubated at room temperature for 2 hours. After the incubation, the plate was washed five times with Extraction Buffer in a plate washer. For detection, 100 µl of substrate from the kit was added to each well and the plate was incubated for 30 minutes. Activity was read and recorded using a micro plate reader at an absorbance of 595 nm.

Absorbance of the ELISA signals from the standards indicated that the absorbance signal was directly proportional to the amounts of PAT present in each well. This data is represented FIG. 5. The samples of the SUPERFECT™ and *Agrobacterium*-mediated transformed plants show strong signals from the ELISA. These amounts are three times as high as the 10 ng/ml standard, the highest standard tested in the assay. These results indicate that PAT is expressed in the *Arabidopsis* transgenic plants that were transformed via SUPERFECT™- mediated plant transformation.

While this invention has been described in certain embodiments, the present invention can be further modified within the spirit and scope of this disclosure. This application is therefore intended to cover any variations, uses, or adaptations of the invention using its general principles. Further, this application is intended to cover such departures from the present disclosure as come within known or customary practice in the art to which this invention pertains and which fall within the limits of the appended claims and their equivalents.

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## CLAIMS

What may be claimed is:

- 5           1.       A method of introducing a molecule of interest into a plant cell having a cell wall to effect stable transformation of a plant and seeds, the method comprising: providing the plant cell having a cell wall; interacting a dendrimer, and optionally one or more CPPs, with a molecule of interest to form an activated dendrimer structure;
- 10       placing the cell having a cell wall and the activated dendrimer structure in contact with each other; and allowing uptake of the dendrimer and the molecule of interest into the cell having the cell wall.
- 15           2.       The method according to claim 1, wherein interacting a dendrimer with a molecule of interest comprises assembly of the molecule of interest onto the surface of the dendrimer.
- 20           3.       The method according to claim 1, wherein interacting a dendrimer with a molecule of interest comprises interacting negatively charged groups of the molecule of interest with charged amino groups at a terminal end of the dendrimer.
4.       The method according to claim 1, further comprising allowing uptake of the dendrimer into a compartment of the plant cell comprising a cell wall.
- 25           5.       The method according to claim 4, 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.

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6. The method according to claim 1, wherein the plant cell comprising a cell wall 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.
- 5
7. The method according to claim 1, 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.
- 10
8. The method according to claim 1, wherein the dendrimer comprises a polyamidoamine dendrimer.
9. The method according to claim 1, further comprising derivatizing the surface of the dendrimer.
- 15
10. The method according to claim 1, wherein the molecule of interest comprises a component selected from the group consisting of nucleic acids, DNA, RNA, RNAi molecules, genes, plasmids, cosmids, YACs, BACs, polypeptides, enzymes, hormones, glyco-peptides, sugars, fats, signaling peptides, antibodies, vitamins, messengers, second messengers, amino acids, cAMP, drugs, herbicides, fungicides, antibiotics, and combinations thereof.
- 20
11. The method according to claim 9, wherein the molecule of interest comprises a gene.
- 25
12. The method according to claim 10, wherein the gene is a foreign protein gene, an agronomic gene, or a marker gene.
13. The method according to claim 10, further comprising selecting cells that have stably integrated the molecule of interest.
- 30

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14. The method according to claim 12, wherein the selected cells are regenerable cells.

15. The method according to claim 13, further comprising regenerating a  
5 plant from the regenerable cells.

16. The method according to claim 1, wherein the dendrimer comprises a dendrimer reagent.

10 17. A method of stably expressing a gene, the method comprising:  
providing a plant cell having a cell wall;  
interacting a dendrimer, and optionally one or more CPPs, with a gene to form an  
activated dendrimer structure;  
placing the plant cell having a cell wall and the activated dendrimer structure in contact  
15 with each other;  
allowing uptake of the dendrimer and the gene into the plant cell comprising a cell  
wall; and  
expressing the gene in progeny of a plant having the plant cell.

20 18. The method according to claim 17, wherein the gene is expressed in a chloroplast.

19. The method according to claim 17, further comprising selecting for  
cells stably expressing the gene.

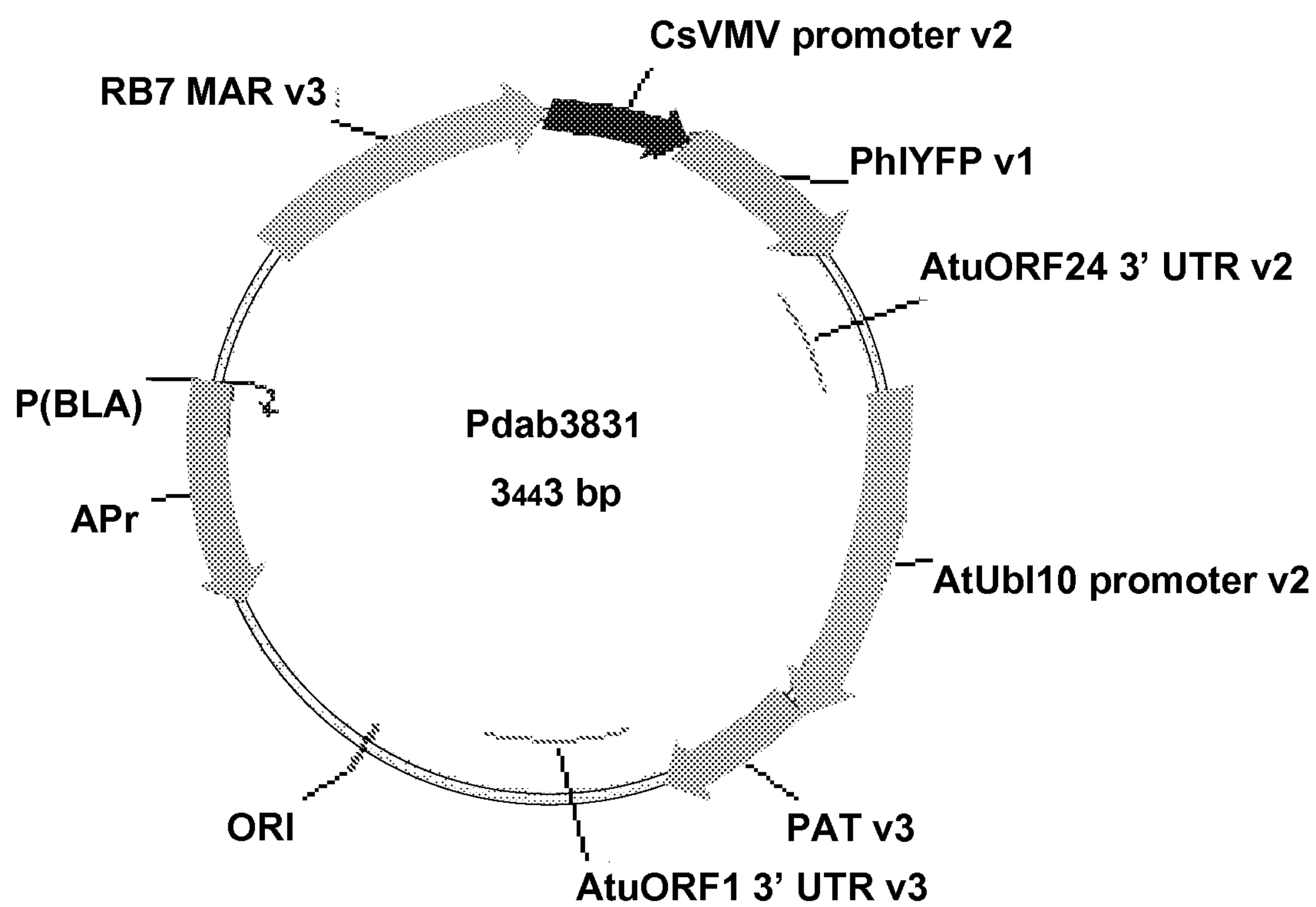
25

20. A method for transferring a molecular substance into a plant cell,  
comprising:  
interacting a dendrimer, and optionally one or more CPPs, with a plasmid DNA to  
form an activated dendrimer structure; and  
30 contacting the activated dendrimer structure with an intact wall-bearing plant cell under  
conditions permitting the uptake of the dendrimer and a gene from the plasmid  
DNA into the plant cell.



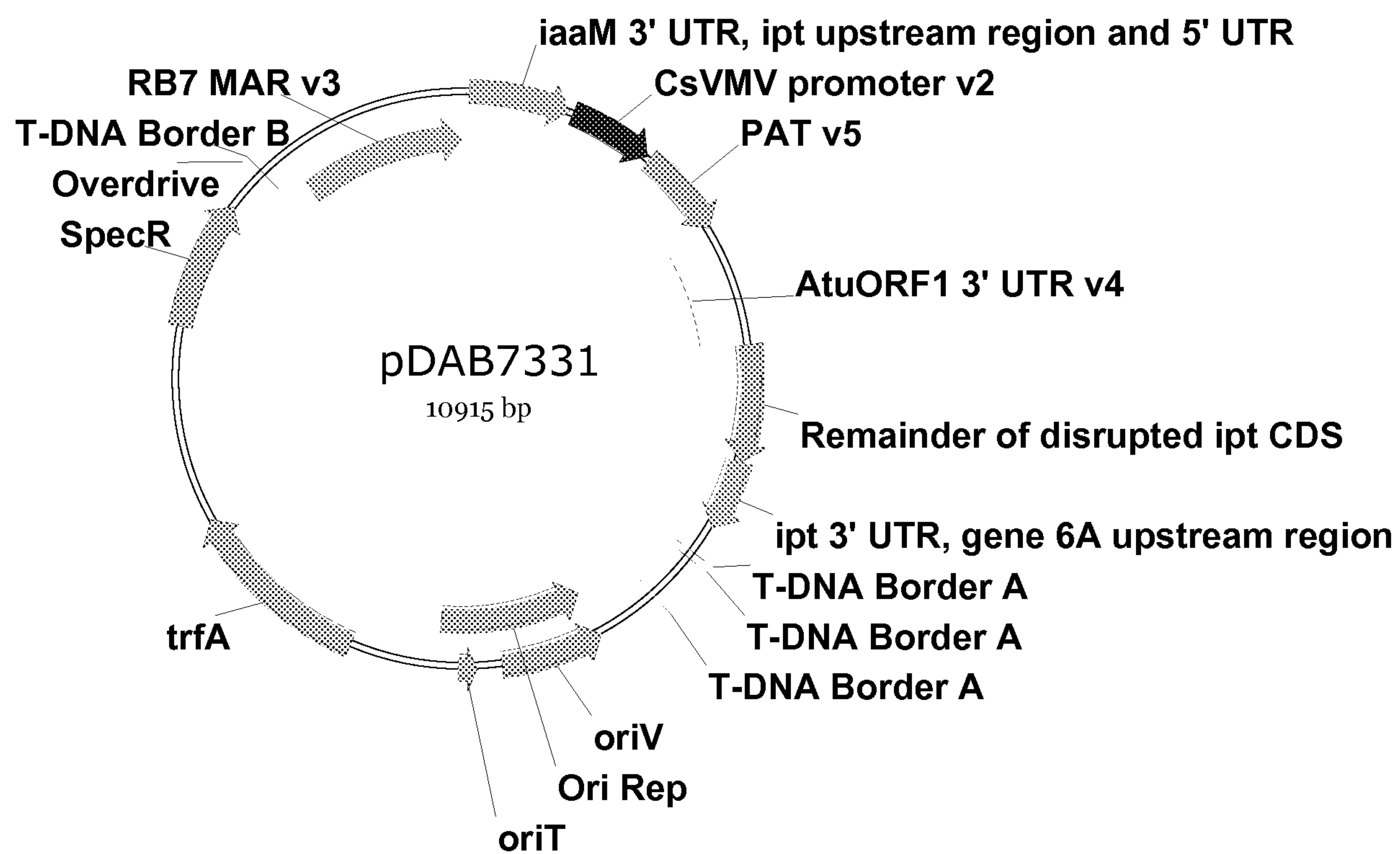
- 32 -

21. The method of claim 20, further comprising stably expressing the gene in progeny of a plant having the plant cell.

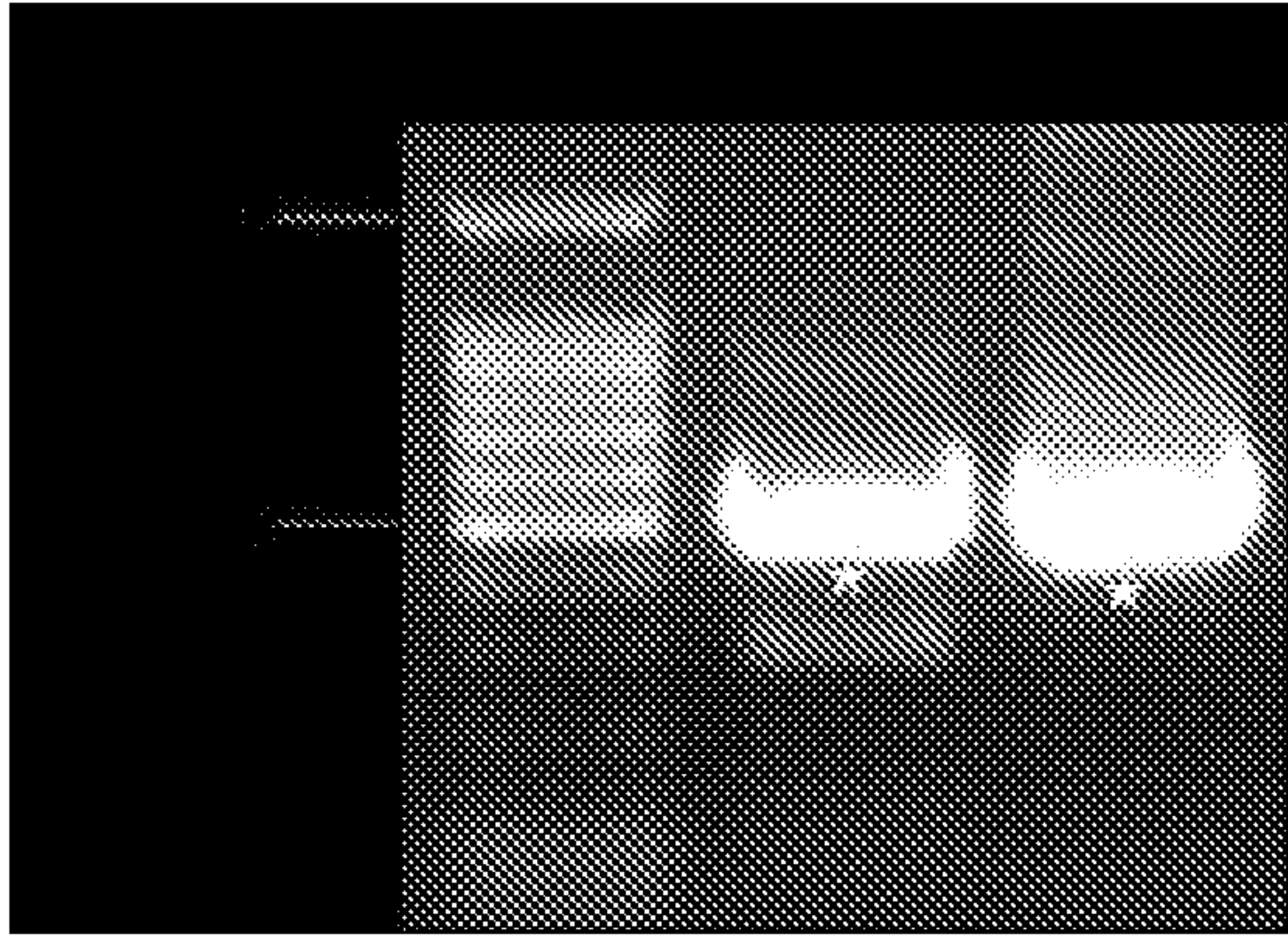


**Figure 1:** Map of plasmid pDAB3831.

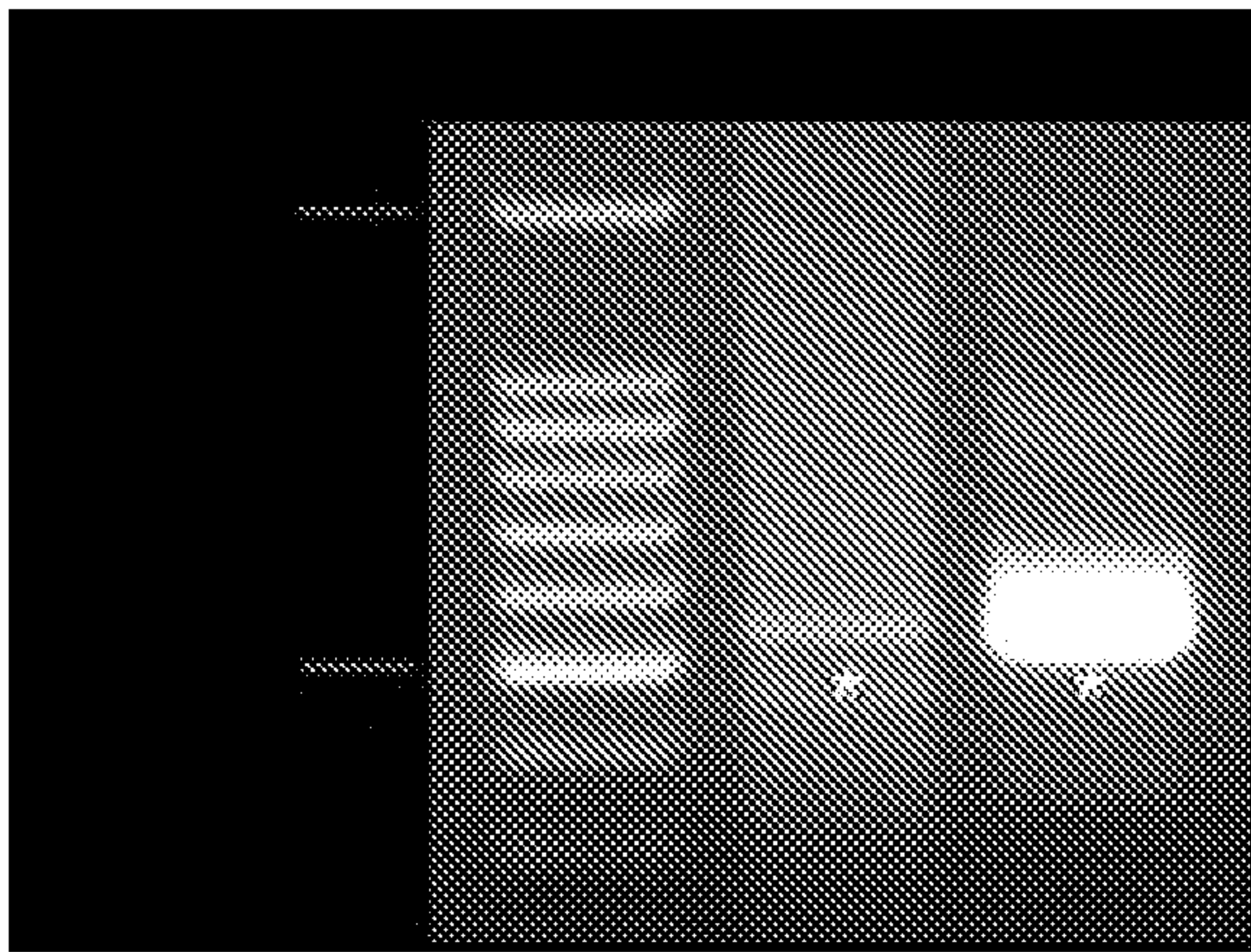




**Figure 2:** Map of plasmid pDAB7331.

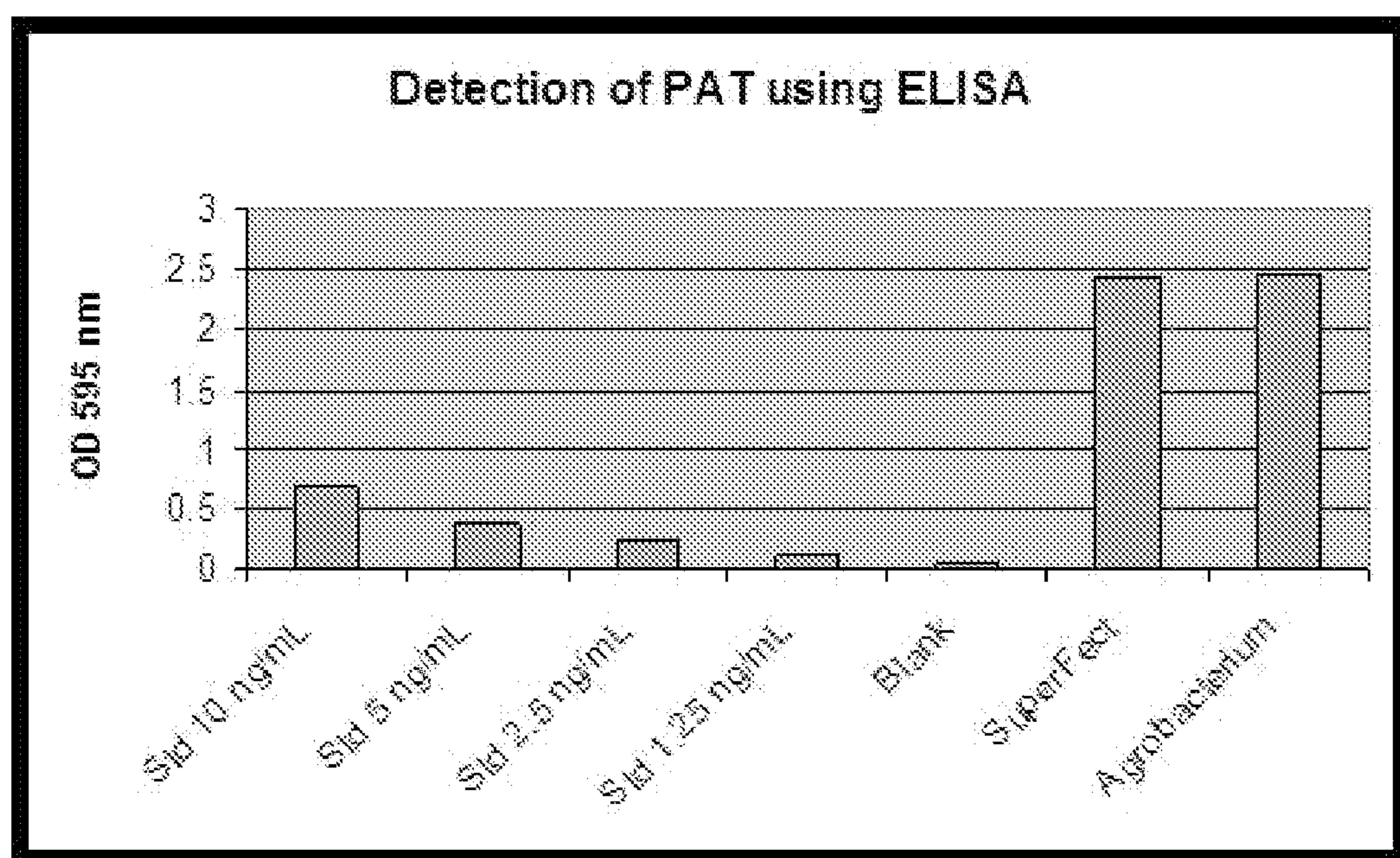


**Figure 3:** Ethidium bromide stained gel Image showing PAT PCR amplification from Genomic DNA of *Arabidopsis* transgenic plant.

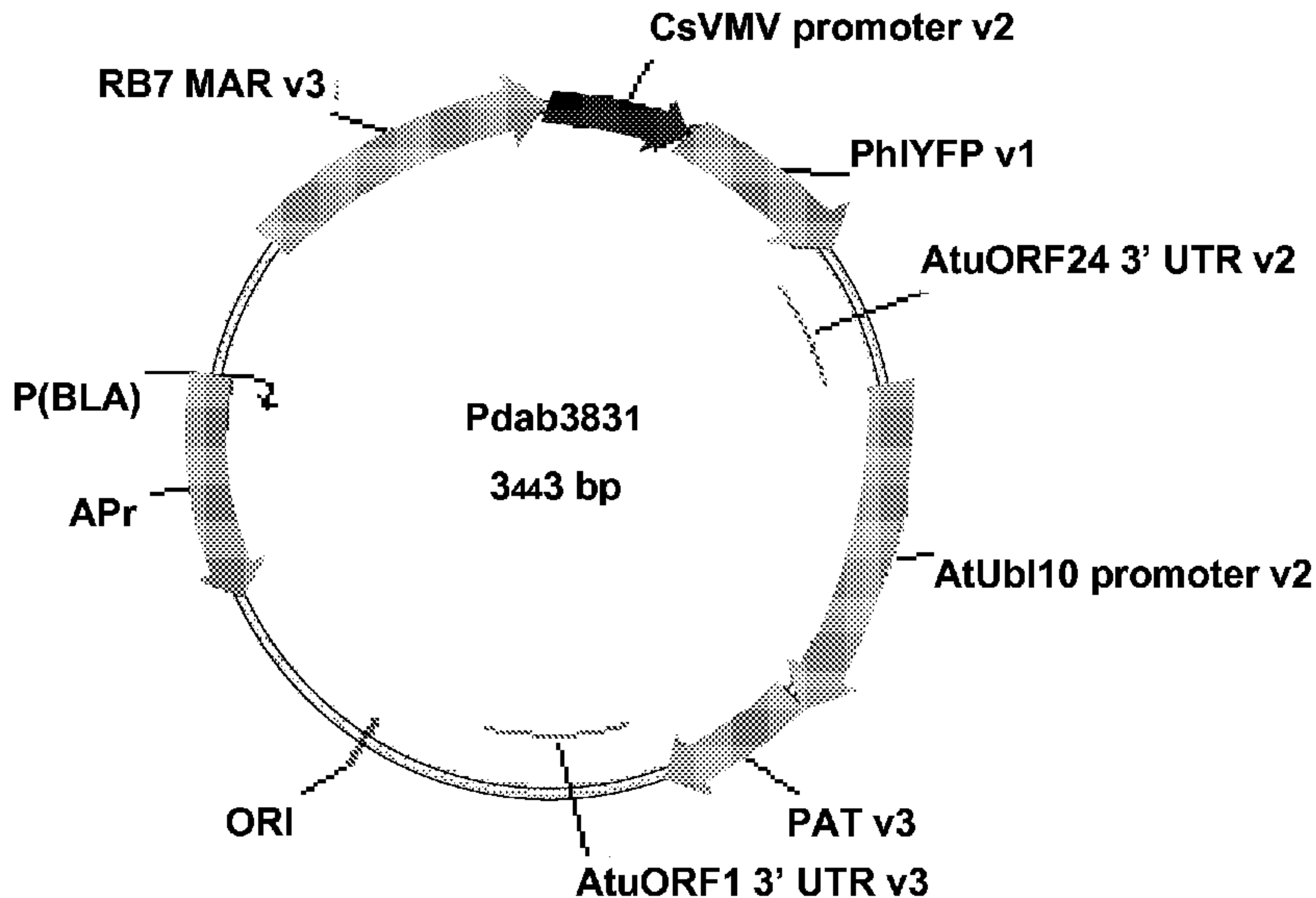


**Figure 4:** Gel Image showing YFP PCR amplification from Genomic DNA of *Arabidopsis* transgenic plant.





**Figure 5:** PAT protein expression levels from leaf tissues of Dendrimer-mediated (SuperFect) transformed *Arabidopsis* plants.



**Figure 1:** Map of plasmid pDAB3831.