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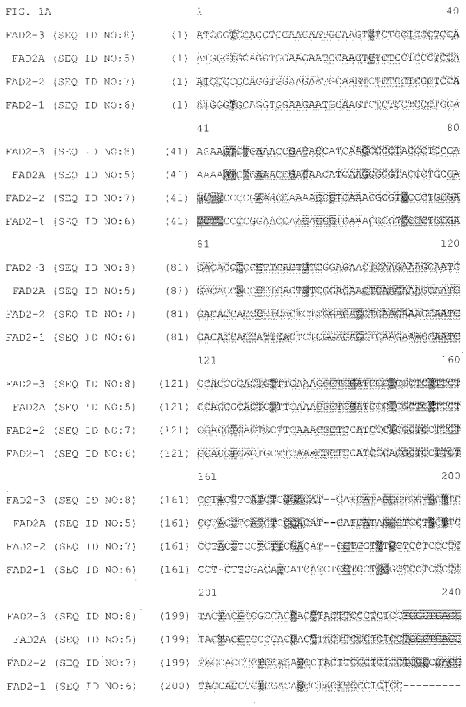
(54) Title: FLUORESCENCE ACTIVATED CELL SORTING (FACS) ENRICHMENT TO GENERATE PLANTS



(57) Abstract: An Engineered Transgene Integration Platform (ETIP) is described that can be inserted randomly or at targeted locations in plant genomes to facilitate rapid selection and detection of a GOI that is perfectly targeted (both the 3? and 5? ends) at the ETIP genomic location. One element in the invention is the introduction of specific double stranded breaks within the ETIP. In some embodiments, an ETIP is described using zinc finger nuclease binding sites, but may utilize other targeting technologies such as meganucleases, TALs, CRISPRs, or leucine zippers. Also described are compositions of, and methods for producing, transgenic plants wherein the donor or payload DNA expresses one or more products of an exogenous nucleic acid sequence (e.g. protein or RNA) that has been stably-integrated into an ETIP in a plant cell. In embodiments, the ETIP facilitates testing of gene candidates and plant expression vectors from ideation through Development phases.

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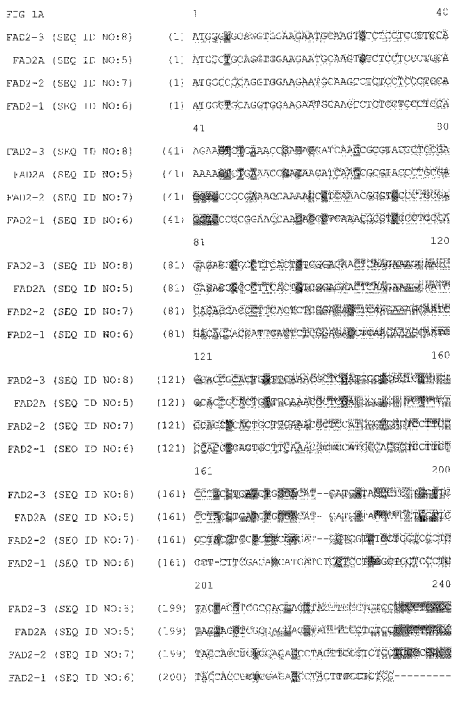

FIG. 1A

(57) Abstract: An Engineered Transgene Integration Platform (ETIP) is described that can be inserted randomly or at targeted locations in plant genomes to facilitate rapid selection and detection of a GOI that is perfectly targeted (both the 3? and 5? ends) at the ETIP genomic location. One element in the invention is the introduction of specific double stranded breaks within the ETIP. In some embodiments, an ETIP is described using zinc finger nuclease binding sites, but may utilize other targeting technologies such as meganucleases, TALs, CRISPRs, or leucine zippers. Also described are compositions of, and methods for producing, transgenic plants wherein the donor or payload DNA expresses one or more products of an exogenous nucleic acid sequence (e.g. protein or RNA) that has been stably-integrated into an ETIP in a plant cell. In embodiments, the ETIP facilitates testing of gene candidates and plant expression vectors from ideation through Development phases.

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FLUORESCENCE ACTIVATED CELL SORTING (FACS) ENRICHMENT TO GENERATE PLANTS

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims priority to the benefit of U.S. Provisional Patent Application Serial No. 61/697,890, filed September 7, 2012.

TECHNICAL FIELD

The disclosure relates to the field of fluorescence activated cell sorting to generate plants. In a preferred embodiment, the disclosure describes FACS enrichment of edited, regenerable protoplasts to generate fertile edited plants.

BACKGROUND

The Fluorescence Activated Cell Sorter (FACS) was invented in the late 1960s by Bonner, Sweet, Hulett, Herzenberg, and others to do flow cytometry and cell sorting of viable cells. Becton Dickinson Immunocytometry Systems introduced the commercial machines in the early 1970s. Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest.

The cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks

from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off.

A wide range of fluorophores can be used as labels in flow cytometry. Fluorophores, or simply "fluors," are typically attached to an antibody that recognizes a target feature on or in the cell; they may also be attached to a chemical entity with affinity for the cell membrane or another cellular structure. Each fluorophore has a characteristic peak excitation and emission wavelength, and the emission spectra often overlap. Consequently, the combination of labels which can be used depends on the wavelength of the lamp(s) or laser(s) used to excite the fluorochromes and on the detectors available.

Fluorescence-activated cell sorting (FACS) provides a rapid means of isolating large numbers of fluorescently tagged cells from a heterogeneous mixture of cells. Collections of transgenic plants with cell type-specific expression of fluorescent marker genes such as green fluorescent protein (GFP) are ideally suited for FACS-assisted studies of individual cell types.

It has been demonstrated that flow cytometric analysis and fluorescence activated cell sorting (FACS) of plant protoplasts is practicable, moreover, this technique has yielded valuable results in a number of different fields of research (Harkins and Galbraith, 1984; Galbraith *et al.*, 1995; Sheen *et al.*, 1995). For instance, FACS of protoplasts from *Arabidopsis* plants expressing tissue-specific fluorescent protein markers has been used to examine both basal and environmentally stimulated transcriptional profiles in particular cell types (Birnbaum *et al.*, 2003; Brady *et al.*, 2007; Gifford *et al.*, 2008; Dinneny *et al.*, 2008) and flow cytometry has been employed to analyze reactive oxygen species production and programmed cell death tobacco protoplasts (*Nicotiana tabacum*; Lin *et al.*, 2006). A broad selection of fluorescent tools is available to study a plethora of physiological parameters in plants, *e.g.*, *cis*-regulatory elements fused to fluorescent proteins (Haseloff and Siemering, 2006), genetically-encoded molecular sensors (Looger *et al.*, 2005) or dye-based sensors (Haugland, 2002) can be used in combination with cytometry to measure diverse biological processes.

However, there are certain inefficiencies with this process due to the sensitivities of the assays and thus there is room for improvement.

DISCLOSURE

A particular embodiment of the disclosure relates to a method for generating a plant from a population of plant cells by isolating a plant protoplast utilizing a polynucleotide of interest by providing a population of plant protoplasts having at least one protoplast comprising a polynucleotide of interest and a fluorescent marker, wherein the population is substantially free of plant protoplasts comprising the fluorescent marker and not comprising the polynucleotide of interest; wherein the plant protoplast is encapsulated by sodium alginate; separating the at least one protoplast comprising the polynucleotide of interest and the fluorescent marker from the remaining plant protoplasts in the population, thereby isolating a plant protoplast comprising the polynucleotide of interest; regenerating a plant from said isolated plant protoplast; and culturing said plant.

In another embodiment of the invention, there can be a plant regenerated by isolating a plant protoplast comprising a polynucleotide of interest integrated into the genome of the plant protoplast by providing a population of plant protoplasts having at least one protoplast comprising a polynucleotide of interest and a fluorescent marker; wherein the plant protoplast is encapsulated by sodium alginate; recovering microcalli from the population of protoplasts comprising the polynucleotide of interest and the fluorescent marker wherein the at least one protoplast comprises the polynucleotide of interest and the fluorescent marker has been transformed with the polynucleotide of interest and a polynucleotide encoding the fluorescent marker; regenerating a plant from said microcalli; and culturing said plant.

Alternative embodiments include methods for producing a transgenic plant, the method can include providing a population of plant protoplasts having at least one protoplast comprising a polynucleotide of interest and a fluorescent marker, wherein the at least one protoplast comprises a site-specific nuclease, such that the polynucleotide of interest is capable of being integrated in the genome of the at least one plant protoplast by homologous recombination at a recognition site of the site-specific nuclease and wherein the plant protoplast is encapsulated by sodium alginate; separating the at least one protoplast comprising the polynucleotide of interest and the fluorescent marker from

the remaining plant protoplasts in the population; regenerating the transgenic plant from the at least one protoplast; and culturing said transgenic plant.

The foregoing and other features will become more apparent from the following detailed description of several embodiments, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A-1E: Shows a sequence alignment of FAD2 gene sequences, generated using ALIGNX®.

FIG. 2: Shows a phylogenetic tree of FAD2 gene sequences generated using JALVIEW® v 2.3 based on neighbor joining distances.

FIGS. 3A-3M′: Shows a sequence alignment of FAD3 gene sequences, generated using ALIGNX®.

FIG. 4: Shows a phylogenetic tree of FAD3 gene sequences generated using JALVIEW® v 2.3 based on neighbor joining distances. The labeled sequences correspond as follows: FAD3A′/A″ is described throughout this application as FAD3A′; Haplotype2 is described throughout the application as FAD3C′; Haplotype 1 is described throughout the application as FAD3C″; and, Haplotype 3 is described throughout the application as FAD3A″.

FIG. 5: Shows a plasmid map of pDAB104010 which that is a representative Zinc Finger Nuclease expression cassette. The lay-out of this construct was similar for the other ZFN expression cassettes, wherein the Zinc Finger domains, 24828 and 24829, were exchanged with alternative Zinc Finger domains that are described above.

FIG. 6: is an example multiple line graph showing number of sequence reads per 10,000 sequence reads with deletions at the target ZFN site. The X axis on the graph denotes number of bases deleted, the Y axis denotes number of sequence reads and the Z axis denotes color-coded sample identity as described to the right of the graph. Specific example shown is for locus 1 of the FAD2 gene family that contains 3 target ZFN sites, A, B and C with the four gene family members and two control transfections assessed as control samples A and B.

FIG. 7: (A) Details of figure axis are as FIG. 6. The figure displays data from ZFN targeting locus 4 of the FAD2 gene family. The locus contains two ZFN sites and two requisite control transfections. FIG. 7:(B) Specific sequence context (SEQ ID NOs

471-480) surrounding the ZFN target site, identifying FAD2A and C containing tri-nucleotide repeats of C, T and G, leading to the observed increase in single base deletions through sequencing of the FAD2A and C loci.

FIG. 8: Shows a plasmid map of pDAS000130.

FIG. 9: Shows a plasmid map of pDAS000271.

FIG. 10: Shows a plasmid map of pDAS000272.

FIG. 11: Shows a plasmid map of pDAS000273.

FIG. 12: Shows a plasmid map of pDAS000274.

FIG. 13: Shows a plasmid map of pDAS000275.

FIG. 14: Shows a plasmid map of pDAS000031.

FIG. 15: Shows a plasmid map of pDAS000036.

FIG. 16: Shows a plasmid map of pDAS000037.

FIG. 17 illustrates an ETIP and payload nucleic acid configuration, as well as the product of the targeted Payload at the ETIP site in the plant cell genome.

FIG. 18 illustrates transformation of protoplast followed by FACS selection of targeted Payload DNA at the ETIP in the host line using reconstruction of truncated scorable and selectable markers at both the 3′ and 5′ ends.

FIGS. 19A and 19B: Illustrates the homology directed repair of the ETIP canola event which results from the double stranded DNA cleavage of the genomic locus by the Zinc Finger Nuclease (pDAS000074 or pDAS000075) and the subsequent integration of the *Ds-red* donor (pDAS000068, pDAS000070, or pDAS000072) into the ETIP locus of the canola chromosome. The integration of the donor into the genomic locus results in a fully functional, highly expressing *Ds-red* transgene.

FIG. 20: Shows the FACS sorting of canola protoplasts and the calculated transfection efficiency of canola protoplasts that were transfected with pDAS000031 ("pDAS31"). In addition, the FACS sorting results of untransformed canola protoplasts are provided as a negative control.

FIG. 21: Shows the FACS sorting of canola protoplasts and the calculated transfection efficiency of canola ETIP protoplast events which were transfected with pDAS000064 / pDAS000074 (top graph) and pDAS000064 / pDAS000075 (bottom graph).

FIG. 22: Shows the FACS sorting of canola protoplasts and the calculated transfection efficiency of canola ETIP protoplast events which were transformed with

pDAS000068 / pDAS000074 (top graph) and pDAS000068 / pDAS000075 (bottom graph).

FIG. 23: Shows the FACS sorting of canola protoplasts and the calculated transfection efficiency of canola ETIP protoplast events which were transformed with pDAS000070 / pDAS000074 (top graph) and pDAS000070 / pDAS000075 (bottom graph).

FIG. 24: Shows the FACS sorting of canola protoplasts and the calculated transfection efficiency of canola ETIP protoplast events which were transformed with pDAS000072 / pDAS000074 (top graph) and pDAS000072 / pDAS000075 (bottom graph).

FIG. 25: Shows a plasmid map of pDAS000074.

FIG. 26: Shows a plasmid map of pDAS000075.

FIG. 27: Shows a plasmid map of pDAS000064.

FIG. 28: Shows a plasmid map of pDAS000068.

FIG. 29: Shows a plasmid map of pDAS000070.

FIG. 30: Shows a plasmid map of pDAS000072.

FIG. 31: Is a schematic showing binding sites of transgene target primers and probe for transgene copy number estimation assay.

FIG. 32: Shows a SEQUENCHER® file showing FAD2A ZFN DNA recognition domain (bc12075_Fad2a-r272a2 and bc12075_Fad2a-278a2), and binding sites of ZFN specific primers (FAD2A.UnE.F1 and FAD2A.UnE.R1) and endogenous primers (FAD2A/2C.RB.UnE.F1 and FAD2A/2C.RB.UnE.R1).

FIG. 33: Shows a schematic showing binding sites of endogenous and transgene target primers used in the detection of transgene integration at FAD2A via perfect HDR.

FIG. 34: Is a schematic showing where Kpn1 restriction endonuclease sites would occur in a perfectly edited FAD2A locus, and where FAD2a 5′, *hph* and FAD2A 3′ Southern probes bind.

FIG. 35: Shows the location and size of Kpn1 fragments, FAD2A 5′, hph, FAD2A 3′ probes and expected outcomes of Southern analysis for plants that have integration of ETIP at FAD2A locus via HDR.

FIG. 36: Shows representative data output from copy number estimation qPCR. The left hand column represents data obtained from a known T_0 transgenic plant with a single random transgene insert and is used as the calibrator sample to which all other

samples are "normalized" against. The right hand column is a known T_0 transgenic plant with 5 transgene integrations. The insert copy numbers for both plants was determined using Southern analysis. The remaining columns provide copy number estimates for the putative transgenic plants. The labels below the columns correspond with the columns in the graph and can be used to determine the estimated copy number for each transgenic plant. When using the software to estimate copy numbers, wild-type plants, non-transformed control plants, and plasmid only controls do not result in a copy number as they do not possess a Cq for both the *hph* and *HMG I/Y* target.

MODE(S) FOR CARRYING OUT THE INVENTION

Transient transformation of protoplasts is a widely utilized tool in plant research that is swift and unproblematic. The technique can be used, for example, to monitor the regulation of promoter elements, to analyze gene expression or enzymatic activity in response to a variety of stimuli, to examine the roles of transcription factors or signal-transduction cascade components or to study the subcellular localization of proteins (Sheen, 2001; Yoo *et al.*, 2007). As opposed to stable transformation of plants (*Arabidopsis thaliana* being the most commonly used platform), which generally takes months and requires the use of a transfecting agent (usually *Agrobacterium tumefaciens*), transfection of protoplasts can be achieved in just one day and entails only raw DNA and either a chemical- or electroporation-based transfection method. Additionally, transient transformation analyses can overcome problems encountered with stable over-expression such as pleiotropic developmental effects or nonviability, when a cell-based assay is appropriate. However, due to the fact that protoplast transformation efficiency is never 100%, results can be convoluted by the non-transformed cells.

Transformation efficiencies are often low and variable (*e.g.* Cummins *et al.*, 2007; <10%) and depend on the employed method as well as properties of the protoplasts and DNA used. The present invention relates to the field of plant biotechnology, but can be used for all biological purposes. In particular, embodiments of the present invention relate to the generation of native or transgenic plant cell lines from a heterogeneous population of plant cells through flow cytometric sorting. Such plant cell line may either be a monocot or a dicot. As will be apparent for a skilled person, the invention also uses the plant cell line for the regeneration of whole fertile plants.

One of the embodiments of the present inventions relates to FACS based sensitivity selection wherein there is better selection to select for successfully transformed cells. It has previously been reported that transformation of a population of plant cells such as a plant suspension culture frequently results in transgenic cultures that heterogeneous and inconsistent expression levels. The present invention is primarily concern with the provision of a plant based system. The ability to isolate and grow single cells has numerous possible applications. For example, methods outlined herein have utility in the improvement of processes related to the productivity of plant cell cultures. However, this application has broad applicability for all cells.

Embodiments of the present invention include the use of flow cytometric sorting such as FACS technology to separate or isolate single, i.e. individualized protoplast that are prepared from a population of plant cells using materials and methods known in the art. These protoplasts can be transformed and are capable of 1) producing a fluorescent marker protein or polypeptide; 2) producing a desired product; and/or 3) surviving in the presence of a selection agent. Sorting criteria for FACS can be selected from the group comprising the genetic background (e.g., ploidy, aneuploidy), mutants, transgenics, gene exchange products, and fluorescence (autofluorescence (chloroplasts, metabolites), fluorescent proteins or enzyme mediation fluorescence). Any fluorescent protein may be used. A selection agent may or may not be used.

After separation or isolation of the single protoplasts by flow cytometric sorting, each single transformed protoplast is regenerated until the formation of a microcolony (microcallus) by co-cultivation. The plant source origin is not limited but is restricted to those lines, varieties and species whose protoplasts have the potential to regenerate until the formation of a microcolony or microcallus. The present invention will thus be applicable to all plant varieties and species for which a regeneration protocol has been established or will be provided. Thus, the present invention can be carried out with all plant varieties and species for which a regeneration portion has been established or will be provided for in the future.

The microcolony itself may be separated or removed from the feeder cell material and cultivated until the formation of a plant cell line.

Embodiments of the present invention can also include the generation of a callus tissue by 1) transferring the microcolony or microcallus to a solid cultivation medium and 2) cultivating the microcolony or microcallus in the presence of at least one selection agent

until the formation of a transgenic callus tissue from which a transgenic plant cell line can be established by transferring the callus tissue to a liquid cultivation medium. The microcolony can also be removed or separated from the feeder cell material by mechanical means, i.e., by clone picking. In this case, no selection agent is needed and the cells comprised by the microcolony do not need to display resistance against any selection agent.

In some embodiments the cells can comprise a heterogeneous population of plant cells that are native or non-transgenic cells that, before being subjected to flow cytometric sorting and are stably or transiently transformed with at least one expression vector comprising at least one heterologous nucleic acid sequence which can be operably linked to a functional promoter, wherein said at least one heterologous nucleic acid sequence codes for a desired product. In additional embodiments the at least one heterologous nucleic acid sequence is operably linked to at least one functional promoter wherein the at least one heterologous nucleic acid sequence codes for a fluorescent marker protein or polypeptide and at least one heterologous nucleic acid sequence for resistance to a selection agent or for a desired product. Additional embodiments can include wherein the cells may additionally comprise a heterologous nucleic acid sequence that codes for a desired product to be accumulated in the transgenic plant cell line as provided.

In other embodiments the genome of the host cell can be expressed so that the recombinant protein or peptide can be modified by recombination, for example homologous recombination or heterologous recombination.

Any (transgenic) monoclonal or diclonal plant cell lines established can be treated or cultivated in the presence of precursors, inducers, hormones, stabilizers, inhibitors, RNAi/siRNA molecules, signaling compounds, enzymes and/or elicitors in addition to or instead of the vector suspension, for the production of recombinant proteins or metabolites.

Heterologous nucleic acids may encode genes of bacterial, fungal, plant or non-plant origin such as fusion proteins, and proteins of animal origin. Polypeptides produced may be utilized for producing polypeptides which can be purified therefrom for use elsewhere. Proteins that can be produced in a process of the invention include heterodimers, immunoglobulins, fusions antibodies and single chain antibodies. Furthermore, the above genes may be altered to produce proteins with altered characteristics.

Embodiments of the present invention include the ability to produce a large variety of proteins and polypeptides. These embodiments can also include methods for the production of at least one desired product selected from the group consisting of heterologous proteins or polypeptides, secondary metabolites, and markers. The method comprises to use the plant cell line as established according to the invention in order to produce and accumulate the at least one desired product which is subsequently obtained or isolated from the producing cells or from the cultivation medium.

Additional methods include methods of generating at least an extracellular heterologous protein comprising the steps of 1) stably introducing into a target cell comprised by the starting population of plant cells a first nucleic acid comprising the nucleotide sequence coding for the heterologous protein or desired product; 2) preparing protoplasts form plant suspension cells provided from said plant suspension culture, wherein the protoplasts are additionally transformed and capable of i) producing a fluorescent marker protein or polypeptide and ii) surviving in presence of a selection agent; 3) separating single transformed protoplasts by subjecting the preparation of protoplasts to FACS; 4) regenerating a separated single transformed protoplast until the formation of a microcolony or microcallus by co-cultivation in the presence of feeder cell material; 5) generating callus tissue by i) transferring the microcolony or microcallus to solid cultivation medium and ii) cultivating the microcolony or microcallus in the presence of at least one selection agent until the formation of a transgenic callus tissue; 6) establishing a transgenic plant cell line by transferring the callus tissue to liquid cultivation medium; 7) causing or permitting expression from the nucleic acid of the heterologous protein or desired product by providing appropriate cultivation conditions; and 8) harvesting the accumulated heterologous protein or desired product from the producing cells. Such isolation can be by entirely conventional means and may or may not entail partial or complete purification.

More than one gene may be used in each construct. Multiple vectors, each including one or more nucleotide sequences encoding heterologous protein of choice, may be introduced into the target cells as described herein or elsewhere. This can also be useful for producing multiple subunits of an enzyme.

The fluorescent marker protein or polypeptide can be a protein detectable by fluorescence such as GUS, fluorescent proteins such as GFP or DsRed, luciferase, etc. Preferably the reported is a non-invasive marker such as DsRed or GFP.

The techniques of this invention may be used to select for certain plants to be grown. Selection of a gene of interest may be handled in a number of ways. A large number of techniques are available for inserting DNA into a plant host cell. These techniques include transformation with T-DNA using Agrobacterium tumefaciens or Agrobacterium rhizogenes as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If Agrobacteria are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in Agrobacteria. The intermediate vector can be transferred into Agrobacterium tumefaciens by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in E. coli and in Agrobacteria. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into Agrobacteria (Holsters, 1978). The Agrobacterium used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with Agrobacterium tumefaciens or Agrobacterium rhizogenes for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

In some preferred embodiments of the invention, genes encoding proteins of interest are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, inter alia. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate (e.g., PAT/bar), glyphosate (EPSPS), ALS-inhibitors (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, et al.), bromoxynil, HPPD-inhibitor resistance, PPO-inhibitors, ACC-ase inhibitors, and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using Agrobacterium technology, see U.S. Pat. Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Pat. Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Pat. No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Pat. No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Pat. Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Pat. Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. See

U.S. Pat. Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Other direct DNA delivery transformation technology includes aerosol beam technology. See U.S. Pat. No. 6,809,232. Electroporation technology has also been used to transform plants. See WO 87/06614 to Boyce Thompson Institute; U.S. Pat. Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Pat. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform Agrobacterium mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses Agrobacterium tumefaciens or Agrobacterium rhizogenes as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where Agrobacterium is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the Agrobacterium host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

In some cases where Agrobacterium is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al. (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed Agrobacterium and transformed

plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using Agrobacterium, explants may be combined and incubated with the transformed Agrobacterium for sufficient time to allow transformation thereof. After transformation, the Agrobacteria are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3′ non-translated transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G41; hygromycin resistance; methotrexate resistance, as well as those genes which encode for resistance or tolerance to glyphosate; phosphinothricin (bialaphos or glufosinate); ALS-inhibiting herbicides (imidazolinones, sulfonylureas and triazolopyrimidine herbicides), ACC-ase inhibitors (e.g., ayryloxypropionates or cyclohexanediones), and others such as bromoxynil, and HPPD-inhibitors (e.g., mesotrione) and the like.

In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising et al., 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the uidA locus of E. coli, the chloramphenicol acetyl transferase gene from Tn9 of E. coli, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the uidA locus of *E. coli* as described by Jefferson et al. (1987), to identify transformed cells.

In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, see U.S. Pat. No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in

specific cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Pat. No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Pat. Nos. 5,316,931 and 5,589,367 to Biosource.

Means of further increasing tolerance or resistance levels. It is shown herein that plants of the subject invention can be imparted with novel herbicide resistance traits without observable adverse effects on phenotype including yield. Such plants are within the scope of the subject invention. Plants exemplified and suggested herein can withstand 2x, 3x 4x and 5x typical application levels, for example, of at least one subject herbicide. Improvements in these tolerance levels are within the scope of this invention. For example, various techniques are known in the art, and can foreseeably be optimized and further developed, for increasing expression of a given gene.

One such method includes increasing the copy number of the subject genes (in expression cassettes and the like). Transformation events can also be selected for those having multiple copies of the genes.

Strong promoters and enhancers can be used to "supercharge" expression. Examples of such promoters include the preferred 35T promoter which uses 35S enhancers. 35S, maize ubiquitin, Arabidopsis ubiquitin, A.t. actin, and CSMV promoters are included for such uses. Other strong viral promoters are also preferred. Enhancers

include 4 OCS and the 35S double enhancer. Matrix attachment regions (MARs) can also be used to increase transformation efficiencies and transgene expression, for example.

Shuffling (directed evolution) and transcription factors can also be used for embodiments according to the subject invention.

Variant proteins can also be designed that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like. See e.g. U.S. Pat. No. 7,058,515; Larson et al., *Protein Sci.* 2002 11: 2804-2813, "Thoroughly sampling sequence space: Large-scale protein design of structural ensembles"; Crameri et al., *Nature Biotechnology* 15, 436-438 (1997), "Molecular evolution of an arsenate detoxification pathway by DNA shuffling"; Stemmer, W. P. C. 1994, DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution, *Proc. Natl. Acad. Sci. USA* 91: 10747-10751; Stemmer, W. P. C. 1994, Rapid evolution of a protein in vitro by DNA shuffling, *Nature* 370: 389-391; Stemmer, W. P. C. 1995, Searching sequence space. *Bio/Technology* 13: 549-553; Crameri, A., Cwirla, S, and Stemmer, W. P. C. 1996, Construction and evolution of antibody-phage libraries by DNA shuffling, *Nature Medicine* 2: 100-103; and Crameri, A., Whitehorn, E. A., Tate, E. and Stemmer, W. P. C., 1996, Improved green fluorescent protein by molecular evolution using DNA shuffling, *Nature Biotechnology* 14: 315-319.

The activity of recombinant polynucleotides inserted into plant cells can be dependent upon the influence of endogenous plant DNA adjacent the insert. Thus, another option is taking advantage of events that are known to be excellent locations in a plant genome for insertions. See e.g. WO 2005/103266 A1, relating to cry1F and cry1Ac cotton events; FAD2, FAD3, wherein genes such as AAD1 or AAD12 or others can be substituted in those genomic loci in place of such inserts. Thus, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (USPA 20030232410), relating to the use of zinc fingers for targeted recombination. The use of recombinases (cre-10xand flp-frt for example) is also known in the art.

Computational design of 5′ or 3′ UTR most suitable for synthetic hairpins can also be conducted within the scope of the subject invention. Computer modeling in general, as well as gene shuffling and directed evolution, are discussed elsewhere herein. More specifically regarding computer modeling and UTRs, computer modeling techniques for

use in predicting/evaluating 5′ and 3′ UTR derivatives of the present invention include, but are not limited to: MFold version 3.1 available from Genetics Corporation Group, Madison, Wis. (see Zucker et al., *Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. In RNA Biochemistry and Biotechnology*, 11-43, J. Barciszewski & B. F. C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, Dordrecht, NL, (1999); Zucker et al., Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure. *J. Mol. Biol.* 288, 911-940 (1999); Zucker et al., RNA Secondary Structure Prediction. In *Current Protocols in Nucleic Acid Chemistry*, S. Beaucage, D. E. Bergstrom, G. D. Glick, and R. A. Jones eds., John Wiley & Sons, New York, 11.2.1-11.2.10, (2000)), COVE (RNA structure analysis using covariance models (stochastic context free grammar methods)) v. 2.4.2 (Eddy & Durbin, *Nucl. Acids Res.* 1994, 22: 2079-2088) which is freely distributed as source code and which can be downloaded by accessing the website genetics.wustl.edu/eddy/software/, and FOLDALIGN, also freely distributed and available for downloading at the website bioinf.au.dk. FOLDALIGN/ (see Finding the most significant common sequence and structure motifs in a set of RNA sequences. J. Gorodkin, L. J. Heyer and G. D. Stormo. *Nucleic Acids Research*, Vol. 25, no. 18 pp 3724-3732, 1997; Finding Common Sequence and Structure Motifs in a set of RNA Sequences. J. Gorodkin, L. J. Heyer, and G. D. Stormo. *ISMB* 5;120-123, 1997).

Embodiments of the subject invention can be used in conjunction with naturally evolved or chemically induced mutants (mutants can be selected by screening techniques, then transformed with other genes). Plants of the subject invention can be combined with various resistance genes and/or evolved resistance genes. Traditional breeding techniques can also be combined with the subject invention to powerfully combine, introgress, and improve selection of traits.

All references, including publications, patents, and patent applications, cited and discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

EXAMPLES

The following Examples are provided to illustrate certain particular features and/or aspects. These Examples should not be construed to limit the disclosure to the particular features or aspects described.

EXAMPLE 1: IDENTIFICATION OF PARALOGOUS FAD2 AND FAD3 TARGET SEQUENCES FROM A BACTERIAL ARTIFICIAL CHROMOSOME LIBRARY

BAC CONSTRUCTION

A Bacterial Artificial Chromosome (BAC) library was sourced from a commercial vendor (Amplicon Express, Pullman, WA). The BAC library consisted of 110,592 BAC clones containing high molecular weight genomic DNA (gDNA) fragments isolated from *Brassica napus* L. var. DH10275. The gDNA was digested with either the *BamHI* or *HinDIII* restriction enzyme. Isolated gDNA fragments of about 135 Kbp were ligated into the pCC1BAC vector (Epicentre, Madison, WI) and transformed into *Escherichia coli* str. DH10B (Invitrogen). The BAC library was made up of an even number of BAC clones that were constructed using the two different restriction enzymes. As such, the *Hind III* constructed BAC library consisted of 144 individual 384-well plates. Likewise, the *BamHI* constructed BAC library consisted of 144 individual 384-well plates. A total of 110,592 BAC clones were isolated and arrayed into 288 individual 384-well plates. Each of the 288 individual 384 well plates were provided by the vendor as a single DNA extraction for rapid PCR based screening. The resulting BAC library covers approximately 15 Gbp of gDNA, which corresponds to a 12-fold genome coverage of *Brassica napus* L. var. DH10275genome (estimate of the *Brassica napus* L. genome is ca. 1.132 Gbp as described in Johnston *et al.* (2005) Annals of Botany 95:229-235).

SEQUENCE ANALYSIS OF FAD2 CODING SEQUENCES ISOLATED FROM THE BAC LIBRARY

The constructed BAC library was used to isolate FAD2 gene coding sequences. Sequencing experiments were conducted to identify the specific gene sequences of four FAD2 gene paralogs from *Brassica napus* L. var. DH10275.

The FAD2 gene sequence was initially identified within the model species *Arabidopsis thaliana*. The gene sequence is listed in Genbank as Locus Tag: At3g12120.

Comparative genomic relationships between the model plant species *Arabidopsis thaliana* and the diploid *Brassica rapa,* one of the progenitors of the tetraploid *Brassica napus,* have been previously described. (Schranz *et al.* (2006) Trends in Plant Science 11(11):535-542). With specific relation to the FAD2 gene the comparative analysis predicted that 3-4 copies of the gene may occur within the diploid *Brassica* genome. Additional genetic mapping studies were completed by Scheffler *et al.* (1997) Theoretical and Applied Genetics 94; 583-591. The results of these genetic mapping studies indicated that four copies of the FAD2 gene were present in *Brassica napus.*

Sequencing analysis of the BAC library which was constructed from *B. napus* L. var. DH12075 resulted in the isolation of four BAC sequences (SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:4) from which the coding sequences for the FAD2A (SEQ ID NO:5), FAD2-1 (SEQ ID NO:6), FAD2-2 (SEQ ID NO:7), and FAD2-3(SEQ ID NO:8) genes were determined. The FAD2A, FAD2-1, FAD2-2, and FAD2-3 gene sequences were identified and genetically mapped. Sequence analysis of the four FAD2 genes was conducted using a sequence alignment program and a neighbor-joining tree using percentage of identity. The sequence alignment was made via the ALIGNX® program from the Vector NTI Advance 11.0 computer program (Life Technologies, Carlsbad, CA) and is shown in FIG. 1. ALIGNX® uses a modified **Clustal W algorithm** to generate multiple sequence alignments of either protein or nucleic acid sequences for similarity comparisons and for annotation. The neighbor-joining tree was created with JALVIEW v2.3® software and is shown in FIG. 2. (Waterhouse *et al.* (2009) Bioinformatics 25 (9) 1189-1191). As shown in FIG. 2, the analysis of the isolated sequences indicated that the FAD2A and FAD2-3 sequences shared high levels of sequence similarity and that, likewise, FAD2-1 and FAD2-2 shared high levels of sequence similarity. The four sequences can be categorized in two clades, wherein FAD2A and FAD2-3 comprise a first clade, and FAD2-1 and FAD2-2 comprise a second clade.

Next, the newly isolated FAD2 sequences from *Brassica napus* were used to BLAST genomic libraries isolated from a *Brassica rapa* genomic BAC library and *Brassica oleracea* shotgun genomic sequence reads. Both, *Brassica rapa* and *Brassica oleracea* are diploid progenitors of *Brassica napus* which is an amphidiploid species (AC genome, n = 19). *Brassica napus* derived from a recent hybridization event between *Brassica rapa* (A sub-genome, n = 10) and *Brassica oleracea* (C sub-genome, n = 9). The diploid progenitor sequences were compared to the four different FAD2 coding sequences

isolated from *Brassica napus* using a BLASTn analysis. This sequence analysis identified specific, annotated gene sequences from *Brassica rapa* and *Brassica oleracea* which shared the highest sequence similarity to the newly discovered *Brassica napus* FAD2 sequences. Table 1 lists the newly identified FAD2 coding sequence and the corresponding progenitor reference sequence accession number and source organism.

Table 1: FAD2 sequences from *Brassica napus* and the corresponding progenitor organism and related FAD sequence accession number.

Isolated gene sequence	**Progenitor organism and sequence accession number**	
FAD2A	*B.rapa*	Genbank Accession No: KBrB063G23 (A05)
FAD2-3	*B.oleracea*	Genbank Accession No: GSS23580801*
FAD2-1	*B.rapa*	Genbank Accession No: KBrB130I19
FAD2-2	*B.oleracea*	Genbank Accession No: GSS 17735412

*The Genbank sequence entry was edited

The FAD2 genes exist in the *Brassica napus* genome as two copies of each gene per sub-genome. One copy of each gene is located on the A sub-genome, and likewise one copy of each gene is located on the C sub-genome. New naming conventions are described to indicate which sub-genome that each gene is located on. The high levels of sequence similarity between the four different FAD2 coding sequences isolated from the *Brassica napus* BAC genomic DNA library and the progenitor sequence data suggest that FAD2-3 is a duplicate of the FAD2 sequence from the C sub-genome and could be relabeled as FAD2C; FAD2-1 is a duplicate of the FAD2 sequence from the A sub-genome and could therefore be labeled as FAD2A'; and finally, FAD2-2 is a second copy that was duplicated from the FAD2 sequence of the C sub-genome and could be labeled as FAD2C'.

SEQUENCE ANALYSIS OF FAD3 CODING SEQUENCES ISOLATED FROM THE BAC LIBRARY

The constructed BAC library was used to isolate FAD3 gene coding sequences. Sequencing experiments were conducted to identify the specific gene sequences of five FAD3 gene paralogs from *Brassica napus* L. var. DH10275.

The FAD3 gene sequence was initially identified within the model species *Arabidopsis thaliana*. The gene sequence is listed in Genbank as Locus Tag: At2g29980. Comparative genomic relationships between the model plant species *Arabidopsis thaliana*

and the diploid *Brassica rapa,* one of the progenitors of the tetraploid *Brassica napus,* have been previously described. (Schranz *et al.* (2006) Trends in Plant Science 11(11):535-542). With specific relation to the FAD gene the comparative analysis predicted that 3-4 copies of the gene may occur within the diploid *Brassica* genome. Additional genetic mapping studies were completed by Scheffler *et al.* (1997) Theoretical and Applied Genetics 94; 583-591. The results of these genetic mapping studies indicated that six copies of the FAD3 gene were present in *Brassica napus*.

Previous sequencing efforts focused on the FAD3 genes from *Brassica napus* had identified and genetically mapped both A and C genome specific copies (Hu *et al.,* (2006) Theoretical and Applied Genetics, 113(3): 497-507). A collection of EST sequences from seed specific cDNA libraries had previously been constructed and sequenced from the plant line DH12075 by Andrew Sharpe of Agriculture and Agri-food Canada, 107 Science Place, Saskatoon, Saskatchewan. As a collection of ESTs from the doubled haploid canola plant DH12075 full length gene sequences were not available, moreover the indications of sequence quality and confidence of correctly called nucleotides was also not available. Consequently, sequence variation between different FAD gene sequence reads could not be unequivocally attributed to different gene copies of the various paralogs of the FAD3 gene family, nor was the genomic sequence available. However, when a combined sequence analysis was performed with the ESTs as well as the two FAD3A and FAD3C full length gene sequences described in Hu *et al.*, (2006), ESTs that matched both of the genes were identified along with an additional 3 haplotypes. As a result, a total of six unique haplotypes of FAD3 were identified. Following the assembly of all available data for the various FAD3 haplotypes, high levels of exon sequence divergence in exon 1 was identified. The divergence of the FAD3 sequence in exon 1 was identified as an opportunity which could be utilized for the design of gene/allele specific PCR primers. In addition, exons were identified that were either minimally differentiated between haplotypes (*e.g.,* exons 5, 6, 7 and 8 had 1-3 bp that varied between FAD3A and FAD3C) or that were devoid of sequence variation (*e.g.,* exons 2 and 3).

Sequencing analysis of the BAC library which was constructed from *B. napus* L. var. DH12075 resulted in the isolation of six BAC sequences (SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, and SEQ ID NO:14) from which the coding sequences for the FAD3A (SEQ ID NO:15), FAD3A′ (SEQ ID NO:16), FAD3A″ (SEQ ID NO:17), FAD3C (SEQ ID NO:18), FAD3C″ (SEQ ID NO:19), and

FAD3C′ (SEQ ID NO:20) genes were determined. The FAD3A, FAD3A′, FAD3A″, FAD3C, FAD3C″, and FAD3C′ gene sequences were identified and genetically mapped.

Sequence analysis of the six FAD3 genes was conducted using a sequence alignment program and a neighbor-joining tree using percentage of identity. The sequence alignment was made via the ALIGNX® program from the Vector NTI Advance 11.0 computer program (Life Technologies, Carlsbad, CA) and is shown in FIG. 3. ALIGNX® uses a modified **Clustal W algorithm** to generate multiple sequence alignments of either protein or nucleic acid sequences for similarity comparisons and for annotation. The neighbor-joining tree was created with JALVIEW v2.3® software and is shown in FIG. 4. (Waterhouse *et al.* (2009) Bioinformatics 25 (9) 1189-1191). The contigs identified as containing FAD3 genes were used as BLASTn queries against a database of *Arabidopsis thaliana* genes. The region of each of the 6 contigs containing the FAD3 gene was identified through comparison to the *Arabidopsis thaliana* FAD3 gene (Genbank Accession No: At2g29980). The FAD3 contigs were then orientated such that all FAD3 genes were in the 5′ to 3′ orientation. FAD3 contigs were trimmed to contain as many as 2 upstream (5′) and 1 downstream (3′) *Arabidopsis thaliana* genes where possible. Once orientated the complete coding region of the FAD3 genes were extracted from each contig and used to generate a Neighbor joining tree to display the relationship between the different FAD3 gene family members. The 6 FAD3 family members were aligned into 3 pairs of FAD3 genes (FIG. 4).

PCR BASED SCREENING

A cohort of PCR primers were design to screen the aforementioned BAC library. The primers were designed as either universal primers, which would amplify all members of the gene family, or as gene specific primers for targeted allele amplification. The PCR primers were designed to be 20 bp long (+/- 1bp) and contain a G/C content of 50% (+/- 8%). Table 2 and Table 3 lists the primers which were designed and synthesized. The clones of the BAC library were pooled and screened via the Polymerase Chain Reaction (PCR).

Table 2: Primer sequences used for PCR amplification of FAD3 sequences.

Primer Name:	SEQ ID NO:	Sequence:
D_uni_F3_F1	SEQ ID NO:21	GAATAAGCCATCGGACACAC
D_spec_F3_F2	SEQ ID NO:22	ATGCGAACGGAGACGAAAGG
D_spec_F3_F3	SEQ ID NO:23	TGTTAACGGAGATTCCGGTG
D_spec_F3_F4	SEQ ID NO:24	GTAGCAATGTGAACGGAGAT
D_uni_F3_R1	SEQ ID NO:25	CAGTGTATCTGAGCATCCG
D_spec_F3_R2	SEQ ID NO:26	GTGGCCGAGTACGAAGATAG
D_spec_F3_R3	SEQ ID NO:27	CAGTAGAGTGGCCAGAGGA

Table 3: PCR primer sequences designed for BAC library screening for FAD2 gene identification.

Primer Name	SEQ ID NO:	Sequence
D_UnivF2_F1	SEQ ID NO:28	ATGGGTGCAGGTGGAAGAATG
D_UnivF2_F2	SEQ ID NO:29	AGCGTCTCCAGATATACATC
D_UnivF2_R1	SEQ ID NO:30	ATGTATATCTGGAGACGCTC
D_UnivF2_R2	SEQ ID NO:31	TAGATACACTCCTTCGCCTC
D_SpecificF2_F3	SEQ ID NO:32	TCTTTCTCCTACCTCATCTG
D_SpecificF2_R3	SEQ ID NO:33	TTCGTAGCTTCCATCGCGTG
D_UnivF2_F4	SEQ ID NO:34	GACGCCACCATTCCAACAC
D_UnivF2_R4	SEQ ID NO:35	ACTTGCCGTACCACTTGATG

A Two different sets of conditions were used for the polymerase chain reactions (PCR). The first series of PCR reactions contained: 1X PCR buffer (containing dNTPs); 1.5 mM $MgCl_2$; 200 μM of 0.25 U IMMOLASE® DNA polymerase (Bioline, London, UK); 250 nM of each primer; and, about 5-10 ng template DNA. A second series of PCR reactions were developed for the amplification of genomic DNA and contained: 5-10 ng of genomic DNA, 1X PCR buffer, 2 mM dNTPs, 0.4 μM forward and reverse primer, and 0.25 U IMMOLASE® DNA polymerase (Bioline, London, UK). Amplifications were pooled into a final volume of 13 μL and amplified using an MJ PTC200® thermocycler (BioRad, Hercules, CA) or an ABI 9700 GENE AMP SYSTEM® (Life Technologies, Carlsbad, CA). PCR based screening of specific plates was conducted using a 4 dimension screening approach based on the screening system described by Bryan *et al* (Scottish Crops Research Institute annual report: 2001-2002) with the above described PCR conditions. Following

PCR based screening of pooled BAC libraries; the amplified PCR product was sequenced using a direct Sanger sequencing method. The amplified products were purified with ethanol, sodium acetate and EDTA following the BIGDYE® v3.1 protocol (Applied Biosystems) and electrophoresis was performed on an ABI3730xl® automated capillary electrophoresis platform.

Following PCR based screening and conformational Sanger sequencing, a collection of plates were identified that contained the various different FAD2 and FAD3 gene family members. A total of four unique FAD2 and FAD3 paralogous gene sequences were identified (Table 4 and Table 5). A total of two plates per each FAD2 and FAD3 paralogous gene sequence were chosen to undergo plate screening to identify the specific well and clone within the plate that contained the FAD2 and FAD3 gene (Table 4 and Table 5). The specific wells were identified for both of the plates and an individual clone was selected for each of the FAD2 and FAD3 gene family members.

Table 4: Identification of the BAC clone plates that provided positive reaction with the detailed PCR primer combinations, along with two plate identities that were taken forward for clone identification within the plate.

Gene Name	Primer Sets	Positive Plate Pools	Chosen Plates	Well Id
FAD2A	F4+R1, F1+R1, F1+R4, F3+R3	8, 27, 30, 83, 109, 147, 180, 199, 209, 251, 288	Plate 199 Plate 27	L23 D20
FAD2-1	F1+R4, F4+R1, F1+R1, F2+R2	12, 89, 123, 148, 269	Plate 123 Plate 148	N17 B15
FAD2-2	F4+R1, F1+R1, F1+R4, F2+R2	24, 44, 46, 47, 80, 91, 104, 110, 119, 121, 124, 248	Plate 44 Plate 121	H03 A17
FAD2-3	F1+R4, F4+R1, F1+R1, F3+R3	8, 62, 113, 205, 276	Plate 62 Plate 205	I16 K11

Table 5: Identification of the BAC clone plates that provided positive reaction with the detailed PCR primer combinations, along with two plate identities that were taken forward for clone identification within the plate.

Gene Name	Primer Sets	Positive Plate Pools	Chosen Plates
FAD3A (FAD3A-1)	F2+R2	16, 231	Plate 16 Plate 231
FAD3C	F4+R2	18, 27, 136, 178, 211, 232	Plate 18 Plate 27
FAD3C″ (Haplotype1)	F4+R2, F4+R3, F3+R3	23, 44, 53, 56, 77, 116, 158, 199, 209, 278, 280, 282, 283, 284, 286	Plate 44 Plate 199
FAD3A′ (FAD3A′/FAD3A″)	F4+R2	52, 121, 139	Plate 121 Plate 139
FAD3C′ (Haplotype2)	F4+R2	144, 188, 235	Plate 144 Plate 188
FAD3A″ (Haplotype3)	F4+R3 and F3+R3	69, 105, 106, 229, 242, 247, 248	Plate 69 Plate 106

The single BAC clone, for each identified FAD gene family member, was further analysed via sequencing. The DNA was isolated for the BAC clone and was prepared for sequencing using a LARGE CONSTRUCT KIT® (Qiagen, Valencia, CA) following the manufacturer's instructions. The extracted BAC DNA was prepared for sequencing using GS-FLX® Titanium Technology (Roche, Indianapolis, IN) following manufacturer's instructions. Sequencing reactions were performed using a physically sectored GS-FLX TI® Pico-titer plate with the BACs pooled in pairs for optimal data output. The BACs were combined in pairs where the FAD2 gene was paired with a FAD3 gene. All generated sequence data was assembled by NEWBLER v2.0.01.14® (454 Life Sciences, Branford, CT). The assembled contigs were manually assessed for the presence of the corresponding FAD gene using SEQUENCHER v3.7® (GeneCodes, Ann Arbor, MI).

After the full genomic sequence of all four FAD2 and six FAD3 genes had been identified and fully characterized, zinc finger nucleases were designed to bind to the sequences for each specific gene family member.

EXAMPLE 2: DESIGN OF ZINC FINGER BINDING DOMAINS SPECIFIC TO FAD2 GENES

Zinc finger proteins directed against DNA sequences encoding various functional sequences of the FAD2 gene locus were designed as previously described. *See, e.g.,* Urnov *et al.* (2005) Nature 435:646-651. Exemplary target sequence and recognition helices are shown in Table 6 and Table 8 (recognition helix regions designs) and Table 7 and Table 9 (target sites). In Table 8 and Table 9, nucleotides in the target site that are contacted by the ZFP recognition helices are indicated in uppercase letters; non-contacted nucleotides indicated in lowercase. Zinc Finger Nuclease (ZFN) target sites were designed to bind five target sites of FAD2A, and seven target sites of FAD3. The FAD2 and FAD3 zinc finger designs were incorporated into zinc finger expression vectors encoding a protein having at least one finger with a CCHC structure. *See*, U.S. Patent Publication No. 2008/0182332. In particular, the last finger in each protein had a CCHC backbone for the recognition helix. The non-canonical zinc finger-encoding sequences were fused to the nuclease domain of the type IIS restriction enzyme FokI (amino acids 384-579 of the sequence of Wah *et al.*, (1998) Proc. Natl. Acad. Sci. USA 95:10564-10569) via a four amino acid ZC linker and an *opaque-2* nuclear localization signal derived from *Zea mays* to form FAD2A zinc-finger nucleases (ZFNs). Expression of the fusion proteins was driven by a relatively strong constitutive promoter such as a promoter derived from the Cassava Vein Mosaic Virus (CsVMV) promoter and flanked by the *Agrobacterium tumefaciens* ORF23 3′ UnTranslated Region (AtuORF23 3′UTR v1). The self-hydrolyzing 2A encoding nucleotide sequence from *Thosea asigna* virus (Szymczak *et al.,* 2004) was added between the two Zinc Finger Nuclease fusion proteins that were cloned into the construct. Exemplary vectors are described below.

The optimal zinc fingers were verified for cleavage activity using a budding yeast based system previously shown to identify active nucleases. *See, e.g.,* U.S. Patent Publication No. 20090111119; Doyon *et al.* (2008) *Nat Biotechnol.* 26:702–708; Geurts *et al.* (2009) *Science* 325:433. Zinc fingers for the various functional domains were selected for *in-vivo* use. Of the numerous ZFNs that were designed, produced and tested to bind to the putative FAD genomic polynucleotide target sites, a ZFNs were identified as having *in vivo* activity at high levels, and selected for further experimentation. These ZFNs were characterized as being capable of efficiently binding and cleaving the unique FAD2 genomic polynucleotide target sites *in planta*.

Table 6: FAD3 Zinc Finger Designs

ZFP	F1	F2	F3	F4	F5	F6
27961	RSDNLAR (SEQ ID NO: 178)	QKKDRSY (SEQ ID NO:179)	RSDNLAR (SEQ ID NO:180)	QRGNRNT (SEQ ID NO:181)	RSDHLSR (SEQ ID NO:182)	RNQDRTN (SEQ ID NO:183)
27962	DRSNLSR (SEQ ID NO:184)	RQDSRSQ (SEQ ID NO:185)	QSSDLSR (SEQ ID NO:186)	DRSALAR (SEQ ID NO:187)	TSGSLTR (SEQ ID NO:188)	N/A
27973	QSSDLSR (SEQ ID NO:189)	AASNRSK (SEQ ID NO:190)	TSGSLSR (SEQ ID NO:191)	RSDALAR (SEQ ID NO:192)	RSDVLST (SEQ ID NO:193)	WGRLRKL (SEQ ID NO:194)
27974	ERGTLAR (SEQ ID NO:195)	RSDDLTR (SEQ ID NO:196)	RSDHLSA (SEQ ID NO:197)	QHGALQT (SEQ ID NO:198)	TSGNLTR (SEQ ID NO:199)	QSGHLSR (SEQ ID NO:200)
27987	TSGSLTR (SEQ ID NO:201)	RSDHLSQ (SEQ ID NO:202)	CTRNRWR (SEQ ID NO:203)	RSDNLSE (SEQ ID NO:204)	ASKTRKN (SEQ ID NO:205)	N/A
27990	TSGSLSR (SEQ ID NO:206)	TSSNRAV (SEQ ID NO:207)	TSGNLTR (SEQ ID NO:208)	DRSALAR (SEQ ID NO:209)	RSDVLSE (SEQ ID NO:210)	RNFSLTM (SEQ ID NO:211)
27991	QSGDLTR (SEQ ID NO:212)	TSGSLSR (SEQ ID NO:213)	QSGNLAR (SEQ ID NO:214)	TSGSLSR (SEQ ID NO:215)	QSGSLTR (SEQ ID NO:216)	N/A
27992	DRSHLAR (SEQ ID NO:217)	TSGSLSR (SEQ ID NO:218)	TSSNRAV (SEQ ID NO:219)	TSGNLTR (SEQ ID NO:220)	DRSALAR (SEQ ID NO:221)	N/A
28004	QSGNLAR (SEQ ID NO:222)	HLGNLKT (SEQ ID NO:223)	RSDHLSQ (SEQ ID NO:224)	TARLLKL (SEQ ID NO:225)	QSGNLAR (SEQ ID NO:226)	QTSHLPQ (SEQ ID NO:227)
28005	RSDNLSV (SEQ ID NO:228)	TSGHLSR (SEQ ID NO:229)	TSGSLTR (SEQ ID NO:230)	RSDALST (SEQ ID NO:231)	DRSTRTK (SEQ ID NO:232)	N/A
28021	QNAHRKT (SEQ ID NO:233)	TSGNLTR (SEQ ID NO:234)	LKQMLAV (SEQ ID NO:235)	RSDNLSR (SEQ ID NO:236)	DNSNRKT (SEQ ID NO:237)	N/A
28022	RSDNLSV (SEQ ID NO:238)	QNANRIT (SEQ ID NO:239)	TSGSLSR (SEQ ID NO:240)	QSSVRNS (SEQ ID NO:241)	DRSALAR (SEQ ID NO:242)	N/A
28023	RSDNLSR (SEQ ID NO:243)	DNSNRKT (SEQ ID NO:244)	DRSNLTR (SEQ ID NO:245)	RSDVLSE (SEQ ID NO:246)	TRNGLKY (SEQ ID NO:247)	N/A
28024	RSDALAR (SEQ ID NO:248)	RSDVLSE (SEQ ID NO:249)	RSSDRTK (SEQ ID NO:250)	RSDNLSV (SEQ ID NO:251)	QNANRIT (SEQ ID NO:252)	N/A
28025	QSSDLSR (SEQ ID NO:253)	QSTHRNA (SEQ ID NO:254)	RSDNLAR (SEQ ID NO:255)	QRGNRNT (SEQ ID NO:256)	RSDHLSR (SEQ ID NO:257)	RNQDRTN (SEQ ID NO:258)

ZFP	F1	F2	F3	F4	F5	F6
28026	DRSNLSR (SEQ ID NO:259)	RQDSRSQ (SEQ ID NO:260)	QSSDLSR (SEQ ID NO:261)	DRSALAR (SEQ ID NO:262)	TSGSLTR (SEQ ID NO:263)	N/A
28035	QSSDLSR (SEQ ID NO:264)	AASNRSK (SEQ ID NO:265)	TSGSLSR (SEQ ID NO:266)	RSDALAR (SEQ ID NO:267)	RSDTLSQ (SEQ ID NO:268)	QRDHRIK (SEQ ID NO:269)
28036	RSDDLTR (SEQ ID NO:270)	QSSDLRR (SEQ ID NO:271)	RSDHLSA (SEQ ID NO:272)	QHGALQT (SEQ ID NO:273)	TSGNLTR (SEQ ID NO:274)	QSGHLSR (SEQ ID NO:275)
28039	TSGSLSR (SEQ ID NO:276)	RSDALAR (SEQ ID NO:277)	RSDTLSQ (SEQ ID NO:278)	QRDHRIK (SEQ ID NO:279)	TSGNLTR (SEQ ID NO:280)	DRGDLRK (SEQ ID NO:281)
28040	DSSDRKK (SEQ ID NO:282)	TSGNLTR (SEQ ID NO:283)	DNYNRAK (SEQ ID NO:284)	DRSHLTR (SEQ ID NO:285)	RSDNLTT (SEQ ID NO:286)	N/A
28051	RSDNLSN (SEQ ID NO:287)	TSSSRIN (SEQ ID NO:288)	RSDNLSE (SEQ ID NO:289)	ASKTRKN (SEQ ID NO:290)	RSDALTQ (SEQ ID NO:291)	N/A
28052	RSDTLST (SEQ ID NO:292)	DRSSRIK (SEQ ID NO:293)	RSDDLSK (SEQ ID NO:294)	DNSNRIK (SEQ ID NO:295)	N/A	N/A
28053	QSSDLSR (SEQ ID NO:296)	QAGNLSK (SEQ ID NO:297)	QSGDLTR (SEQ ID NO:298)	TSGSLSR (SEQ ID NO:299)	QSGNLAR (SEQ ID NO:300)	N/A
28054	TSGSLSR (SEQ ID NO:301)	LRQTLRD (SEQ ID NO:302)	TSGNLTR (SEQ ID NO:303)	DRSALAR (SEQ ID NO:304)	RSDVLSE (SEQ ID NO:305)	RNFSLTM (SEQ ID NO:306)
28055	QSGDLTR (SEQ ID NO:307)	TSGSLSR (SEQ ID NO:308)	QSGNLAR (SEQ ID NO:309)	TSGSLSR (SEQ ID NO:310)	QSGSLTR (SEQ ID NO:311)	N/A
28056	DRSHLAR (SEQ ID NO:312)	TSGSLSR (SEQ ID NO:313)	LRQTLRD (SEQ ID NO:314)	TSGNLTR (SEQ ID NO:315)	DRSALAR (SEQ ID NO:316)	N/A

Table 7: Target Sites of FAD3 Zinc Fingers

ZFP	Target Site (5′ to 3′)	SEQ ID NO:
27961	cgCCGGAGAAAGAGAGAGAGctttgagg	SEQ ID NO:36
27962	tgGTTGTCGCTATGGACcagcgtagcaa	SEQ ID NO:37
27969	tcTCCGTTcGCATTGcTACGCTggtcca	SEQ ID NO:38
27970	gaAAGGTTtGATCCGAGCGCAcaaccac	SEQ ID NO:39
27973	ctTGAACGGTGGTTgTGCGCTcggatca	SEQ ID NO:40
27974	tcGGAGATATAAGGGCGGCCattcctaa	SEQ ID NO:41
27987	taGCCCAGAACAGGGTTccttgggcggc	SEQ ID NO:42
27988	ctTCGTACTCGGCCACGactggtaattt	SEQ ID NO:43
27989	ttGAAGTTGCAaTAAGCTttctctcgct	SEQ ID NO:44
27990	acTTGCTGGTCGATCATGTTggccactc	SEQ ID NO:45
27991	aaGTAGTTGAAGTTGCAataagctttct	SEQ ID NO:46
27992	tgGTCGATCATGTTGGCcactcttgttt	SEQ ID NO:47
28004	aaCGAGAATGAAGGAATGAAgaatatga	SEQ ID NO:48
28005	atACCATGGTTGGTAAGtcatttatttt	SEQ ID NO:49
28021	ccAACGAGgAATGATAGAtaaacaagag	SEQ ID NO:50
28022	caGTCACAGTTcTAAAAGtctatggtgt	SEQ ID NO:51
28023	tgTGACTGGACcAACGAGgaatgataga	SEQ ID NO:52
28024	tcTAAAAGTCTATGGTGttccttacatt	SEQ ID NO:53
28025	cgCCGGAGAAAGAGAGAGCTttgaggga	SEQ ID NO:54
28026	tgGTTGTCGCTATGGACcagcgtagcaa	SEQ ID NO:55
28035	ctTAAACGGTGGTTgTGCGCTcggatca	SEQ ID NO:56
28036	tcGGAGATATAAGGGCTGCGattcctaa	SEQ ID NO:57
28039	tcTCCGATctTAAACGGTGGTTgtgcgc	SEQ ID NO:58
28040	atAAGGGCTGCGATTCCtaagcattgtt	SEQ ID NO:59
28051	agATGGCCCAGAAAAGGgttccttgggc	SEQ ID NO:60
28052	cgTACTCGGCCACGactggtaatttaat	SEQ ID NO:61
28053	ttGAAGTTGCAaTAAGCTttctctcgct	SEQ ID NO:62
28054	acTTGCTGGTCGATCGTGTTggccactc	SEQ ID NO:63
28055	aaGTAGTTGAAGTTGCAataagctttct	SEQ ID NO:64
28056	tgGTCGATCGTGTTGGCcactcttgttt	SEQ ID NO:65

Table 8: FAD2 Zinc Finger Designs

ZFP	F1	F2	F3	F4	F5	F6
24800	RSDNLST (SEQ ID NO:317)	HSHARIK (SEQ ID NO:318)	HRSSLRR (SEQ ID NO:319)	RSDHLSE (SEQ ID NO:320)	QNANRIT (SEQ ID NO:321)	N/A
24801	DRSNLSR (SEQ ID NO:322)	HRSSLRR (SEQ ID NO:323)	TSGNLTR (SEQ ID NO:324)	MSHHLRD (SEQ ID NO:325)	DQSNLRA (SEQ ID NO:326)	N/A
24794	QSGNLAR (SEQ ID NO:327)	RSDNLSR (SEQ ID NO:328)	DNNARIN (SEQ ID NO:329)	DRSNLSR (SEQ ID NO:330)	RSDHLTQ (SEQ ID NO:331)	N/A
24795	RSDNLRE (SEQ ID NO:332)	QSGALAR (SEQ ID NO:333)	QSGNLAR (SEQ ID NO:334)	RSDVLSE (SEQ ID NO:335)	SPSSRRT (SEQ ID NO:336)	N/A
24810	RSDSLSR (SEQ ID NO:337)	RKDARIT (SEQ ID NO:338)**	RSDHLSA (SEQ ID NO:339)**	WSSSLYY (SEQ ID NO:340)**	NSRNLRN (SEQ ID NO:341)**	N/A
24811	DQSTLRN (SEQ ID NO:342)	DRSNLSR (SEQ ID NO:343)	DRSNLWR (SEQ ID NO:344)	DRSALSR (SEQ ID NO:345)	RSDALAR (SEQ ID NO:346)	N/A
24814	RSDALSR (SEQ ID NO:347)	DRSDLSR (SEQ ID NO:348)	RSDHLTQ (SEQ ID NO:349)	QSGALAR (SEQ ID NO:350)	QSGNLAR (SEQ ID NO:351)	N/A
24815	DRSNLSR (SEQ ID NO:352)	DSSARNT (SEQ ID NO:353)	DRSSRKR (SEQ ID NO:354)	QSGDLTR (SEQ ID NO:355)	LAHHLVQ (SEQ ID NO:356)	N/A
24818	RSDNLST (SEQ ID NO:357)	HSHARIK (SEQ ID NO:358)	TSGHLSR (SEQ ID NO:359)	RSDNLSV (SEQ ID NO:360)	IRSTLRD (SEQ ID NO:361)	N/A
24819	TSGHLSR (SEQ ID NO:362)	DRSNLSR (SEQ ID NO:363)	HRSSLRR (SEQ ID NO:364)	TSGNLTR (SEQ ID NO:365)	MSHHLRD (SEQ ID NO:366)	N/A
24796	RSDALSR (SEQ ID NO:367)	DRSDLSR (SEQ ID NO:368)	RSDHLTQ (SEQ ID NO:369)	QSGALAR (SEQ ID NO:370)	QSGNLAR (SEQ ID NO:371)	N/A
24797	RSAVLSE (SEQ ID NO:372)	TNSNRIT (SEQ ID NO:373)	LKQHLNE (SEQ ID NO:374)	QSGALAR (SEQ ID NO:375)	QSGNLAR (SEQ ID NO:376)	N/A
24836	DRSNLSR (SEQ ID NO:377)	QSGDLTR (SEQ ID NO:378)	QSGALAR (SEQ ID NO:379)	DRSNLSR (SEQ ID NO:380)	QRTHLTQ (SEQ ID NO:381)	N/A
24837	RSDNLSN (SEQ ID NO:382)	TNSNRIK (SEQ ID NO:383)	QSSDLSR (SEQ ID NO:384)	QSSDLRR (SEQ ID NO:385)	DRSNRIK (SEQ ID NO:386)	N/A
24844	RSANLAR (SEQ ID NO:387)	RSDNLTT (SEQ ID NO:388)	QSGELIN (SEQ ID NO:389)	RSADLSR (SEQ ID NO:390)	RSDNLSE (SEQ ID NO:391)	DRSHLAR (SEQ ID NO:392)

ZFP	F1	F2	F3	F4	F5	F6
24845	DRSHLAR (SEQ ID NO:393)	RSDNLSE (SEQ ID NO:394)	SKQYLIK (SEQ ID NO:395)	ERGTLAR (SEQ ID NO:396)	RSDHLTT (SEQ ID NO:397)	N/A
24820	QSGALAR (SEQ ID NO:398)	QSGNLAR (SEQ ID NO:399)	DRSHLAR (SEQ ID NO:400)	DRSDLSR (SEQ ID NO:401)	RSDNLTR (SEQ ID NO:402)	N/A
24821	DRSHLAR (SEQ ID NO:403)	RSDNLSE (SEQ ID NO:404)	SKQYLIK (SEQ ID NO:405)	ERGTLAR (SEQ ID NO:406)	RSDHLTT (SEQ ID NO:407)	N/A
24828	DRSDLSR (SEQ ID NO:408)	RSDNLTR (SEQ ID NO:409)	QRTHLTQ (SEQ ID NO:410)	RSDNLSE (SEQ ID NO:411)	ASKTRKN (SEQ ID NO:412)	N/A
24829	RSDTLSE (SEQ ID NO:413)	QSHNRTK (SEQ ID NO:414)	QSDHLTQ (SEQ ID NO:415)	RSSDLSR (SEQ ID NO:416)	QSSDLSR (SEQ ID NO:417)	RSDHLTQ (SEQ ID NO:418)
24832	RSDSLSR (SEQ ID NO:419)	RKDARIT (SEQ ID NO:420)	DRSHLSR (SEQ ID NO:421)	QSGNLAR (SEQ ID NO:422)	QSSDLSR (SEQ ID NO:423)	DRSALAR (SEQ ID NO:424)
24833	RSDDLSK (SEQ ID NO:425)	RSDTRKT (SEQ ID NO:426	DRSNLSR (SEQ ID NO:427)	DRSNLWR (SEQ ID NO:428)	RSDSLSR (SEQ ID NO:429)	NNDHRKT (SEQ ID NO:430)

Table 9: Target Sites of FAD2 Zinc Fingers

ZFP	Plasmid No.	Target Site (5′ to 3′)	ZFP target/binding site present in SEQ ID Nos.
24800	pDAB104001	ccCAAAGGGTTGTTGAGgtacttgccgt	SEQ ID NO:66
24801	pDAB104001	cgCACCGTGATGTTAACggttcagttca	SEQ ID NO:67
24794	pDAB104002	taAGGGACGAGGAGGAAggagtggaaga	SEQ ID NO:68
24795	pDAB104002	ttCTCCTGGAAGTACAGtcatcgacgcc	SEQ ID NO:69
24810	pDAB104003	gtCGCTGAAGGcGTGGTGgccgcactcg	SEQ ID NO:70
24811	pDAB104003	caGTGGCTgGACGACACCgtcggcctca	SEQ ID NO:71
24814	pDAB104004	gaGAAGTAAGGGACGAGgaggaaggagt	SEQ ID NO:72
24815	pDAB104004	gaAGTACAGTCATCGACgccaccattcc	SEQ ID NO:73
24818	pDAB104005	tcCCAAAGGGTtGTTGAGgtacttgccg	SEQ ID NO:74
24819	pDAB104005	acCGTGATGTTAACGGTtcagttcactc	SEQ ID NO:75
24796	pDAB104006	gaGAAGTAAGGGACGAGgaggaaggagt	SEQ ID NO:76
24797	pDAB104006	tgGAAGTAcAGTCATCGAcgccaccatt	SEQ ID NO:77
24836	pDAB104007	gtAGAGACcGTAGCAGACggcgaggatg	SEQ ID NO:78
24837	pDAB104007	gcTACGCTGCTgTCCAAGgagttgcctc	SEQ ID NO:79
24844	pDAB104008	gaGGCCAGGCGAAGTAGGAGagagggtg	SEQ ID NO:80
24845	pDAB104008	acTGGGCCTGCCAGGGCtgcgtcctaac	SEQ ID NO:81
24820	pDAB104009	gaGAGGCCaGGCGAAGTAggagagaggg	SEQ ID NO:82
24821	pDAB104009	acTGGGCCTGCCAGGGCtgcgtcctaac	SEQ ID NO:83

ZFP	Plasmid No.	Target Site (5′ to 3′)	ZFP target/binding site present in SEQ ID Nos.
24828	pDAB104010	agGCCCAGtAGAGAGGCCaggcgaagta	SEQ ID NO:84
24829	pDAB104010	ccAGGGCTGCGTCCTAACCGgcgtctgg	SEQ ID NO:85
24832	pDAB104011	taGTCGCTGAAGGCGTGGTGgccgcact	SEQ ID NO:86
24833	pDAB104011	agTGGCTGGACGACaCCGTCGgcctcat	SEQ ID NO:87

EXAMPLE 3: EVALUATION OF ZINC FINGER NUCLEASE CLEAVAGE OF FAD2 GENES

CONSTRUCT ASSEMBLY

Plasmid vectors containing ZFN expression constructs of the exemplary zinc finger nucleases, which were identified using the yeast assay, as described in Example 2, were designed and completed using skills and techniques commonly known in the art. Each zinc finger-encoding sequence was fused to a sequence encoding an opaque-2 nuclear localization signal (Maddaloni *et al.* (1989) *Nuc. Acids Res.* 17(18):7532), that was positioned upstream of the zinc finger nuclease.

Next, the opaque-2 nuclear localization signal::zinc finger nuclease fusion sequence was paired with the complementary opaque-2 nuclear localization signal::zinc finger nuclease fusion sequence. As such, each construct consisted of a single open reading frame comprised of two opaque-2 nuclear localization signal::zinc finger nuclease fusion sequences separated by the 2A sequence from *Thosea asigna* virus (Mattion *et al.* (1996) *J. Virol.* 70:8124-8127). Expression of the fusion proteins was driven by a relatively strong constitutive promoter such as a promoter derived from the Cassava Vein Mosaic Virus (CsVMV) promoter and flanked by the *Agrobacterium tumefaciens* ORF23 3′ UnTranslated Region (AtuORF23 3′UTR).

The vectors were assembled using the IN-FUSION™ Advantage Technology (Clontech, Mountain View, CA). Restriction endonucleases were obtained from New England BioLabs (NEB; Ipswich, MA) and T4 DNA Ligase (Invitrogen) was used for DNA ligation. Plasmid preparations were performed using NUCLEOSPIN® Plasmid Kit (Macherey-Nagel Inc., Bethlehem, PA) or the Plasmid Midi Kit (Qiagen) following the instructions of the suppliers. DNA fragments were isolated using QIAquick Gel Extraction Kit™ (Qiagen) after agarose Tris-acetate gel electrophoresis. Colonies of all assembled plasmids were initially screened by restriction digestion of miniprep DNA. Plasmid DNA of selected clones was sequenced by a commercial sequencing vendor

(Eurofins MWG Operon, Huntsville, AL). Sequence data were assembled and analyzed using the SEQUENCHER™ software (Gene Codes Corp., Ann Arbor, MI). Before delivery to *B. napus* protoplasts, Plasmid DNA was prepared from cultures of *E. coli* using the Pure Yield PLASMID MAXIPREP System® (Promega Corporation, Madison, WI) or PLASMID MAXI KIT® (Qiagen, Valencia, CA) following the instructions of the suppliers.

The resulting eleven plasmid constructs; pDAB104008 (containing the ZFN24845 and ZFN24844 construct), pDAB104009 (containing the ZFN24820 and ZFN24821 construct), pDAB104010 (containing the ZFN24828 and ZFN24829 construct) (FIG. 5), pDAB104003 (containing the ZFN24810 and ZFN24811 construct), pDAB104011 (containing the ZFN24832 and ZFN24833 construct), pDAB104002 (containing the ZFN24794 and ZFN24795 construct), pDAB104006 (containing the ZFN24796 and ZFN24797 construct), pDAB104004 (containing the ZFN24814 and ZFN24815 construct), pDAB104001 (containing the ZFN24800 and ZFN24801 construct), pDAB104005 (containing the ZFN24818 and ZFN24819 construct), and pDAB104007 (containing the ZFN24836 and ZFN24837 construct) were confirmed via restriction enzyme digestion and via DNA sequencing. Table 10 lists the different constructs and the specific FAD sequence which each ZFN was designed to cleave and bind.

The resulting plasmid constructs; pDAB107824 (ZFNs 28025-2A-28026), pDAB107815 (ZFNs 27961-2A-27962), pDAB107816 (ZFNs 27969-2A-27970), pDAB107817 (ZFNs 27973-2A-27974), pDAB107825 (ZFNs 28035-2A-28036), pDAB107826 (ZFNs 28039-2A-28040), pDAB107818 (ZFNs 27987-2A-27988), pDAB107827 (ZFNs 28051-2A-28052), pDAB107821 (ZFNs 28004-2A-28005), pDAB107819 (ZFNs 27989-2A-27990), pDAB107828 (ZFNs 28053-2A-28054), pDAB107829 (ZFNs 28055-2A-28056), pDAB107820 (ZFNs 27991-2A-27992), pDAB107822 (ZFNs 28021-2A-28022) and pDAB107823 (ZFNs 28023-2A-28024) were confirmed via restriction enzyme digestion and via DNA sequencing.

Table 10: lists the Zinc Finger protein binding motif and the corresponding construct number. Each Zinc Finger was designed to bind and cleave the FAD2A which is described in the table.

ZFN Design	Construct No.	Locus ID.	Target Cut Site in FAD2A Sequence
24844-2A-24845	pDAB104008	FAD2_ZFN_Locus1_F2A	263-265
24820-2A-24821	pDAB104009	FAD2_ZFN_Locus1_F2B	265
24828-2A-24829	pDAB104010	FAD2_ZFN_Locus1_F2C	275
24810-2A-24811	pDAB104003	FAD2_ZFN_Locus2_F1D	343-345
24832-2A-24833	pDAB104011	FAD2_ZFN_Locus2_F1E	345-346
24794-2A-24795	pDAB104002	FAD2_ZFN_Locus3_F2F	402
24796-2A-24797	pDAB104006	FAD2_ZFN_Locus3_F2G	408
24814-2A-24815	pDAB104004	FAD2_ZFN_Locus3_F2H	408-410
24800-2A-24801	pDAB104001	FAD2_ZFN_Locus4_F1J	531
24818-2A-24819	pDAB104005	FAD2_ZFN_Locus4_F1K	532-534
24836-2A-24837	pDAB104007	FAD2_ZFN_Locus5_F1L	724

PREPARATION OF DNA FOR TRANSFECTION

Plasmid DNA of the above described vectors was sterilized by precipitation and washing in 100% (v/v) ethanol and dried in a laminar flow hood. The DNA pellet was suspended in 30 µL of sterile double-distilled water at a final concentration of 0.7 µg/µl for transfection into protoplast cells as described below. The preparation of the plasmid DNA was undertaken to result in supercoiled plasmid DNA for transient transfection and linearized plasmid DNA for stable transfection. The addition of carrier DNA (e.g. fish-sperm DNA) to the transforming plasmid was not required for the transient transfection of protoplast cells. For transient studies about 30 µg of plasmid DNA per 10^6 protoplasts was used per transformation.

TRANSFECTION

Transfection of *Brassica napus* L. var. DH10275 was completed as described in Spangenberg *et al.*, (1986) Plant Physiology 66: 1-8, the media formulations are described in Spangenberg G. and Protrykus I. (1995) Polyethylene Glycol-Mediated Direct Gene Transfer in Tobacco Protoplasts. In*: Gene Transfer to Plants*. (Protrykus I. and Spangenberg G. Eds.) Springer-Verlag, Berlin. *Brassica napus* seeds were surface sterilized in 70% ethanol. The seeds were immersed in 12 mL of the 70% ethanol solution and mixed by gently rocking the cocktail for 10 minutes. The 70% ethanol

solution was removed by decanting the solution and exchanged with a seed sterilization solution consisting of 1% w/v calcium hypochlorite and 0.1% v/v Tween-20. The seeds were immersed in the seed sterilization solution and mixed by gently rocking the cocktail for 25 minutes. The seed sterilization solution was decanted and the sterilized seeds were rinsed three times in 50 mL of sterile water. Finally, the seeds were transferred to a sterile 80 mm WHATMAN® filter paper disc (Fisher-Scientific, St. Louis, MO) that had been laid within a Petri dish and the seeds were lightly saturated with sterile water. The Petri dish was sealed with PARAFILM® (Fisher-Scientific, St. Louis, MO) and the plates were incubated at 25°C under complete darkness for one to two days. After signs of seedling emergence were observed from the seeds, the seedlings were transferred to Petri dish containing solidified GEM medium to encourage further seed germination. The seedlings were incubated on the GEM medium at 25°C for four to five days.

A volume of liquid PS medium (about 10 mL) was decanted into a sterile Petri dish. Using sterile forceps and a scalpel, an aerial portion of the four to five day old seedling in the 4-leaf stage of growth and development, was removed and discarded. Hypocotyl segments in lengths of 20-40 mm were determined to produce the highest population of small, cytoplasmic-rich protoplasts. The hypocotyl segments were aseptically excised and transferred to liquid PS medium. The excised hypocotyl segments were grouped together and cut transversely into 5-10 mm segments. Next, the hypocotyl segments were transferred to fresh PS medium and incubated at room temperature for 1 hour. The plasmolyzed hypocotyls were transferred to a Petri dish containing enzyme solution. Care was taken to immerse all of the hypocotyl segments into the solution. The Petri dishes were sealed with PARAFILM® and incubated overnight for sixteen to eighteen hours at 20 - 22°C with gentle rocking.

Protoplast cells were released from the hypocotyl segments. The overnight hypocotyl digests were gently agitated to release protoplasts into the enzyme solution. The Petri dish was angled slightly to aid the transfer of the digesting suspension which consisted of enzyme solution and plant debris. Using a 10 mL pipette the digesting suspension was transferred to a sterilized protoplast filtration (a filter of 100 micron mesh) unit to further separate the protoplasts from the plant debris. The filtration unit was tapped gently to release the excess liquid that had been caught in the sieve. The protoplast suspension, about 8 to 9 mL, was gently mixed and distributed into 14 mL

sterile plastic round-bottomed centrifuge tubes. Each suspension was overlaid with 1.5 mL of W5 solution. The W5 solution was carefully dispensed over the protoplast suspension at an angle and dispensed drop-by-drop with minimal agitation. The addition of the W5 solution to the protoplast suspension resulted in the production of a protoplast rich interface. This interface was collected using a pipette. Next, the collected protoplasts were transferred into a new 14 mL centrifuge tube, and gently mixed. The yield or obtained protoplasts were determined using a hemocytometer to determine the number of protoplasts per milliliter. The method was repeated, wherein leaf tissue was digested to produce mesophyll protoplasts.

Next, W5 solution was added to a volume of 10 mL and the protoplasts were pelleted at 70 g, before removing the W5 solution. The remaining protoplast suspension was resuspended by gentle shaking. Each tube containing the protoplast suspension was filled with 5 mL of W5 solution and incubated at room temperature from one to four hours. The protoplast suspensions were pelleted at 70 g, and all of the W5 solution was removed. Next, 300 µL of transformation buffer was added to each of the pelleted protoplast suspensions which contained the isolated protoplasts. To each of the tubes, 10 µg of plasmid DNA was added to the protoplast suspensions. The plasmid DNA consisted of the Zinc Finger Nuclease constructs described above (*e.g.*, pDAB104010). Next, 300 µL of pre-warmed PEG 4000 solution was added to the protoplast suspension and the tubes were gently tapped. The protoplast suspensions and transformation mixture was allowed to incubate at room temperature for fifteen minutes without any agitation. An additional 10 mL of W5 solution was added to each tube in sequential aliquots of 1 mL, 1 mL, 1 mL, 2 mL, 2 mL, and 3 mL with gentle inversion of the tubes between each addition of W5 solution. The protoplasts were pelleted by spinning in a centrifuge at 70 g. All of the W5 solution was removed leaving a pure protoplast suspension.

Next, 0.5 mL of K3 medium was added to the pelleted protoplast cells and the cells were resuspended. The resuspended protoplast cells were placed in the center of a Petri dish and 5 mL of K3 and 0.6 mL Sea Plaque™ agarose (Cambrex, East Rutherford, NJ) in a 1:1 concentration. The Petri dishes were shaken in a single gentle swirling motion and left to incubate for 20-30 minutes at room temperature. The Petri dishes were sealed with PARAFILM® and the protoplasts were cultured for twenty-four hours in complete darkness. After the incubation in darkness, the Petri dishes were

cultured for six days in dim light (5 μMol m^{-2} s^{-1} of Osram L36 W/21 Lumilux white tubes). After the culture step, a sterile spatula was used to divide the agarose containing the protoplasts into quadrants. The separated quadrants were placed into a 250 mL plastic culture vessel containing 20 mL of A medium and incubated on a rotary shaker at 80 rpm and 1.25 cm throw at 24 °C in continuous dim light for 14 days and then analyzed to determine the level of activity of each Zinc Finger Nuclease construct.

GENOMIC DNA ISOLATION FROM CANOLA PROTOPLASTS

Transfected protoplasts were supplied in individual 1.5 or 2.0 mL microfuge tubes. The cells were pelleted at the base of the tube in a buffer solution. DNA extraction was carried out by snap freezing the cells in liquid nitrogen followed by freeze drying the cells, for about 48 hours in A LABCONCO FREEZONE 4.5® (Labconco, Kansas City, MO) at -40°C and about 133 x 10^{-3} mBar pressure. The lyophilized cells were subjected to DNA extraction using the DNEASY® (QIAGEN, Carlsbad, CA) plant kit following manufactures instructions, with the exception that tissue disruption was not required and the protoplast cells were added directly to the lysis buffer.

TESTING OF FAD2A AND FAD3 ZFNs FOR GENOMIC DNA SEQUENCE CLEAVAGE IN CANOLA PROTOPLASTS

The design of the ZFN target sites for the FAD2A and FAD3 gene loci were clustered, so that multiple pairs of ZFN were design to overlap the target sites. The clustering of ZFN target sites enabled PCR primers to be designed that would amplify the surrounding genomic sequence from all FAD2A and FAD3 gene family members within a 100 bp window as to encapsulate all of the overlapping ZFN target sites. As such, the Illumina short read sequence technology could be used to assess the integrity of the target ZFN site of the transfected protoplasts. In addition, the PCR primers designed needed to include specific nucleotide bases that would attribute sequence reads to the specific gene member of the FAD2A and FAD3 family. Therefore, all of the PCR primers would be required to bind 5-10 nucleotides away from any ZFN target cut site as non-homologous end joining (NHEJ) activity is known to cause small deletions that could remove a priming site, inhibit amplification and therefore distort the assessment of NHEJ activity.

Primers were designed to bind to all of the ZFN target loci for the FAD2A and FAD3 gene families (Table 11) and were empirically tested for amplification of all gene family members through Sanger based sequencing of PCR amplification products. In several instances primers could not be developed that would distinguish all gene family members (Table 12 and Table 13), however in all instances the target gene sequences of FAD2A and FAD3, could be distinguished. Following PCR primer design custom DNA barcode sequences were incorporated into the PCR primers that were used to distinguish the different ZFN target loci and identify specific sequence reads to a transfection and ZFN (Tables 11, 12 and 13).

Table 11: Primer sequences designed for FAD2 and FAD3 ZFN assessment of activity. Primers include custom barcodes, along with both requisite Illumina adaptor sequences for construction of Illumina library for sequencing-by-synthesis analysis. Purchased primer was the sum of all three columns presented.

Locus ID	SEQ ID NO:	Illumina Adaptor Primer Sequence	Barcode	Locus Primer
FAD2_ZFN_Locus1_F	88	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CGGG	CCCTCTCYCYTACYTCGCC
FAD2_ZFN_Locus1_F2A	89	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	ACGTA	CCCTCTCYCYTACYTCGCC
FAD2_ZFN_Locus1_F2B	90	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CGTAC	CCCTCTCYCYTACYTCGCC
FAD2_ZFN_Locus1_F2C	91	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GTACG	CCCTCTCYCYTACYTCGCC
FAD2_ZFN_Locus2_F1D	92	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TACGT	GTCATAGCCCACGAGTGCGGC
FAD2_ZFN_Locus2_F1E	93	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CTGAC	GTCATAGCCCACGAGTGCGGC
FAD2_ZFN_Locus3_F2F	94	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TGACT	GTCGGCCTCATCTTCCACTCC
FAD2_ZFN_Locus3_F2G	95	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GACTG	GTCGGCCTCATCTTCCACTCC
FAD2_ZFN_Locus3_F2H	96	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	ACTGA	GTCGGCCTCATCTTCCACTCC
FAD2_ZFN_Locus4_F1J	97	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GCTAG	CAGACATCAAGTGGTACGGC
FAD2_ZFN_Locus4_F1K	98	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CTAGC	CAGACATCAAGTGGTACGGC
FAD2_ZFN_Locus5_F1L	99	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TAGCT	ATCTCCGACGCTGGCATCCTC

Locus ID	SEQ ID NO:	Illumina Adaptor Primer Sequence	Barcode	Locus Primer
FAD2_ZFN_Locus1_R1A	100	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	ACGTA	CTGGTAGTCGCTGAAGGCGT
FAD2_ZFN_Locus1_R1B	101	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CGTAC	CTGGTAGTCGCTGAAGGCGT
FAD2_ZFN_Locus1_R1C	102	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GTACG	CTGGTAGTCGCTGAAGGCGT
FAD2_ZFN_Locus2_R1D	103	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TACGT	GGACGAGGAGGAAGGAGTGGA
FAD2_ZFN_Locus2_R1E	104	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CTGAC	GGACGAGGAGGAAGGAGTGGA
FAD2_ZFN_Locus3_R1F	105	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TGACT	AGTGTTGGAATGGTGGCGTCG
FAD2_ZFN_Locus3_R1G	106	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GACTG	AGTGTTGGAATGGTGGCGTCG
FAD2_ZFN_Locus3_R1H	107	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	ACTGA	AGTGTTGGAATGGTGGCGTCG
FAD2_ZFN_Locus4_R1J	108	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GCTAG	CCCGAGACGTTGAAGGCTAAG
FAD2_ZFN_Locus4_R1K	109	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CTAGC	CCCGAGACGTTGAAGGCTAAG
FAD2_ZFN_Locus5_R1L	110	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TAGCT	GAAGGATGCGTGTGCTGCAAG
FAD3_ZFN_Locus1A_F3	111	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	ACGTA	CCTTTCTTCACCACATTYCA
FAD3_ZFN_Locus1B_F3	112	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CGTAC	CCTTTCTTCACCACATTYCA
FAD3_ZFN_Locus2C_F1	113	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CTGAC	GATGGTTGTCGCTATGGACC
FAD3_ZFN_Locus3D_F1	114	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TGACT	CGAAAGGTTTGATCCRAGCG
FAD3_ZFN_Locus3E_F1	115	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GACTG	CGAAAGGTTTGATCCRAGCG
FAD3_ZFN_Locus3F_F1	116	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	ACTGA	CGAAAGGTTTGATCCRAGCG
FAD3_ZFN_Locus4G_F1	117	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GCTAG	CCGTGTATTTTGATAGCTGGTTC
FAD3_ZFN_Locus4H_F1	118	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CTAGC	CCGTGTATTTTGATAGCTGGTTC
FAD3_ZFN_Locus5J_F1	119	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TAGCT	GGAGCTTCTCAGACATTCCTCT
FAD3_ZFN_Locus6K_F1	120	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TCAGT	GTTTATTTGCCCCAAGCGAGAG
FAD3_ZFN_Locus6L_F1	121	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	CAGTC	GTTTATTTGCCCCAAGCGAGAG

Locus ID	SEQ ID NO:	Illumina Adaptor Primer Sequence	Barcode	Locus Primer
FAD3_ZFN_Locus6M_F1	122	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	AGTCA	GTTTATTTGCCCCAAGCGAGAG
FAD3_ZFN_Locus6N_F1	123	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GTCAG	GTTTATTTGCCCCAAGCGAGAG
FAD3_ZFN_Locus7P_F3	124	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	GTACG	ACTTCAACTACTTGCTGGTCSAT
FAD3_ZFN_Locus7Q_F3	125	ACACTCTTTCCCTACACGACGCTCTTCCGATCT	TACGT	ACTTCAACTACTTGCTGGTCSAT
FAD3_ZFN_Locus1A_R1	126	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	ACGTA	CGTTCACATTGSTRCGYTGG
FAD3_ZFN_Locus1B_R1	127	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CGTAC	CGTTCACATTGSTRCGYTGG
FAD3_ZFN_Locus2C_R1	128	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CTGAC	CCGATCTTAAACGGYGGTTGT
FAD3_ZFN_Locus3D_R1	129	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TGACT	TAGCTCATGGATCTCAAAGGACT
FAD3_ZFN_Locus3E_R1	130	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GACTG	TAGCTCATGGATCTCAAAGGACT
FAD3_ZFN_Locus3F_R1	131	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	ACTGA	TAGCTCATGGATCTCAAAGGACT
FAD3_ZFN_Locus4G_R_uni	132	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GCTAG	TTAAATTACCAGTCGTGGCC
FAD3_ZFN_Locus4H_R_uni	133	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CTAGC	TTAAATTACCAGTCGTGGCC
FAD3_ZFN_Locus5J_R2	134	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TAGCT	CTTTTTTCTTCGATKCTAAAGATT
FAD3_ZFN_Locus6K_R1	135	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TCAGT	CTGTGACTGGACCAACGAGG
FAD3_ZFN_Locus6L_R1	136	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	CAGTC	CTGTGACTGGACCAACGAGG
FAD3_ZFN_Locus6M_R1	137	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	AGTCA	CTGTGACTGGACCAACGAGG
FAD3_ZFN_Locus6N_R1	138	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GTCAG	CTGTGACTGGACCAACGAGG
FAD3_ZFN_Locus7P_R1	139	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	GTACG	ACTTACAATGTAAGGAACRCCRTA
FAD3_ZFN_Locus7Q_R1	140	CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT	TACGT	ACTTACAATGTAAGGAACRCCRTA

Table 12: Amplification performance of the designed PCR primers on the FAD2 gene families. An "X" indicates gene copy detection specificity, grey shading and an "+" indicates that at the specific locus in question the sequence reads designed by the two primers were unable to be distinguished and an "N/A" indicates that the locus was unable to be amplified from those specific gene copies.

ZFN Locus	FAD Gene Copy			
	FAD2A	FAD2C	FAD2A′	FAD2C′
Locus 1	X	X	X	X
Locus 2	X	X	X	X
Locus 3	+	+	X	X
Locus 4	X	X	X	X
Locus 5	X	X	X	X

Table 13: Amplification performance of the designed PCR primers on the FAD3 gene families. An "X" indicates gene copy detection specificity, grey shading and an "+" indicates that at the specific locus in question the sequence reads designed by the two primers were unable to be distinguished and an "N/A" indicates that the locus was unable to be amplified from those specific gene copies.

ZFN Locus	FAD Gene Copy					
	FAD3A	FAD3C	FAD3A′	FAD3C′	FAD3A″	FAD3C″
Locus 1	X	X	X	X	X	X
Locus 2	X	X	X	X	N/A	X
Locus 3	X	X	+	+	X	X
Locus 4	X	X	X	X	+	+
Locus 5	X	X	N/A	N/A	N/A	N/A
Locus 6	X	X	X	X	X	X
Locus 7	X	X	X	X	X	X

Following DNA extraction of canola protoplasts transfected with the ZFN, PCR amplification of the target ZFN loci was performed to generate the requisite loci specific DNA molecules in the correct format for Illumina based sequencing by synthesis technology. Each assay was optimised to work on 25 ng starting DNA (about 12,500 cell equivalents of the *Brassica napus* genome). Multiple reactions were performed, per

sample to provide the coverage required to assess NHEJ efficiency and specificity at the appropriate level, about sixteen PCR reactions equivalent to 200,000 copies of the *Brassica napus* genome taken from individual protoplasts. PCR amplification master-mixes were made for all samples to be tested with the same assay and one reaction, performed in triplicate, was assayed using a quantitative PCR method that was used to determine the optimal number of cycles to perform on the target tissue, to ensure that PCR amplification had not become reagent limited and was still in an exponential amplification stage. The experimentation with the necessary negative control reactions, was performed in 96 well format using a MX3000P THERMOCYCLER® (Stratagene, LaJolla, CA). From the output gathered from the quantitative PCR platform, the relative increase in fluorescence was plotted from cycle-to-cycle and the cycle number was determined per assay that would deliver sufficient amplification, while not allowing the reaction to become reagent limited, in an attempt to reduce over cycling and the amplification of common transcripts or molecules. The unused master mix, remained on ice until the quantitative PCR analysis was concluded and the cycle number determined and was then aliquoted into the desired number of reaction tubes (about 16 per ZFN assay) and the PCR reaction was performed. Following amplification, samples for a single ZFN locus were pooled together and 200 µL of pooled product per ZFN was cleaned using the MINELUTE® PCR purification kit (Qiagen) following manufacturer's instructions. To enable the sample to be sequenced using the Illumina short read technology additional paired end primers were required to be attached by amplification onto the generated fragments. This was achieved by PCR amplification using primers that would be, in part complementary to the sequence added in the first round of amplification, but also contain the paired end sequence required. The optimal number of PCR cycles to perform, that would add the paired end sequences without over amplifying common fragments to the template was again determined using a sample pass through a quantitative PCR cycle analysis, as described previously. Following PCR amplification, the generated product was cleaned using a MINELUTE® column (Qiagen) following manufacturer's instructions and was resolved on a 2.5% agarose gel. DNA fragments visualised using SYBER SAFE® (Life Technologies, Carlsbad, CA) as bands of the correct size were gel extracted to remove any residual PCR generated primer-dimer or other spurious fragments, the DNA was extracted from the gel slice using a MINELUTE® gel extraction kit (Qiagen) following manufacturer's instructions. After

completion of the gel extraction an additional clean up of the DNA was performed using AMPURE® magnetic beads (Beckman-Coulter, Brea, CA) with a DNA to bead ratio of 1:1.7. The DNA was then assessed for concentration using a quantitative PCR based library quantification kit for Illumina sequencing (KAPA) with a 1/40,000 and a 1/80,000 dilution and with the reaction being performed in triplicate. Based on the quantitative PCR results the DNA was diluted to a standard concentration of 2 nM and all libraries were combined for DNA sequencing. The samples were prepared for sequencing using a CBOT CLUSTER® generation kit (Illumina, San Diego, CA) and were sequenced on an ILLUMINA GA2x® with 100 bp paired-end sequencing reads following manufacturer's instructions.

METHOD OF DATA ANALYSIS FOR DETECTION OF NON-HOMOLOGOUS END JOINING AT TARGET ZINC FINGER SITES

Following completion of the sequencing reaction and primary data calling performed using the Illumina bioinformatic pipeline for base calling, full analysis was performed to identify deleted bases at the target ZFN site in each instance. A custom PERL script was designed to extract and sort barcodes from DNA sequences computationally following a list of input sequences. The barcode had to match the reference sequence at a Phred score of greater than 30 to be accepted, to reduce misattributing sequence reads. After the sequence reads had been binned into the different barcode groups that had been used, a quality filter was passed across all sequences. The quality filter was a second custom developed PERL script. Sequence reads were excluded if there were more than three bases called as "N," or if the median Phred score was less than 20, or if there were 3 consecutive bases with a Phred score of less than 20, or if the sequence read was shorter than 40 bp in length. The remaining sequences were merged where both of the paired sequence reads were available using the NEXTGENE® (SoftGenetics, State College, PA) package. The remaining merged sequence reads were then reduced to a collection of unique sequence reads using a third custom PERL script with a count of the number of redundant sequences that had been identified recorded on the end of the remaining sequence identifier. The unique sequence reads were then aligned to the FAD2 and FAD3 reference sequence using the NEXTGENE® software that created a gapped FASTA aligned file.

Using the gapped FASTA file a conversion of the gapped base position number to the input reference was performed using a fourth custom PERL script. This enabled bases that discriminate the different gene family members (either homoeologous or paralogous sequence variation between the different gene family members) to be identified in the assembled data. Once the conversion of base numbering had been performed it was possible to generate haplotype reports for each unique sequence reads and assign the reads to specific gene family members. Once the reads had been grouped by gene a 10 bp window was identified and assessed that surrounded the ZFN target site. The number of sequences with deletions was recorded per gene along with the number of missing bases.

The data was then graphically displayed as a multiple line graph, with the number of sequences with 1 through 10 bases deleted at the target ZFN site per 10,000 sequence reads (FIG. 6). This analysis was performed for all ZFN transfections along with control transfections. In several instances, repeats in the native DNA sequence lead to an increase in sequencing error in the target ZFN site, such an error can be commonly seen as an increase in the prevalence of single base deletions that were reported in all samples, both transfected with ZFN or controls (FIG. 7).

From these results highest level of ZFN activity at a FAD2 target site, as determined by the greater activity of NHEJ, was identified at locus E. The ZFNs which were encoded on plasmid pDAB104010 (*i.e.,* ZFN24828 and 24829) were selected for *in planta* targeting of an Engineered Transgene Integration Platform (ETIP) given its characteristics of significant genomic DNA cleavage activity and minimal non-target activity.

EXAMPLE 4: DNA CONSTRUCTS FOR ENGINEERED TRANSGENE INTEGRATION PLATFORM (ETIP) CANOLA PLANT LINES

The plasmid vector constructs described below were built using methods and techniques commonly known by one with skill in the art. The application of specific reagents and techniques described within this paragraph are readily known by those with skill in the art, and could be readily interchanged with other reagents and techniques to achieve the desired purpose of building plasmid vector constructs. The restriction endonucleases were obtained from New England BioLabs (NEB; Ipswich, MA). Ligations were completed with T4 DNA Ligase (Invitrogen, Carlsbad, CA). Gateway

reactions were performed using GATEWAY® LR CLONASE® enzyme mix (Invitrogen) for assembling one entry vector into a single destination vector. IN-FUSION™ reactions were performed using IN-FUSION™ Advantage Technology (Clontech, Mountain View, CA) for assembling one entry vector into a single destination vector Plasmid preparations were performed using NUCLEOSPIN® Plasmid Kit (Macherey-Nagel Inc., Bethlehem, PA) or the Plasmid Midi Kit® (Qiagen) following the instructions of the suppliers. DNA fragments were isolated using QIAquick Gel Extraction Kit™ (Qiagen) after agarose Tris-acetate gel electrophoresis. Colonies of all assembled plasmids were initially screened by restriction digestion of miniprep DNA. Plasmid DNA of selected clones was sequenced by a commercial sequencing vendor (Eurofins MWG Operon, Huntsville, AL). Sequence data were assembled and analyzed using the SEQUENCHER™ software (Gene Codes Corp., Ann Arbor, MI).

DIRECT-DELIVERY VECTORS FOR PRECISION INTEGRATION OF ETIP IN THE FAD2A LOCUS OF CANOLA

Standard cloning methods were used in the construction of the ETIP-containing vectors pDAS000130 (FIG. 8, T-strand insert as SEQ ID NO:141), for specific integration into the FAD2A gene of *B. napus*. This construct has been designed to be delivered into canola protoplasts with the Zinc Finger Nuclease construct pDAB1004010. The Zinc Finger Nuclease Construct will cleave the FAD2A locus and then the pDAS000130 construct will integrated within the canola genome via a homology directed repair mechanism. The ETIP consists of four expression cassettes (two incomplete) separated by additional ZFN recognition sequences and an Engineered Landing Pad (ELP) containing another ZFN recognition sequences. The additional ZFN recognition sequences are unique and have been designed to be targeted for the introduction of polynucleotide sequences within the ETIP and ELP transgene insertions. Similarly, the ZFN recognition sequences can be utilized for excision of polynucleotide sequences. The first gene expression cassette was an incomplete dsRED expression cassette and contained the promoter, 5′ untranslated region and intron from the *Arabidopsis thaliana* Polyubiquitin 10 (AtUbi promoter) gene (Callis, *et al.*, (1990) *J. Biol. Chem.*, 265: 12486-12493) followed by 210 bp of a dsRed gene from the reef coral *Discosoma* sp. (Clontech, Mountain View, CA) codon-optimized for expression in

dicot plants (ds RED (dicot optimized)exon 1) followed by an intron from the *Arabidopsis thaliana* thioreductase-like gene (Intron 1 from At thioreductase: Accession No: NC_00374) and the 3′ untranslated region comprising the transcriptional terminator and polyadenylation site of the Zea mays Viviparous-1 (Vp1) gene (Zmlip terminator: Paek *et al.*, (1998) Molecules and Cells , 8(3): 336-342). The second expression cassette contained the 19S promoter including 5′ UTR from cauliflower mosaic virus (CaMV 19S: Cook and Penon (1990) *Plant Molecular Biology* 14(3): 391-405) followed by the *hph* gene from *E. coli*, codon-optimized for expression in dicots (hph(HygR): Kaster *et al.*, (1983) Nucleic Acids Research 11(19): 6895-6911) and the 3′UTR comprising the transcriptional terminator and polyadenylation site of open reading frame 1 of *A. tumefaciens* pTi15955 (At-ORF1 terminator: Barker *et al.*, (1983) Plant Molecular Biology 2(6): 335-50). The third expression cassette was an incomplete PAT expression cassette and contained the first intron from Arabidopsis 4-coumaryl-CoA synthase (intron#2 4-coumaryl-CoA synthase v: Accession No: At3g21320/NC003074) followed by the last 256 bp of a synthetic, plant-optimized version of phosphinothricin acetyl transferase gene, isolated from *Streptomyces viridochromogenes*, which encodes a protein that confers resistance to inhibitors of glutamine synthetase comprising phophinothricin, glufosinate, and bialaphos (PAT(v6) 3′end: Wohlleben *et al.*, (1988) Gene 70(1): 25-37). This cassette was terminated with the 3′ UTR comprising the transcriptional terminator and polyadenylation sites of open reading frame 23 of *A. tumefaciens* pTi15955 (AtuORF23 terminator: Barker *et al.*, (1983) Plant Molecular Biology 2(6): 335-50). The fourth Expression Cassette was the *ipt* gene cassette and contained a 588 bp truncated version of the promoter and 5′ UTR from the Arabidopsis DNA-binding protein MYB32 gene (U26933) (AtMYB32(T) promoter: Li *et al.*, (1999) Plant Physiology 121: 313) followed by the isopentyl transferase (*ipt*) gene from *A. tumefaciens* and the 35s terminator comprising the transcriptional terminator and polyadenylation sites from cauliflower mosaic virus (CaMV 35S terminator: Chenault *et al.*, (1993) Plant Physiology 101 (4): 1395-1396). For delivery to FAD2A, each end of the ETIP sequence was flanked by 1kb of FAD2A genomic sequence from either side of the location of the double-stranded break induced by delivery of the ZFN encoded in pDAB104010 to the FAD2A gene of *B. napus*.

The ETIP sequence was synthesized by a commercial gene synthesis vendor (GeneArt, Life Technologies). The 1 kb segments of FAD2A genome sequence were

amplified from genomic DNA purified from leaf tissue of *B. napus* DH12075 using a Qiagen DNEASY® plant mini kit (Qiagen, Hilden) following instructions supplied by the manufacturer. The 1 kb FAD2A sequences were ligated into the ETIP vector using T4 ligase (NEB, Ipswich, MA). Colonies of all assembled plasmids were initially screened by restriction digestion of miniprep DNA. Restriction endonucleases were obtained from New England BioLabs (NEB, Ipswich, MA) and Promega (Promega Corporation, WI). Plasmid preparations were performed using the QIAPREP *SPIN MINIPREP*® Kit (Qiagen) or the Pure Yield Plasmid MAXIPREP® system (Promega Corporation, WI) following the instructions of the suppliers. Plasmid DNA of selected clones was sequenced using ABI Sanger Sequencing and BIG DYE TERMINATOR® v3.1 cycle sequencing protocol (Applied Biosystems, Life Technologies). Sequence data were assembled and analyzed using the SEQUENCHER™ software (Gene Codes Corp., Ann Arbor, MI).

DIRECT-DELIVERY VECTORS FOR PRECISION INTEGRATION OF ETIP IN THE FAD 3 LOCUS OF CANOLA

Standard cloning methods were used in the construction of the ETIP-containing vectors pDAS000271 (FIG. 9, T-strand insert as SEQ ID NO:142), pDAS000272 (FIG. 10, T-strand insert as SEQ ID NO:143), pDAS000273 (FIG. 11, T-strand insert as SEQ ID NO:144), pDAS000274 (FIG. 12, T-strand insert as SEQ ID NO:145), and pDAS000275 (FIG. 13, T-strand insert as SEQ ID NO:146) for specific integration into the FAD3A or FAD3C gene locus of *B. napus*. This construct has been designed to be delivered into canola protoplasts with the Zinc Finger Nuclease construct pDAB107828 or pDAB107829. A specific Zinc Finger Nuclease Construct will cleave the FAD3A locus and then the pDAS000273 or pDAS275 construct will integrate within the canola genome via a homology directed repair mechanism. Likewise, a specific Zinc Finger Nuclease Construct will cleave the FAD3C locus and then the pDAS000271, pDAS000272 or pDAS000274 construct will integrate within the canola genome via a homology directed repair mechanism. The ETIP consists of four expression cassettes (two incomplete) separated by additional ZFN recognition sequences and an Engineered Landing Pad (ELP) containing another ZFN recognition sequences. The additional ZFN recognition sequences are unique and have been designed to be targeted for the introduction of polynucleotide sequences within the ETIP and ELP transgene insertions.

Similarly, the ZFN recognition sequences can be utilized for excision of polynucleotide sequences. The first gene expression cassette was an incomplete dsRED expression cassette and contained the promoter, 5′ untranslated region and intron from the *Arabidopsis thaliana* Polyubiquitin 10 (AtUbi promoter) gene (Callis, *et al.,* (1990) *J. Biol. Chem.*, 265: 12486-12493) followed by 210 bp of a dsRed gene from the reef coral *Discosoma* sp. (Clontech, Mountain View, CA) codon-optimized for expression in dicot plants (ds RED (dicot optimized)exon 1) followed by an intron from the *Arabidopsis thaliana* thioreductase-like gene (Intron 1 from At thioreductase: Accession No: NC_00374) and the 3′ untranslated region comprising the transcriptional terminator and polyadenylation site of the Zea mays Viviparous-1 (Vp1) gene (Zmlip terminator: Paek *et al.,* (1998) Molecules and Cells , 8(3): 336-342). The second expression cassette contained the 19S promoter including 5′ UTR from cauliflower mosaic virus (CaMV 19S: Cook and Penon (1990) *Plant Molecular Biology* 14(3): 391-405) followed by the *hph* gene from *E. coli*, codon-optimized for expression in dicots (hph(HygR): Kaster *et al.,* (1983) Nucleic Acids Research 11(19): 6895-6911) and the 3′UTR comprising the transcriptional terminator and polyadenylation site of open reading frame 1 of *A. tumefaciens* pTi15955 (At-ORF1 terminator: Barker *et al.,* (1983) Plant Molecular Biology 2(6): 335-50). The third expression cassette was an incomplete PAT expression cassette and contained the first intron from Arabidopsis 4-coumaryl-CoA synthase (intron#2 4-coumaryl-CoA synthase v: Accession No: At3g21320/NC003074) followed by the last 256 bp of a synthetic, plant-optimized version of phosphinothricin acetyl transferase gene, isolated from *Streptomyces viridochromogenes*, which encodes a protein that confers resistance to inhibitors of glutamine synthetase comprising phophinothricin, glufosinate, and bialaphos (PAT(v6) 3′ end: Wohlleben *et al.,* (1988) Gene 70(1): 25-37). This cassette was terminated with the 3′ UTR comprising the transcriptional terminator and polyadenylation sites of open reading frame 23 of *A. tumefaciens* pTi15955 (AtuORF23 terminator: Barker *et al.,* (1983) Plant Molecular Biology 2(6): 335-50). The fourth Expression Cassette was the *ipt* gene cassette and contained a 588 bp truncated version of the promoter and 5′ UTR from the Arabidopsis DNA-binding protein MYB32 gene (U26933) (AtMYB32(T) promoter: Li *et al.*, (1999) Plant Physiology 121: 313) followed by the isopentyl transferase (*ipt*) gene from *A. tumefaciens* and the 35s terminator comprising the transcriptional terminator and polyadenylation sites from cauliflower mosaic virus

(CaMV 35S terminator: Chenault *et al.*, (1993) Plant Physiology 101 (4): 1395-1396). For delivery to FAD3A or FAD3C, each end of the ETIP sequence was flanked by 1 kb of FAD3A or FAD3C genomic sequence from either side of the location of the double-stranded break induced by delivery of the ZFN encoded in FAD3A or FAD3c gene of *B. napus*.

The ETIP sequence was synthesized by a commercial gene synthesis vendor (GeneArt, Life Technologies). The 1 kb segments of FAD3A and FAD3C genome sequence were amplified from genomic DNA purified from leaf tissue of *B. napus* DH12075 using a Qiagen DNEASY® plant mini kit (Qiagen, Hilden) following instructions supplied by the manufacturer. The 1 kb FAD3A or FAD3C sequences were ligated into the ETIP vector using T4 ligase (NEB, Ipswich, MA). Colonies of all assembled plasmids were initially screened by restriction digestion of miniprep DNA. Restriction endonucleases were obtained from New England BioLabs (NEB, Ipswich, MA) and Promega (Promega Corporation, WI). Plasmid preparations were performed using the QIAPREP *Spin Miniprep*® Kit (Qiagen) or the Pure Yield Plasmid MAXIPREP System® (Promega Corporation, WI) following the instructions of the suppliers. Plasmid DNA of selected clones was sequenced using ABI Sanger Sequencing and BIG DYE TERMINATOR® v3.1 cycle sequencing protocol (Applied Biosystems, Life Technologies). Sequence data were assembled and analyzed using the SEQUENCHER™ software (Gene Codes Corp., Ann Arbor, MI).

CONTROL VECTORS

A control vector was used to develop a Fluorescence Activated Cell Sorting (FACS) cell based sorting method. Standard cloning methods were used in the construction of a control vector, pDAS000031 (FIG. 14: T-strand insert as SEQ ID NO:147) consisting of two gene expression cassettes. The first gene expression cassette contained the Cauliflower mosaic virus 19s promoter (CaMV 19S promoter; Shillito, *et al.*, (1985) *Bio/Technology* 3; 1099-1103):: hygromycin resistance gene (hph(HygR);US Patent No. 4,727,028) :: and the *Agrobacterium tumefaciens* Open Reading Frame 1 3′ UnTranslated Region (AtORF1 terminator; Huang *et al.*, (1990) *J. Bacteriol.* 1990 *172*:1814-1822). The second gene expression cassette contained the *Arabidopsis thaliana* Ubiquitin 10 promoter (AtUbi10 promoter; Callis, *et al.*, (1990) *J. Biol. Chem.*, 265: 12486-12493):: dsRED (dsRED(D); US Patent No. 6,852,849) and an intron from

Arabidopsis (intron #1; GenBank: AB025639.1) :: *Agrobacterium tumefaciens* Open Reading Frame 23 3′ UnTranslated Region (AtORF23 terminator; US Patent No. 5,428,147) as an in-frame fusion with a *trans* orientation (*e.g.*, head to head orientation). The plasmid vector was assembled using the IN-FUSION™ Advantage Technology (Clontech, Mountain View, CA).

CONSTRUCTION OF BINARY VECTORS FOR RANDOM INTEGRATION OF ETIP IN CANOLA

Two binary vectors were constructed for random integration of an ETIP T-Strand sequence within the genome of *Brassica napus*. Standard cloning methods were used in the construction of the ETIP-containing vectors pDAS000036 (FIG. 15, T-strand insert as SEQ ID NO:148) and pDAS000037 (FIG. 16, T-strand insert as SEQ ID NO:149). The ETIP vectors consist of four expression cassettes (two incomplete expression cassettes) separated by ZFN recognition sequences and an Engineered Landing Pad (ELP) containing further ZFN recognition sequences. The first gene expression cassette was an incomplete dsRED expression cassette and contained the promoter, 5′ untranslated region and intron from the Arabidopsis thaliana polyubiquitin 10 (AtUbi10 promoter) gene (Norris *et al.*, (1993) Plant Molecular Biology, 21(5): 895-906) followed by 210 bp of a dsRed gene from the reef coral *Discosoma* sp. (Clontech, Mountain View, CA) codon-optimized for expression in dicot plants followed by an intron from the Arabidopsis thioreductase-like gene (Accession No: NC_00374) and the 3′ untranslated region (UTR) comprising the transcriptional terminator and polyadenylation site of the *Zea mays* Viviparous-1 (Vp1) gene (Zm Lip terminator: Paek *et al.*, (1998) Molecules and Cells, 8(3): 336-342). The second expression cassette contained the 19S promoter including 5′ UTR from cauliflower mosaic virus (CsVMV 19 promoter: Cook and Penon (1990) Plant Molecular Biololgy 14(3): 391-405) followed by the *hph* gene from *E. coli* codon-optimized for expression in dicots (hph(D): Kaster *et al* (1983) Nucleic Acids Research, 11(19): 6895-6911) and the 3′ UTR comprising the transcriptional terminator and polyadenylation site of open reading frame 1 (ORF1) of *A. tumefaciens* pTi15955 (AtORF1 terminator: Barker *et al.*, (1983) Plant Molecular Biology, 2(6): 335-50). The third expression cassette was an incomplete PAT expression cassette and contained the first intron from Arabidopsis 4-coumaryl-CoA synthase (Accession No: At3g21320 (NC003074)) followed by the last

256 bp of a synthetic, plant-optimized version of phosphinothricin acetyl transferase (PAT) gene, isolated from *Streptomyces viridochromogenes*, which encodes a protein that confers resistance to inhibitors of glutamine synthetase comprising phophinothricin, glufosinate, and bialaphos (PATv6(exon2); Wohlleben *et al.*, (1988) Gene, 70(1): 25-37). This cassette was terminated with the 3′ UTR comprising the transcriptional terminator and polyadenylation sites of open reading frames 23 (ORF23) of *A. tumefaciens* pTi15955 (AtORF23 terminator; Barker *et al.*, (1983) Plant Molecular Biology, 2(6): 335-50). The fourth Expression Cassette was the *ipt* gene cassette and contained a 588 bp truncated version of the promoter and 5′ UTR from the Arabidopsis DNA-binding protein MYB32 gene (U26933) (AtMYB32(T)promoter; Li *et al.*, (1999) Plant Physiology, 121: 313) followed by the isopentyl transferase (*ipt*) gene from *A. tumefaciens* and the 35s terminator comprising the transcriptional terminator and polyadenylation sites from cauliflower mosaic virus (CaMV 35 S terminator; Chenault *et al.*, (1993) Plant Physiology, 101 (4): 1395-1396).

The expression cassettes and ELP were synthesized with Multi-Gateway sites by a commercial gene synthesis vendor (GeneArt, Life Technologies). Entry clones were constructed of each expression cassette and ELP using BP clonase II enzyme mix™ (Invitrogen, Life Technologies) and the pDONR221 vector suite™ (Invitrogen, Life Technologies). The Entry clones were then used in a Multi-Gateway reaction with a Gateway-enabled binary vector using LR Clonase II Plus Enzyme mix™ (Invitrogen, Life Technologies). Colonies of all assembled plasmids were initially screened by restriction digestion of miniprep DNA. Restriction endonucleases were obtained from New England BioLabs (NEB; Ipswich, MA) and Promega (Promega Corporation, WI). Plasmid preparations were performed using the QIAprep *Spin Miniprep* Kit™ (Qiagen, Hilden) or the Pure Yield Plasmid Maxiprep System™ (Promega Corporation, WI) following the instructions of the suppliers. Plasmid DNA of selected clones was sequenced using ABI Sanger Sequencing and Big Dye Terminator v3.1 cycle sequencing protocol™ (Applied Biosystems, Life Technologies). Sequence data were assembled and analyzed using the SEQUENCHER™ software (Gene Codes Corporation, Ann Arbor, MI).

EXAMPLE 5: GENERATION OF ETIP CANOLA PLANT LINES

TRANSFORMATION OF *Brassica napus*

The ETIP constructs (pDAS000036, pDAS000037), the DS-Red control construct (pDAS000031), and the FAD2A, FAD3A, and FAD3C site specific constructs (pDAS000130, and pDAS000271-pDAS000275) and accompanying Zinc Finger Nuclease (pDAB104010, pDAB10728, and pDAB10729) described in Example 4. The binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101: PM90. Transformation of *Brassica napus* protoplast cells was completed using the transfection protocol described in Example 3 with some modification.

The modifications to the protocol included the use of sodium alginate instead of Sea Plaque™ agarose. The transfection experiments in which both the Zinc Finger Nuclease construct and the ETIP construct were co-delivered into *Brassica napus* protoplast cells were completed at DNA concentrations comprising a 5:1 molar ratio of plasmid DNA. The other ETIP and control plasmid constructs were transformed at concentrations of 30 µg of plasmid DNA. As such, pDAS000130 consisted of a concentration of 27.8 µg of plasmid DNA and pDAB104010 consisted of a concentration of 2.2 µg of plasmid DNA. The other ETIP and control plasmid constructs were transformed at concentrations of 30 µg of plasmid DNA.

Additional modifications to the protocol included the propagation of whole plants from the transformed protoplast cells in medium containing 1.5 mg/mL of hygromycin. The propagation of whole plants required that the A medium was replaced every two weeks and the growth of the protoplast-derived colonies was monitored. After the protoplast-derived colonies had grown to approximately 2-3 mm in diameter, the colonies were transferred into individual wells of a 12-well COSTAR® plate (Fisher Scientific, St. Louis, MO) containing solidified MS morpho medium. The plates were incubated for one to two weeks at 24 °C under continuous dim light until the calli had proliferated to a size of 8-10 mm in diameter. After the protoplast cells had reached a diameter of 1-2 cm in diameter, the protoplast cells were transferred to individual 250 mL culture vessels containing MS morpho medium. The vessels were incubated at 24 °C under 16 h light (20 µMol m^{-2} s^{-1} of Osram L36 W/21 Lumilux white tubes) and 8 h dark conditions. Within one to two weeks, multiple shoots were visible. The shoots were transferred into 250 mL culture vessels containing MS medium after they reached a length of 3-4 cm. The 250 mL culture vessels were incubated at 24 °C under 16 h

light (20 μMol m^{-2} s^{-1} of Osram L36 W/21 Lumilux white tubes) and 8h dark conditions. The shoots were maintained in the culture vessels until they developed into plantlets at which time they were transferred to a greenhouse to grow to maturity.

EXAMPLE 6: MOLECULAR CONFIRMATION OF INTEGRATION OF T-DNAS CONTAINING ETIPS IN CANOLA

Genomic DNA was extracted from leaf tissue of all putative transgenic plants using a DNEASY® 96 Plant DNA extraction kit™ or a DNEASY® Plant Mini Kit™ (Qiagen). The genomic DNA from each plant was analyzed by PCR using primers designed to amplify *vir*C from pTiC58 Forward (SEQ ID NO:150 CGAGAACTTGGCAATTCC) and pTiC58 Reverse (SEQ ID NO:151 TGGCGATTCTGAGATTCC) to test for persistence of *A.tumfaciens,* primers designed to amplify actin from *B. napus*; Actin Forward (SEQ ID NO:152 GACTCATCGTACTCTCCCTTCG) and Actin Reverse (SEQ ID NO:153 GACTCATCGTACTCTCCCTTCG) to check the quality of the genomic DNA. Primers were designed to amplify the *hph* gene; HPH Forward (SEQ ID NO:154 TGTTGGTGGAAGAGGATACG) and HPH Reverse (SEQ ID NO:155 ATCAGCAGCAGCGATAGC) encoded by the ETIP. Plants that did not give a product from *vir*C primers but from which products of the correct size were amplified with primers to actin and *hph* were classified as transgenic.

A second screen was completed, where gDNA from each transgenic plant was analyzed by PCR using five sets of primers designed to amplify the binary vector outside of the T-DNA region [(1F SEQ ID NO:156 ATGTCCACTGGGTTCGTGCC; 1R SEQ ID NO:157 GAAGGGAACTTATCCGGTCC) (2F SEQ ID NO:158 TGCGCTGCCATTCTCCAAAT; 2R SE ID NO:159 ACCGAGCTCGAATTCAATTC) (3F SEQ ID NO:160 CCTGCATTCGGTTAAACACC; 3R SEQ ID NO:161 CCATCTGGCTTCTGCCTTGC) (4F SEQ ID NO:162 ATTCCGATCCCCAGGGCAGT; 4R SEQ ID NO:163 GCCAACGTTGCAGCCTTGCT) (5F SEQ ID NO:164 GCCCTGGGATGTTGTTAAGT; 5R SEQ ID NO:165 GTAACTTAGGACTTGTGCGA)]. Plants from which PCR products of the correct and expected size were amplified with primer sets 3 and 4 were considered to have backbone integration.

DNA from plants with no backbone integration was purified from 20 g of leaf tissue using a modified CTAB method (Maguire *et al,.* (1994) Plant Molecular Biology Reporter, 12(2): 106-109). The isolated gDNA was digested with several restriction enzymes and 10 µg of gDNA was separated by electrophoresis on an agarose gel and transferred to membrane using a standard Southern blotting protocol. Membranes were probed using the DIG Easy Hyb System™ (Roche, South San Francisco, CA) following the manufacturer's instructions. Probes to each expression cassette to the ELP and to an endogenous control gene, actin, were amplified from the ETIP construct using the following primers: (IPT-F SEQ ID NO:166 TCTCTACCTTGATGATCGG; IPT-R SEQ ID NO:167 AACATCTGCTTAACTCTGGC; dsRED-F SEQ ID NO:168 ATGGCTTCATCTGAGAACG; dsRED-R SEQ ID NO:169 TTCCGTATTGGAATTGAGG; PAT-F SEQ ID NO:170 TTGCTTAAGTCTATGGAGGCG; PAT-R SEQ ID NO:171 TGGGTAACTGGCCTAACTGG; ELP-F SEQ ID NO:172 ATGATATGTAGACATAGTGGG; ELP-R SEQ ID NO:173 AGGGTGTAAGGTACTAGCC; Hph-F SEQ ID NO:174 TGTTGGTGGAAGAGGATACG; Hph-R SEQ ID NO:175 ATCAGCAGCAGCGATAGC; actin-F SEQ ID NO:176 GTGGAGAAGAACTACGAGCTACCC; actin-R SEQ ID NO:177 GACTCATCGTACTCTCCCTTCG).

The ETIP sequence was amplified and sequenced from all plants containing only a single copy of the ETIP. The sequence of each T-DNA insert was analyzed by direct sequencing of PCR products using the ABI3730xI™ (Applied Biosystems, Life Teachnologies). The T-DNA insert was amplified from genomic DNA, using Phusion Hot Start II Polymerase™ (Finnzymes, Thermo Fisher Scientific). The amplification reactions of the T-DNA were completed with multiple primer pairs to amplify overlapping sequences of approximately 2 Kbp in length. Each PCR product was sequenced with multiple primers to ensure complete coverage. The PCR reactions were treated with shrimp alkaline phosphatase and exonuclease I (Applied Biosystems, Life Technologies) to inactivate excess primer prior to the sequencing PCR reaction. The sequences flanking the T-DNA insert of each single copy ETIP line were identified by digestion of purified genomic DNA with eight restriction endonucleases followed by ligation of double-stranded adapters specific for the overhangs created by the restriction

endonucleases. Following this ligation step a PCR was performed with a biotinylated primer to either the 3′ or 5′ end of the ETIP and a primer to each adapter. The PCR products were captures and cleaned on Ampure Solid Phase Reversible Immobilization (SPRI) beads™ (Agencourt Bioscience Corporation, Beckman Coulter Company). A nested PCR was performed and all products were sequenced using ABI Sanger Sequencing and Big Dye Terminator v3.1 cycle™ sequencing protocol (Applied Biosystems, Life Technologies). Sequence data were assembled and analyzed using the SEQUENCHER™ software (Gene Codes Corp., Ann Arbor, MI).

SOUTHERN BLOT ANALYSIS

Specific restriction enzymes were selected to digest gDNA samples prior to Southern probing. The putative transgenic plants were analyzed by digesting the genomic DNA with *EcoR*I and *Swa*I. Next, the digested gDNA and uncut gDNA samples were probed with either polynucleotide fragments comprising PATv6, IPT or ELP gene elements as these polynucleotide probe fragments enabled differentiation of multiple inserts in *EcoR*I digests as well as in the *Swa*I digests. Identified single copy transgenic plant lines were then further analyzed with all six probes to identify the presence of all essential elements of the inserted vector.

Accordingly, 67 independent events transformed with ETIP-pDAS000036 were sampled and tested for the presence of the transgene (*hph*), and the presence of vector backbone. Of the 67 plants tested, 47 were found to have the transgene integrated within the genome. From the 47 transgenic plants, 17 of the plants were found to contain vector backbone (Table 14). The remaining 30 plants that contained no significant portion of vector backbone (absence of Ori or SpecR) were sampled for Southern analysis. As a general rule, the plants were screened initially with the IPT probe, and plant lines identified as putative single copy lines were further tested with probes comprising the dsRED, PAT, ELP and *hph* gene elements in order to confirm the presence of the whole cassette.

Likewise, 52 independent events transformed with ETIP-pDAS000037 and surviving in soil were sampled and tested for the presence of the transgene (*hph*), and the presence of vector backbone. Of the 52 plants tested, 48 were found to have the transgene integrated within the genome. From the 48 transgenic plants, 23 of the plants were found to contain vector backbone as well and 3 plants were not tested (Table 14).

The remaining 22 plants that contained no significant portion of vector backbone (absence of Ori or SpecR) were sampled for Southern analysis. These transgenic plants were initially screened with the IPT probe, and the plant lines were identified as putative single copy lines, and were further tested with the dsRED, PAT, ELP, *hph* and actin probes in order to confirm results. Once the identification of 5 independent single copy lines were obtained, Southern analysis was terminated on the remaining plants. In total, 11 ETIP-pDAS000037 lines underwent Southern analysis.

Table 14: Summary of +/- transgene and +/- vector backbone PCRs results

Confirmation of transgene – Endpoint PCR				
	Independent Events Surviving in Soil	**Independent Events Sampled**	**Independent Events Tested**	**Independent Events Positive for Transgene**
pDAS000036	67	67	67	47
pDAS000037	52	52	52	48
Presence of Backbone – Endpoint PCR				
	Independent Events Tested		**Independent Events with no Ori or Spec^R**	
pDAS000036	47		30	
pDAS000037	48		22	

RESULTS OF ETIP TRANSGENIC CANOLA TRANSFORMED WITH PDAS000036 AND PDAS000037

The transgenic *Brassica napus* events which were produced via transformation of pDAS000036 and pDAS000037 resulted in the production of single copy, full length T-strand insertions. Three to four events for each plant were fully characterized, and were putatively mapped to specific chromosomes within the *Brassica napus* genome. Although a few single base-pair rearrangements occurred during the T-strand integration, the selected events contained full length expression cassettes which are capable of driving robust expression of the transgene. The selected T_0 events were grown to the T_1 stage of development. The T_1 were res-screened using the above

described PCR assays to determine the zygosity of the integrated T-strand. Screened events were categorized as homozygous, hemizygous, or null.

The ETIP sequence was amplified and sequenced from all transgenic events containing only a single copy of the integrated ETIP sequence. The sequence of each T-DNA insert was analyzed by direct sequencing of PCR products. The T-DNA insert was amplified from genomic DNA, using Phusion Hot Start II Polymerase™ (Finnzymes, Thermo Fisher Scientific). Next, the T-DNA was amplified with multiple primer pairs to amplify overlapping sequences of approximately 2 Kb in length. Each PCR product was sequenced with multiple primers to ensure complete coverage. The PCR reactions were treated with Shrimp Alkaline Phosphotase and Exonuclease I (Applied Biosystems, Life Technologies) to inactivate excess primer prior to the sequencing PCR reaction.

The sequences flanking the T-DNA insert of each single copy ETIP line was identified by digestion of purified genomic DNA with eight restriction endonucleases followed by ligation of double-stranded adapters specific for the overhangs created by the restriction endonucleases. Following this step a PCR reaction was performed with a biotinylated primer to either the 3′ or 5′ end of the ETIP and a primer to each adapter. The PCR products were captured and cleaned on Ampure Solid Phase Reversible Immobilization™ (SPRI) beads (Agencourt Bioscience Corporation, Beckman Coulter Company). A nested PCR was performed and all products were sequenced using ABI Sanger Sequencing and Big Dye Terminator v3.1 cycle sequencing protocol (Applied Biosystems, Life Technologies). Sequence data were assembled and analyzed using the SEQUENCHER™ software (Gene Codes Corp., Ann Arbor, MI). Eight ETIP lines were identified and selected for flanking sequence analysis (Table 15). The left and right flanking sequences (also described as border or junction sequences) are provided as SEQ ID NO:431 – SEQ ID NO:446, the underlined sequences indicated plasmid vector, the non-underlined sequences indicate genomic flanking sequence.

Table 15: Details of single copy events used in flanking sequence studies

Plasmid Description	Event name	Barcode	Left Hand Border SEQ ID NO:	Right Hand SEQ ID NO:
pDAS000036	em02-5788-1-1	228688	431	432
	ad58-5784-2-1	232502	433	434
	ad58-5898-10-1	237143	435	436
pDAS000037	lf31-6139-2-3	234576	437	438
	bm56-6315-1-1	234703	439	440
	ad58-6372-1-1	240653	441	442
	ad58-6620-4-1	242268	443	444
	ad58-6620-17-1	242293	445	446

pDAS000036 Event details: em02-5788-1-1 Left border flanking (SEQ ID NO:431)

TCGAGATTGTGCTGAAGTAAACCATTTTACTTCAAATCTATTTTTAAC
TATTTACTTTTATTAAGGAGAGAAACTTTGCTGATTAATTCAAATTAGTGAT
CATTAAGATTCCAAAGATTCCGATTTAGAAAAGTCAAAGATTCAAAGAACA
AGTCTAGGTCCTCATGGCTCATGTTGCATCCGATTCACCATCCACTCATCTTT
CATATCTTCCTCCACTGTCTCTCTAGAAACAACTCATTTAATTTAGAAAACTC
CTTTTTCAATTTAGAAATATTAAAGTTTATCACAATGTATCAATTAAATATTA
TCCGATGACTCATTCATAGTCAGGACCTTGCTGTCTGTGTCGTCCGTAATTAT
TATTTCAATACAAAACAAATATATGTTCACTCAGAAAATTACGGCGCAATCA
TCTAATTTTGTGGACCAAAATAAATAGCGTAGCTTCGAGATTTCAAAGTTGT
GTTCAAATTTAATTTTGATTTCCGTTCCTCGTATACTCTTTTATGTATAGAAA
ATAATAATATCCACTACTAGTAGTTGATAACTACATTACATATATTAAAATT
ATGATGTCACATTGCGGACGTTTTTAATGTACTGAATTAACGCCGAATTGAA
TTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAG
CTTAGCTTGAGCTTGGATC

em02-5788-1-1 Left border flanking (SEQ ID NO:432)

CATAACCACCATCTCAAACAATAGAACTTCCTAAGTGAAGCAATGAC
TTCAAATCTACTTGAAGGCATGGAGTATAAGCCATGTTCCTTTCAGAGGGGA
CTGTACTTCTGTAGATTACTTTCCCTCATTAACCAGATCTGGCCGGCCTACCC

AGCTTTCTTGTACACATAGCGACCGAGCTCGAGCCGAAGTTCGGTCGCTGTT
TCACTGTTTGGGAAAGCATCAGTAACGCAGAAGACATAATTAAAAATTAAT
TATATATGGTAGTTTTTCTAGATTCTCCACTATACCTCATTGTGATTGAAAAA
CAACTATATATATATATATATATGTATTTAAAATTAAGAAATCATTAAATCG
TACCATAATGCAGAAAACTTTATAAGTTCCTATTCTTTTGTCAAGATGAGTA
GATGACACATCATGTACCACATAACATAACCATAATGGTGCGATACCATAC
CAAAACTTAATTTAGAAACTAATTAAAATTTTGGTAGTTTAAGATTCCTCCT
AATGGTTGTCAAAAAAAAAAAAAGATTCCTCCTAATACATGGTAGAATATA
TTTTTGAGTTAAAATGAAAGTAAAATCTTCAAATTAGTCATAAGGAAATTCT
AAAAAACATCGACTTTCTTTATAAAGATCCCATTGTAATTTTAGATGATTAA
TTTTATCCCAATCCAATTAAGAATTGTACACATCGGCCTCTATATATATCAA
ACACCTAAAA

pDAS000036 Event details: ad58-5784-2-1 Left border flanking (SEQ ID NO:433)

CGATTTGCAGCTATAATCAATCACACCTTATCGTTCTTTCAAAGAAAA
ATCGAAAGTTGTAAACTTTATCAGCCTGTGTAGTGATTATTTCAATTTGATA
AAGAAAAAAAAAAGGCTTAGCTTTATTTGGTTTTTTGTTACAATCTTGATTAA
TTTTAGATTAGCACTCTGATTCTAGCGGAACATGAGAGTGGTTCCATCAAAC
CTCAGACAGTGAGCACAGTGGTTGCAGCAAACCATTTGGGTGAGAGCTCTT
CAGTTTCTTTGCTATTAGCTGGTTCTGGCTCTTCTCTTCAAGAAGCTGCTTCT
CAAGCTGCCTCTTGTCATCCTTCTGTTTCCGAGGTAACTAACTACATTCTTCA
TTTGTCCTTTTTTCTTGTGGGTCTTAAATGTTYGTGCTTTTCTTTATAGGTACT
TGTTGCTGATTCAGATAGATTTGAGTACCCTTTAGCTGAACCTTGGGCTAAA
CTGGTTGACTTTGTTCGCCAACAAAGAGATTACTCTCACATCCTTGCCTTCCT
CTAGCTCATTTGGCAAGAACATACTTCCTCTAGACAACTTAATAACACATTG
CGGACGTTTTTAATGTACTGAATTAACGCCGAATTGAATTCGAGCTCGGTAC
CCGGGGATCCTCTAGAGTC

ad58-5784-2-1 Right border flanking (SEQ ID NO:434)

CCTGTCATAACCACCATCTCAAACAATAGAACTTCCTAAGTGAAGCA
ATGACTTCAAATCTACTTGAAGGCATGGAGTATAAGCCATGTTCCTTTCAGA
GGGGACTGTACTTCTGTAGATTACTTTCCCTCATTAACCAGATCTGGCCGGC

CTACCCAGCTTTCTTGTACAAAGTGGTGATAAACTATCGCCGGCCTACCTCG
CGTTGCTGCTCTTTTAGATGTCTCTCCTGTTACTGATGTTGTCAAAATCTTAG
GATCCAATGAGTTTATCAGGTATACTTCTATCATGTATTGCTTGAGATTTTGG
AGTGTTAGTAAAGATTTCAATAAAAGAATTTTTTCAAACAAAATTTTGGGGG
CTTGAAGCTAATGTTTGGAAATATGTAACGGAGTTTAAATCTTTTGGCAGGC
CTATATATGCGGGAAACGCCTTGTGTAGAGTTCGCTATACTGGTGCTGGTCC
TTGTGTGTTGACTATTAGAACTACATCTTTTCCTGTTACCCCAATAACTGAGT
CAAAGAAAGCTACTATCTCTCAGATTGATCTCTCGAAATTCAAAGAAGGTTT
GTTTAGTATTATTCTCTTGTGCATAGCCTTTTTGCTTTTTTTTTTTTTATAAAA
AAAGTTGAGTATGCTTATTGCCCATTGCA

pDAS000036 Event details: ad58-5898-10-1 Left border flanking (SEQ ID NO:435)

TAATTTCATTTTCGTCATTTTGGTAAAGTAACAAAAGACAGAATATTG
GTTAGTCGTGTTGGTTAGGAATAAAATAAAGAACGTGGACATCGTGGAATA
AAAATATTCAGACAAGGAAACTAACAATAAAACAGTAATGAACATGGTTCT
GAATCTCATCTTTGTGTATCTCCAATGGAATCCACCGCCACGAATCAGACTC
CTTCTCCAAGCTCCACCGTCGACGATGACAATGGCGACGGTGTCTATACCGA
CGAATTTACCAAACTACCCCCGGACCCGGGGATCCTCTAGAGTCAACAAATT
GACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATT
AACGCCGAATTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCT
GCAGGCATGCAAGCTTAGCTTGAGCTTGGATCAGATTGTC

ad58-5898-10-1 Right border flanking (SEQ ID NO:436)

CGGCCTACCCAGCTTTCTTGTACAAAGTGGTGATAAACTATCAGTGTT
TGATTAAAGATAAAATTTGATTTTTCATTACATAATAATCCATTAATTTTCAC
GCACGTGGAACCCATTTGGTGTACTTCCACGTCCTCTAAGAGAGCACTGACC
CACTCATCAAAAAGATACATCTTTATAAGCCCCTTCATCGTCACACAGACAC
ACACTTCCCTCTCAATTATTCCATATTCTCCTAATTTCTAATTGTTACACCTC
AACACATTAGATTCGTACCAAACAAAAACGATTAGCTCCCAAGCCTAAGCT
TTTATTTCCTTATAATTTTTCTTGGGTTTCTCTCTATAAAGAATGCAAATGAC
TGAGAGAGGCCGAGCCATGTGGCACACGTCCCTAGCCTCGGCATTCCGCAC
AGCTCTAGCTTGCACAATCGTTGGTGCGGCTACGCTCTACGGACCCGAGTGG

ATCCTCCGTTTTGTGGCATTCCCGGCGTTTTCTTACGTCACGGTCATTCTCAT
CATTACGGACGCCACGCTAGGCGACACACTACGTGGCTGCTGGCTAGCCCTT
TACGCCACATGTCAGAGCGTTGCACCGGCTATCATTACACTAAGGCTTATAG
GACCAGCTCGGCTCACGGCCGG

pDAS000037 Event details: lf31-6139-2-3 Left border flanking (SEQ ID NO:437)

TTTCTTTCCATCAGTTCTTCGGCACCTTCCTGGCTCTGCGTCTATCTTT
CTTTCCATCCCGGCCCATCTCGTACACATTCCACCCAACTACACAAACACGT
TATAGTCTTTTACATTATGACCAAATCAACCCTAAAGATACAGCCTTTATAA
AAAATATAGGGGTCAAAGCAAAGAAGAGAAAGTTTGCTTACAGTGTGGAGA
AAAGAAGTTGGAAGATGAGGAGTAAGAAGAAGAAGAGAAGAGAAAGGGTC
TTCTGATGAGGAATAGGAGATAAGGTGGAACTGGAATGTTTGCCGCTAAAT
ACTTGAAGACAAGAGCTTGATTTTCAAGCTCTTCCCATTGTGACTCAGTGAA
AGGGATCCTAGTCGTCATGAAGAGATAGAGAAAATTGATGATGAAGGAGGT
CTGATGAAGAGAAGAGAGAGAGAGAGATATTACACAAAGAGGGTTGTATT
GCAAAATTGAAAGTGTAGAGAGAGTAGTAGGTAAGTTTTTATTAATAATGTT
GTTCACACCTGCAGTCTGCAGAATATTGTGGTGTGAACAAATTGACGCTTAG
ACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATTAACGCCGAA
TTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATG
CAAGCTTA

lf31-6139-2-3 Right border flanking (SEQ ID NO:438)

TTCAATCTACTTGAAGGCATGGAGTATAAGCCATGTTCCTTTCAGAGG
GGACTGTACTTCTGTAGATTACTTTCCCTCATTAACCAGATCTGGCCGGCCT
ACCCAGCTTTCTTGTACAAAGTGGTGATAAACTATCAGTGTTTGAACATATA
TATACGCATAATATTCTCAGAACCCGACCCATTGGTTGACTCGGATCAAGAT
CGACCCGATCCGACCCGGTTAAAAGCACGTCGTCTCCTTTGGTTCGCCCCTT
ATATTCGACGAGTGAGTTCGATTGGATCGCTGTTTGTCATTTCTCACTACCTT
AAGAAAAAAAAAAAAGGTGCGTCTCTCTCTCACCTTTACCGCTCACTTACCTC
TCAGATCTGACATCGATTTTCCAAATCTTC:TCCAGGTACTCTCTCTCCTGGC
CGTTGACGGTCCCGTCCCGGCCGTGGATCTGATTTCGCCGCGATCTGAGGTC

AAGCATGGCGGCAGCTAACGCCCCCATCACGATGAAGGAGGTCCTAACGGT
GAGTCCCGCCTCCATTTTTAGTAACATA

pDAS000037 Event details: bm56-6315-1-1 Left border flanking (SEQ ID NO:439)

TACATCGCGATTCATCCTGGTTTGATTAGAATGACGAGGAAGTTGTC
ATATTCCCAAACAGGAAAATTGGGATCGCCTTATTTGAAAGTGGGATAACTT
CTTCATCTTAATTCTTATGAGAATTATTCCACTTCCTGGTGATTCTCCACTAC
TTTTTGTATATAAATACAGCTTCTTACATCGCGATTCATCCTGATTTGATTAG
AATGACGAGAAAGTTGTCATATTCCCAAACAGGAAAACTGGGATCACCTGA
TTTGAAAGTGGGATAACTTCTTCATCCTAATTCTTATGAGATTTATTCCACTT
CCTGGTGATTCTCCACTTCTTTATGTATCCAAATACATCTTCTTACATCGCGA
TTCATCCTGGTTTGATTAGAATGACGAGGAAGTTGTCATATTCCCAAACATG
AAAACTGGGAAAGTGGGATTGACGCTTAGACAACTTAATAACACATTGCGG
ACGTTTTTAATGTACTGAATTAACGCCGAATTGAATTCGAGCTCGGTACCCG
GGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTAGCTTGAGCTTGGAT

bm56-6315-1-1 Right border flanking (SEQ ID NO:440)

CCACCATCTCAAACAATAGAACTTCCTAAGTGAAGCAATGACTTCAA
ATCTACTTGAAGGCATGGAGTATAAGCCATGTTCCTTTCAGAGGGGACTGTA
CTTCTGTAGATTACTTTCCCTCATTAACCAGATCTGGCCGGCCTACCCAGCTT
TCTTGTACAAAGTGACGATAAACTATCAGTCTTTCAAAGCGCATCTATGGCT
AGTCATCACGGTTTTTAACTGTTTTACGAAGTCGCGGAGAGGCTCGTCTTCT
CCCTAGGATAAACTGCAGAGGTCGACATCGGAGGTTTCTCGATCTATGAAC
ATAGAGTACTGTTTGAGAAACTCCGAGGCGAGTTGGCGGAAACTCCCTATA
GAGTTTTTCTTTAGACAAGAGAACCACTCAAGCGCTGCTTCGCGGAGGTTCT
CGACGAAGAGGCGGCATTCGCCGGCATCTCTTTCTCTATCTTTAAACTTGGC
TCTTCCCATCGCGATCTGGAAAGCCTGCAAGTGTGCCTTAGGATCGGTTGTA
CCATCGTAAGGAGCCACTTTGACTTTATCAGGATCCGAAACCGTAGTTTCGG
TGATGCGGGTGGTGAAGGGCGTTTTTCGAGACTCCTCGAGGAGTAGGGTGA
TATCGAGTGCAGTACTAGTAGCGTGATGATTTGGGATTTCACCGCTCTAACC
TCCGCAGCCGTTTTCA

pDAS000037 Event details: ad58-6372-1-1 Left border flanking (SEQ ID NO:441)

TTTTACAGTGTTAGAAGAAGTGGATGAAGCTGAAATTGAATTTTCAA
ACTCGTTCAGCTTGACTAGAGGTGGGAGAGAGTCAAAGCCTCCCATCAAGT
ACCAAAAACATGGAATAGAAGACAGTCCGAGGGAGAGGAAACCGTGGCCGA
CGAGGCCGTGGCTCCTATCATTAACTAGTGTCTTTCTTACTATTAATGGTTTA
TTAAGTCTCAGCCTTTGTTGTTTCATTGGTTTGAGATTCACTCATACATGAAA
CTTGTTTCATTCCAGCTTTTCCAAACTATAAGAATATTTCCAATCTTATCTTG
TAATAGTTTAAGTTTTAAATTGAAAGCCCTTAGTTCAAAAAACAAAAAAAA
AAAATTAGCCCTTGAATTTATATATAATCACGACGGCCATATTTGGCAGCTA
CACTGATATGTTTTCAATTGGCTGACAGGCCTTGAGCAGGGTTTGCTGGGTA
TATTGGTAGGAAGATGTGTTGCGAGGTTGAAGCCTCATTTAGGCAATATAAA
CATGATCATTAGCGTTATGTCATTAGTTATACTTATACGTAGACTAAGTAAC
CCACTAAAGGTTGCTGATTCCTTTTGTATCGACTAACACATTGCGGACGTTTT
TAATGTACTGAATTAACGCCGAATTGAATTCGAGCTCGGTACCCGGGGATCC
TCTAGAGTCGACCTGCAGGCATGCAAGCTTAGCTTGAGCTTGGATCAGATTG
TCGTTTCCCGC

ad58-6372-1-1 Right border flanking (SEQ ID NO:442)

CATGTTCCTTTCAGAGGGGACTGTACTTCTGTAGATTACTTTCCCTCATTAAC
CAGATCTGGCCGGCCTACCCAGCTTTCTTGTACAAAGTGGTGATAAACTATC
AGTGTTTGACTGAATTTTAATTTCTAATTTTTGTAAAAAATTTGTATAACCTC
AAATTATTAAAAGGCGGATTTTATTAGAATTATAACTAAATTATCTATAACT
CCAAAATTTTGACAATCAATCATGTCTATATCTTTATTTTTTTGCTAAATTAT
CATGTCTATATCTTTTCTTTCTTCCAAACTTACTTGAGACTAAAAGTCTTTAT
AAATTTTGATAGGAGTTCCACACACAAACAAAAACAAAACAAATATTTTTC
ATCAAGGGATACTTATTTAACATCACGGATTCACAGTTTATTAACAAAAATC
CAAACAAAGACTGAAAGACAGAAGATTCAATCTAACAATAGTCGGCAAACA
CCAGTGATTAACTAACGAAATAAATTAACAAGTGGTCAGATCTTCGGGAAA

pDAS000037 Event details: ad58-6620-4-1 Left border flanking (SEQ ID NO:443)

TAATTTCATTTTCGTCATTTTGGTAAAGTAACAAAAGACAGAATATTG
GTTAGTCGTGTTGGTTAGGAATAAAATAAAGAACGTGGACATCGTGGAATA
AAAATATTCAGACAAGGAAACTAACAATAAAACAGTAATGAACATGGTTCT
GAATCTCATCTTTGTGTATCTCCAATGGAATCCACCGCCACGAATCAGACTC
CTTCTCCAAGCTCCACCGTCGACGATGACAATGGCGACGGTGTCTATACCGA
CGAATTTACCAAACTACCCCCGGACCCGGGGATCCTCTAGAGTCAACAAATT
GACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATT
AACGCCGAATTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCT
GCAGGCATGCAAGCTTAGCTTGAGCTTGGATCAGATTGTC

ad58-6620-4-1 Right border flanking (SEQ ID NO:444)

CATAACCACCATCTCAAACAATAGAACTTCCTAAGTGAAGCAATGAC
TTCAAATCTACTTGAAGGCATGGAGTATAAGCCATGTTCCTTTCAGAGGGGA
CTGTACTTCTGTAGATTACTTTCCCTCATTAACCAGATCTGGCCGGCCTACCC
AGCTTTCTTGTACAAAGTGGTGATAAACTATCAGTGTTTGAAATAATCGGAT
ATTTAATTTTCTTAGACAGTTCATTAGTAGTTGATCTTAAACATTCACGTTTT
ATTTTCTTTTCTTTTCGAATGCTAGACTCTAGTTTGGTACCCATAGGATTCGA
GTTACATGAAGCTATTGACACTGGGAGTGCTTCAAGATCTGTAAGAGGCAA
AGATTCACAAACAGAACGTGATTTCTTGGATAGTGATGTGGAGATTGTGATA
AGAACCAGCATGAGTATTACTTTTACTGCCCCTGCTGTGGTGAAGACATCAC
CAAAACAGTCAAGCTCGTGAAGAAATCAGATATTCAACCCGCAAAAAAATC
TGACAATGCAAATAAACCTATTGACACTAAGAATGGTTCAAGATCCGAAGA
CAAGAAGACAAAAAATTTGTCCTGGCTCCCTGCTTATCTCCAGAAGCTGTTT
CTTTCTGTTTATGGCCACATCAAAGACAAAGGTACCCTTTCTTTTGGTGCCTT
TGGTGTGGACAGGTGTGATTGACTTTGGTTTCTTGGCAGATTCAGGCAAGAT
AGAGGTTGATTCAAAGTCAACTAACAATGATCTTGGTACCAATAGTGAGGA

pDAS000037 Event details: ad58-6620-17-1 Left border flanking (SEQ ID NO:445)

CCGCCTTGAACAACCGCTCCGCCGTTGCTCAGTACATTATCGAGGTTA
CCAAAAAGTTCAATCCTTTATGCTTGTTTTGGCGTGTGATTCGTTACGAATG

AGTAAAATTGATTTGGTTTTTTTCTTTGAACAGCATGGTGGAGATGTGAATG
CGACGGATCATACGGGGCAGACTGCGTTGCATTGGAGTGCGGTTCGTGGTG
CGGTGCAAGTTGCGGAGCTTTTGCTTCAAGAGGGTGCAAGGGTTGATGCTAC
GGATATGTATGGATATCAGGTTCTAACCACTTCCTCTTTCTTTGTGGAGATTG
TCTTTTTGTTTCAATGCTAGTCAACTTCTTTCTTTCTTTCACAAAACTAAGTA
GTATTGCTTGTTTTGTTGTCGTTGCATTTTTTCTTATGGCTGTGTTCTGAGGTT
CATGTAGGTATATAAGCACTTCGTACTTTGCCACTTGTTTCATTTAGGCAAC
ATTGTGCACATATCTAAGTAGTTGGTCTTTGTAAAATTAGTTTGTTTGTCTTC
AAAGTATATTGAGCAGTTTCATGACTCATTATTCAAAGGTTTGTCTAAATTA
GAGAGAACTTTCATTTTGCCTGGATTTAATCAGCATTTAGAATGTTTATAGC
GATATCATTTTTAGTTGAAAAAATCTCAACAAATTGACGCTTAGACAACTTA
ATAACACATTGCGGACGTTTTTAATGTACTGAATTAACGCCGAATTGAATTC
GAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTT
AGCTTGAGCTTGGATCAGATTGTCGTTTC

ad58-6620-17-1 Right border flanking (SEQ ID NO:446)

GTATAAGCCATGTTCCTTTCAGAGGGGACTGTACTTCTGTAGATTACT
TTCCCTCATTAACCAGATCTGGCCGGCCTACCCAGCTTTCTTGTACAAAGTG
GTGATAAACTATCAGTGTTAGATCCCCGACCGACCGCCCATCCTGGACGGCC
TCGTGCATGCTGATGTTGTCAAAATCTTAGGATCCAATGAGTTTATCAGGTA
TACTTCTATCATGTATTGCTTGAGATTTTGGAGTGTTAGTAAAGATTTCAATA
AAAGAATTTTTTCAAACAAAATTTTGGGGGCTTGAAGCTAATGTTTGGAAAT
ATGTAACGGAGTTTAAATCTTTTGGCAGGCCTATATATGCGGGAAACGCCTT
GTGTAGAGTTCGCTATACTGGTGCTGGTCCTTGTGTGTTGACTATTAGAACT
ACATCTTTTCCTGTTACCCCAATAACTGAGTCAAAGAAAGCTACTATCTCTC
AGATTGATCTCTCGAAATTCAAAGAAGGTTTGTTTAGTATTATTCTCTTGTGC
ATAGCCTTTTTGSTTTTTTTTTTTTTATAAAAAAAGTTGAGTATGCTTATTGCC
CATTGC

MAPPING OF ETIPS

For each transgenic event containing a single copy insertion of the ETIP, the flanking sequence was taken following manual assembly and used as the query in a local BLAST analysis. There were a total of eight plants that had single copy

integrations identified by this process (Table 16 and Table 17). A collection of 595,478 genomic derived shotgun sequences from *Brassica oleracea* were downloaded from the NCBI GSS database and formatted as a nucleotide BLAST database. The flanking ETIP sequences were then BLASTn compared to the database and all matches were manually examined. The most significant sequence match to the flanking ETIP sequence from the *B. oleracea* database was then taken and aligned against the online *Brassica rapa* genome sequence (http://brassicadb.org/brad/blastPage.php) where the position in the genome that had the most significant sequence match was also retrieved. In instances where a only the 5′ or 3′ flanking sequences provided significant matches with the *B. oleracea* genome sequences, it was assumed that the unaligned or unmatched sequence had either; identified missing sequence from the database, or that there had been significant genome rearrangements generated during the integration of the ETIP. For the samples that generated significant BLASTn matches from the analysis the flanking ETIP sequence, the most significant *B. oleracea* GSS matching sequence along with the most significant matching sequence from the *B. rapa* genome, were then manually aligned in Sequencher™ v5.0 software (Gene Codes Corp., Ann Arbor, MI) for each of the eight single copy ETIP plants. The three sequences were then compared and the most similar sequence from either of the diploid *Brassica* species compared to the flanking ETIP was designated the genome that the ETIP was located in. For the majority of the samples significant variation did exist between the two diploid *Brassica* genome sequences and the *B. napus* derived flanking ETIP sequence showed a predominant association with one or other of the diploid sequences. There were instances however, where there was insufficient sequence variation between the diploids and a linkage group assignment may have been possible but a sub-genome assignment was not possible. The specific genome location was then predicted from the location from the *Brassica rapa* genome sequence. In instances where the ETIP was identified as being integrated into the *B. oleracea* C genome, the comparative synteny between the diploid Brassica genomes described in Parkin *et al.* (Genetics 2005, 171: 765-781) was used to extrapolate the genomic location into the *Brassica napus* C sub-genome. In addition the sequences identified were BLASTn compared to the *Arabidopsis thaliana* genomes coding sequences (TAIR 9 CDS downloaded from http://arabidopsis.org/index.jsp) and the identity of any gene sequences disrupted were identified, as well as a confirmation

of genomic location following the *Arabidopsis Brassica* synteny described in Schranz *et al.* (Trend in Plant Science 2006, 11,11: 535-542).

Table 16: BLAST search and predicted the location of these above sequences (predicted locations in *Brassica napus* genome).

Event/Vector Name	One copy of each cassette detected by Southern	LB Flanking Sequence	RB Flanking Sequence	Predicted location
pDAS000036				
em02-5788-1-1 228688	yes	yes	yes	A6
ad58-5784-2-1 232502	yes	yes	yes	A8
ad58-5898-10-1 237143	yes	yes	yes	C7
pDAS000037				
lf31-6139-2-3 234576	yes	yes	yes	A5
bm56-6315-1-1 234703	yes	yes	yes	Genomic Repeat
ad58-6372-1-1 240653	yes	yes	yes	A/C8
ad58-6620-4-1 242268	yes	yes	yes	C1
ad58-6620-17-1 242293	yes	yes	yes	A/C 3 or 8

Table 17: description of single copy ETIP containing plant from the two constructs pDAS000036 and 37, BLASTn result to a *Brassica oleracea* genome sequence data base, potential disruption of gene sequence identified through *Arabidopsis thaliana* gene comparison and predicted genome location.

pDAS000036	**BLASTn match to the C genome**	**Predicted gene disrupted**	**Predicted Location**
em02-5788-1-1 228688	Left border only value e-175	At3g30775: proline oxidase	A6
ad58-5784-2-1 232502	Left border only value e-134	At1g50940: Electron transfer flavoprotein alpha	A8
ad58-5898-10-1 237143	Left border generated two significant matches at value 0 and e-107	No significant match to *Arabidopsis* gene	C7
pDAS000037	**BLASTn match to the C genome**	**Predicted gene disrupted**	**Predicted Location**
lf31-6139-2-3 234576	Right border only value e-105	At3g08530: Clathrin	A5
bm56-6315-1-1 234703	Both borders had large numbers of matches e value 0	N/A	Genomic Repeat
ad58-6372-1-1 240653	Left and right border value e-103 and -80	No significant match to Arabidopsis genes	Equivocal location: subgenome A or C on linkage group 8
ad58-6620-4-1 242268	Left and right border value e-154 and e-48	At4g27860: Vacuolar ion transporter	C1
ad58-6620-17-1 242293	Left and right border value e-167 and e-94	Borders identified At5g20340 and At1g50930	Equivocal location: potentially sub-genome A or C and linkage group 3 or 8

The homozygous events are used to produce protoplasts via the previously described method. The protoplasts are subsequently co-transformed with a Zinc Finger Nuclease that is designed to target a Zinc Finger binding site which is incorporated within the ETIP sequence and a donor plasmid which shares homology with specific regions of the ETIP. The Zinc Finger Nuclease cleaves the ETIP locus and the donor plasmid is integrated within the genome of *Brassica napus* cells via homology directed repair. As a result of the integration of the donor plasmid, the partial *DS-red* transgene is repaired to a full length *DS-red* transgene. The expression of the now fully operational *DS-red* transgene is used to sort protoplast cells with a FACS method.

Putative transgenic plants are sorted using the FACS method described in Example 7 and the isolated protoplasts are regenerated into mature plants. The integration of the donor plasmid is confirmed within the ETIP-targeted plants using molecular confirmation methods. As such, the ETIP locus serves as a site-specific locus for gene targeted integration of a donor polynucleotide sequence.

The genomic targeting locations provide genomic locations that do not alter the plants normal phenotype. The resulting events, wherein a transgene is targeted within an ETIP present no agronomically meaningful unintended differences when the ETIP events are compared to the control plants. In addition, the protein expression levels of transgenes integrated within the ETIP locus are robustly expressed and consistent and stable across multiple genomic locations. The disclosed genomic sequences of SEQ ID NO:431 to SEQ ID NO:446 provide genomic locations within the brassica genome that are targetable for the integration of gene expression cassettes comprising a transgene.

MOLECULAR CONFIRMATION OF FAD2A INTEGRATION OF ETIPS IN CANOLA

Genomic DNA was extracted from leaf tissue of all putative transgenic plants using a DNeasy Plant Mini Kit™ (Qiagen) following the manufacturer's instructions, with the exception that tissue was eluted in 80 µl of AE buffer. Thirty milligrams of young leaf tissue from regenerated plants was snap frozen in liquid nitrogen before being ground to a powder.

Molecular characterization of the FAD2A locus was performed using three independent assays. Assays were designed and optimized using the following controls; characterized transgenic events comprising a single randomly integrated transgene, characterized transgenic event with five randomly integrated transgenes, wild-type canola c.v. DH12075 plants and non-template control reactions. The results from the three following molecular analyses are considered together in order to provide evidence for integration of the ETIP at FAD2A via HDR.

IDENTIFYING TRANSGENE INTEGRATION BY REAL-TIME POLYMERASE CHAIN REACTION

Four replicates of each plant were analyzed using primers specific to the *hph* (also described as *hpt*) target gene (SEQ ID NO:447, hpt F791 5'

CTTACATGCTTAGGATCGGACTTG 3′; SEQ ID NO:448, hpt R909 5′ AGTTCCAGCACCAGATCTAACG 3′; SEQ ID NO:449, hpt Taqman 872 5′ CCCTGAGCCCAAGCAGCATCATCG 3′ FAM) (FIG. 31) and reference gene encoding High Mobility Group protein I/Y *(HMG I/Y)* (SEQ ID NO:450, F 5′ CGGAGAGGGCGTGGAAGG 3′; SEQ ID NO:451, R 5′ TTCGATTTGCTACAGCGTCAAC 3′; SEQ ID NO:452, Probe 5′ AGGCACCATCGCAGGCTTCGCT 3′ HEX). The reactions were amplified using the following conditions: 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, with amplification data being captured at the end of each annealing step. Copy number was calculated using the ΔCq method, where ΔCq = Cq(target gene) – Cq(reference gene). Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. 25(4): p. 402-8. Plants with amplification of *hph* and *HMG I/Y* and a copy number of 0.5 or more were considered transgenic, while plants with a copy number of ≥ 0.5 and ≤ 1.2 were scored as putatively single copy. Amplification was performed on a BioRad CFX96 Touch™ Real-Time PCR Detection System with FastStart Universal Probe Master (ROX), (Roche, Basel, Switzerland).

DETECTION OF DISRUPTED FAD2A ZFN SITE

Each plant was analyzed for presence or absence of amplification of endogenous target in the disrupted locus test, which is a dominant assay. The assay is a SYBR® Green I qPCR assay and in singleplex, but with each reaction run simultaneously on the same PCR plate, targets an endogenous locus (FAD2A/2C.RB.UnE.F1, SEQ ID NO:453, 5′ CTTCCACTCCTTCCTCCTCGT*C 3′ and FAD2A/2C.RB.UnE.R1, 5′ SEQ ID NO:454, GCGTCCCAAAGGGTTGTTGA*G 3′) and the ZFN locus (locus at which the ZFN pDAB104010 binds and cuts the genome) (FAD2A.UnE.F1, SEQ ID NO:455, 5′ TCTCTACTGGGCCTGCCAGGG*C 3′ and FAD2A.UnE.R1, SEQ ID NO:456, 5′ CCCCGAGACGTTGAAGGCTAAGTACAA*A 3′) (FIG. 32). Both primer pairs were amplified using the following conditions: 98°C for 30 seconds followed by 35 cycles of (98°C for 10 seconds, 65°C for 20 seconds, 72°C for 90 seconds) then followed by 95°C for 10 seconds then a melt analysis from 50°C to 95°C with 0.5°C

increments for 0.05 seconds and a plate read at each increment. The reaction conditions are listed in Table 18.

Table 18: Single reaction reagent components and concentrations for PCR amplification.

Reaction Components	**Volume (µl)**
10 mM dNTP	0.40
5X Phusion HF Buffer	4.00
Phusion Hot Start II High-Fidelity DNA Polymerase (2U/µl) (Thermo Scientific)	0.25
Forward Primer 10 µM	0.40
Reverse Primer 10 µM	0.40
1:10000 dilution of SYBR Green I dye (Invitrogen)	1.00
Molecular Biology Grade H_2O	11.55
Genomic DNA template (~20 ng/µl)	2.00
Total Volume	20.00

Plants that had amplification of the endogenous target but no amplification of the ZFN target, were scored as positive for the disrupted locus test and were considered to have a disrupted ZFN locus. This assay was considered to be positive when the ZFN binding site on both alleles at the FAD2A locus have been disrupted.

PCR DETECTION OF TRANSGENE INTEGRATION AT FAD2A VIA HOMOLOGY DIRECTED REPAIR

Each putative plant transformant was analyzed using endpoint with PCR primers designed to amplify the transgene target *hph* (*hph*_ExoDigPC_F1, SEQ ID NO:457, 5′ TTGCGCTGACGGATTCTACAAGGA 3′ and *hph*_ExoDigPC_R1, SEQ ID NO:458, 5′ TCCATCAGTCCAAACAGCAGCAGA 3′), the FAD2A endogenous locus (FAD2A.Out.F1, SEQ ID NO:459, 5′ CATAGCAGTCTCACGTCCTGGT*C 3′ and FAD2A.Out.Rvs3, SEQ ID NO:460, 5′ GGAAGCTAAGCCATTACACTGTTCA*G 3′), the region spanning the 5′ end of any transgene inserted into the FAD2A locus via HDR, upstream of the transgene into the FAD2 A locus (FAD2A.Out.F1, SEQ ID NO:461, 5′ CATAGCAGTCTCACGTCCTGGT*C 3′ and QA520, SEQ ID NO:462, 5′ CCTGATCCGTTGACCTGCAG 3′) and the region spanning the 3′ end of any

transgene inserted into the FAD2A locus via HDR, downstream of the transgene into the FAD2 A locus (QA558, SEQ ID NO:463, 5′ GTGTGAGGTGGCTAGGCATC 3′ and FAD2A.Out.Rvs3, SEQ ID NO:464, 5′ GGAAGCTAAGCCATTACACTGTTCA*G 3′) (FIG. 33). All primer pairs were amplified using the following conditions 98°C for 30 seconds followed by 35 cycles of (98°C for 10 seconds, 65°C for 20 seconds, 72°C for 90 seconds). Reaction reagent conditions are as described in **Table 19.**

Table 19: Single reaction reagent components and concentrations for PCR amplification.

Reaction Components	**Volume (µl)**
5x Phusion HF Buffer	6.00
10 mM dNTPs	0.60
Forward Primer 10 µM	0.60
Reverse Primer 10 µM	0.60
Phusion Hot Start II High-Fidelity DNA Polymerase (2U/µl) (Thermo Scientific)	0.25
Molecular Biology Grade H_2O	19.95
Genomic DNA template (~20 ng/µl)	2.00
Total Volume	30.0

Amplification of the 5′ transgene-genome flanking target and/or amplification of the 3′ transgene-genome flanking target indicated a putative insertion event. It must be noted that due to the approximately 1,000 bp FAD2A homology arms in the pDAS000130 cassette (comprising polynucleotide sequences with 100% sequence identity to the FAD2A regions immediately upstream and downstream of the ZFN cut site), the PCR reactions were subject to false positive PCR product amplification due to PCR chimerism arising from amplification of off-target ETIP integration events. Amplification of the *hph* target confirmed transgene integration had occurred. Amplification of the FAD2A target suggests that the FAD2A locus is intact or contains only a partial insertion. Due to the size of the ETIP (11,462 bp for the ETIP cassettes or 13,472 bp including the FAD2A homologous arms and the ETIP cassettes) it is expected that the FAD2A primers would not amplify a product when an intact ETIP is integrated into the FAD2A locus.

SOUTHERN DETECTION OF FAD2A EDITING

Plants that had amplification of either a 5′ genome-transgene flanking target product and/or amplification of a 3′ transgene-genome flanking target, or no amplification of the ZFN locus target, or both, were subject to Southern analysis for detection of transgene integration at the FAD2A locus. Genomic DNA was purified from 5 g of leaf tissue using a modified CTAB method (Maguire, T.L., G.G. Collins, and M. Sedgley *A modified CTAB DNA extraction procedure for plants belonging to the family proteaceae.* Plant Molecular Biology Reporter, 1994. 12(2): p. 106-109). Next, 12 µg of genomic DNA was digested with *Kpn1*-HF (New England BioLabs) and digestion fragments were separated by electrophoresis on a 0.8% agarose gel before transfer to membrane using a standard Southern blotting protocol. Primers to *FAD2A* 5′ target region (F, SEQ ID NO:465, 5′ AGAGAGGAGACAGAGAGAGAGT 3′ and R, SEQ ID NO:466, 5′ AGACAGCATCAAGATTTCACACA 3′), *FAD2A* 3′ target region (F, SEQ ID NO:467, 5′ CAACGGCGAGCGTAATCTTAG 3′ and R, SEQ ID NO:468, 5′ GTTCCCTGGAATTGCTGATAGG 3′) and *hph* (F, SEQ ID NO:469, 5′ TGTTGGTGGAAGAGGATACG 3′ and R, SEQ ID NO:470, 5′ ATCAGCAGCAGCGATAGC 3′) were used to generate probes to detect the presence of the ETIP within the FAD2A locus using the DIG EASY HYB SYSTEM® (Roche, South San Francisco, CA) following the manufacturer's instructions (FIG. 34). Hybridization was performed at 42° C for *FAD2A* 5′ region, 45°C for *FAD2A* 3′ region and 42° C for detection of *hph*.

Membrane-bound genomic DNA was probed in a specific order; firstly *FAD2A* 5′ sequences were probed, then the *FAD2A* 3′ sequences were probe, and finally the *hph* sequences were probed (FIG. 35). The rational for this is as follows. The first probe (FAD2A 5′) is the diagnostic probe, and if the ETIP has integrated into FAD2A via perfect HDR, a 5,321 bp fragment will be visible on the membrane. The resulting band size is easily differentiated during electroporation and will sit close to the 5,148 bp fragments in the DIG labeled Roche DNA MOLECULAR WEIGHT MARKER III® (Roche, Indianapolis, IN). The second probe of the membrane is with the FAD2A 3′ probe and an edited plant will have a 22,433 bp fragment whereas an unedited plant will have a 16,468 bp fragment. The same 22,433 bp fragment identified with the FAD2A 3′ probe should also be bound by and identified with the *hph* probe. These fragments are difficult to differentiate on a gel as they are extremely large and it may be difficult to determine

any difference between a fragment occurring above or below the largest, 21,226 bp fragment in the DIG labeled Roche DNA MOLECULAR WEIGHT MARKER III®. As such, these probes provide evidence that may strengthen the identification of ETIP integration into FAD2A via homology directed repair (HDR), by visualization of a 5 kb fragment using the FAD2A 5′ probe. The restriction enzyme, KpnI was the only suitable restriction endonuclease for use in this assay, as *KpnI* sites occurred in a single locus of the cut the ETIP cassette in a single locus, and was present in two sites of the FAD2A ZFN locus. One site was located upstream and the second site located downstream of the FAD2A homology arms. In addition, *KpnI* is not methylation sensitive, and is available as a recombinant enzyme with increased fidelity (New England Biolabs).

RESULTS OF MOLECULAR AND SOUTHERN ANALYSIS

Following transfection, culturing, and selection the transgenic plants were transferred to soil. From this process, 139 plants survived and had tissue sampled for gDNA extraction and analysis. All 139 plants were analyzed for copy number estimation. Of these 139 plants, 56 were positive for the ETIP and 11 of the 56 positive plants had a putative single copy integration (FIG. 36) (Table 20). Of the 56 plants that were positive for ETIP integration, amplification of the FAD2A 5′-genome-transgene flanking sequence occurred in 7 plants. Amplification of the FAD2A 3′-transgene-genome flanking sequence did not occur in any of the 56 plants that were positive for ETIP integration. Additionally, of the 56 plants that were positive for transgene integration, 11 plants were positive for the disrupted locus qPCR test. Fourteen plants that were positive for amplification of the FAD2A 5′ genome-transgene flanking sequence and/or positive for the disrupted locus qPCR test were subject to Southern analysis, with the 3 probes described above. Of the 14 plants advanced for Southern analysis, all of the plants showed partial integration within the FAD2A locus, but none of these plants showed evidence of a complete full-length integration of the ETIP at the FAD2A locus via HDR when probed with the FAD2A 5′ probe, FAD2A 3′ and *hph* probes. No bands that appeared to be i) larger than WT and ii) identical to bands observed for those samples when probed with FAD2A 3′ probe (**Table 20**).

Table 20: Overview of outcomes from analysis of ETIP integration.

Number of plants surviving in soil	Number of plants sampled	Number of plants for which qPCR copy number analysis was completed	Number of plants positive for ETIP integration	Number of plants comprising a putative single copy insert	Number of ETIP/ *FAD2* in-out 5′ reaction	Number of ETIP/ *FAD2* in-out 3′ reactions	Number of locus disrupted qPCR tests	ETIP on-target (Southern)
139	139	139	56	11	7 (from 56)	0 (from 56)	9 (from 56)	0 (from 14)

RESULTS OF ETIP TRANSGENIC CANOLA TRANSFORMED WITH PDAS000130 AND PDAB104010.

The transgenic *Brassica napus* events which are produced via transformation of pDAS000130 and pDAB104010 result in the integration of a single copy, full length T-strand insertion of the ETIP polynucleotide sequence from pDAS000130 within the FAD2A locus. Three to four events are fully characterized and confirmed to contain the integrated ETIP. The confirmation is completed using an in-out PCR amplification method, and further validated via Southern blot. The selected T_0 events are grown to the T_1 stage of development. The T_1 plants are re-screened to determine the zygosity of the integrated T-strand. Screened events are categorized as homozygous, hemizygous, or null.

The homozygous events are used to produce protoplasts via the previously described method. The protoplasts are subsequently co-transformed with a Zinc Finger Nuclease that is designed to target a Zinc Finger binding site which is incorporated within the ETIP sequence and a donor plasmid which shares homology with specific regions of the ETIP wherein the donor is integrated within the ETIP via an HDR mechanism. Likewise, the protoplasts are subsequently co-transformed with a Zinc Finger Nuclease that is designed to target a Zinc Finger binding site which is incorporated within the ETIP sequence and a donor plasmid which does not share homology with specific regions of the ETIP, wherein the donor is integrated within the ETIP via an non-homologous end joining mechanism. The Zinc Finger Nuclease cleaves the ETIP locus and the donor plasmid is integrated within the genome of *Brassica napus* cells via homology directed repair or non-homologous end joining. As a

result of the integration of the donor plasmid, the partial *DS-red* transgene is repaired to a full length *DS-red* transgene. The expression of the now fully operational *DS-red* transgene is used to sort protoplast cells with a FACS method. Putative transgenic plants are sorted using the FACS method described in Example 7 and the isolated protoplasts are regenerated into mature plants. The integration of the donor plasmid is confirmed within the ETIP-targeted plants using molecular confirmation methods. As such, the ETIP locus serves as a site-specific locus for gene targeted integration of a donor polynucleotide sequence.

RESULTS OF ETIP TRANSGENIC CANOLA TRANSFORMED WITH ZINC FINGER NUCLEASE AND PDAS000271-PDAS000275 ETIP CONSTRUCTS

The transgenic *Brassica napus* events which are produced via transformation of ETIP and Zinc Finger Nuclease constructs result in the integration of a single copy, full length T-strand insertion of the ETIP polynucleotide sequence from pDAS000273 or pDAS275 within the FAD3A locus, and from pDAS000271, pDAS000272 or pDAS000274 into the FAD3C locus. Three to four events are fully characterized and confirmed to contain the integrated ETIP. The confirmation is completed using an in-out PCR amplification method, and further validated via Southern blot. The selected T_0 events are grown to the T_1 stage of development. The T_1 plants are res-screened to determine the zygosity of the integrated T-strand. Screened events are categorized as homozygous, hemizygous, or null.

The homozygous events are used to produce protoplasts via the previously described method. The protoplasts are subsequently co-transformed with a Zinc Finger Nuclease that is designed to target a Zinc Finger binding site which is incorporated within the ETIP sequence and a donor plasmid which shares homology with specific regions of the ETIP. The Zinc Finger Nuclease cleaves the ETIP locus and the donor plasmid is integrated within the genome of *Brassica napus* cells via homology directed repair. As a result of the integration of the donor plasmid, the partial *DS-red* transgene is repaired to a full length *DS-red* transgene. The expression of the now fully operational *DS-red* transgene is used to sort protoplast cells with a FACS method. Putative transgenic plants are sorted using the FACS method described in Example 7 and the isolated protoplasts are regenerated into mature plants. The integration of the donor plasmid is confirmed within the ETIP-targeted plants using molecular

confirmation methods. As such, the ETIP locus serves as a site-specific locus for gene targeted integration of a donor polynucleotide sequence.

EXAMPLE 7: FACS BASED SORTING OF PROTOPLAST CELLS

Brassica napus protoplasts that were transfected with the DS-Red control construct, pDAS000031, were sorted via FACS-mediated cell sorting using a BD Biosciences Influx-Cell sorter™ (San Jose, CA). The protoplast cells were isolated and transfected as described in Example 3. After the cells had been transfected with pDAS000031, the cells were sorted using the FACS sorter with the conditions described in Table 21.

Table 21: Conditions used for sorting protoplast cells transfected with pDAS000031.

Parameters	
Drop frequency	6.1 KHz
Nozzle diameter	200 μm
Sheath pressure	4 psi
Recovery media	W5 media
Culture conditions	Bead type culture using sea-plaque agarose and sodium alginate
Sort criteria	Sorting based on chlorophyll autofluorescence, reporter gene expression (Ds-Red)
Sort recovery (%)	50-75
Viability post sorting (%)	>95

The protoplasts which expressed the *DS-red* transgene were sorted and isolated. The FACS isolated protoplasts were counted using the sorter. About $1x10^5$ to $1.8x10^5$ of cells were placed in a well of a 24-well micro titer plate on the first day after the FACS isolation. The cells were transferred to a bead culture for 5 to 20 days. Similar conditions were tested, wherein about $1x10^4$ of cells were placed in a well of a 2 or 4-well micro titer plate on the second day after the FACS isolation. The various conditions that were tested resulted in the recovery of cells at a viability or 95 – 98% of

the total isolated protoplast cells. The FACS sorted protoplast cells were transferred to a bead culture for 3 – 20 days. The FACS sorted protoplast cells were regenerated into plants on media which contained 1.5 mg/mL of hygromycin using the above described protocol. The putative transgenic plants were confirmed to contain an intact T-strand insert from pDAS000031 via molecular conformation protocols.

TARGETING OF ETIP LINES WITH ZFN MEDIATED HOMOLOGOUS RECOMBINATION OF DS-RED

A canola line containing the T-strand insert from pDAS000036 was obtained and confirmed via molecular characterization to contain a full length, single copy of the T-strand. This canola event was labeled as pDAS000036 – 88 and was used to produce protoplasts via the previously described method. The protoplasts were isolated and ~50,000 canola protoplast cells were subsequently co-transformed with a Zinc Finger Nuclease, either pDAS000074 (FIG. 25) or pDAS000075 (FIG. 26), that was designed to target the Zinc Finger binding sites incorporated within the ETIP sequence and a donor plasmid, pDAS000064, pDAS000068, pDAS000070, or pDAS000072 (FIG. 27, FIG. 28, FIG. 29, and FIG. 30, respectively), which shares homology with specific regions of the ETIP. FIG. 19 and FIG. 20 provide illustrations of the homology directed repair which results in the site-specific integration of the *Ds-red* transgene via Zinc Finger Nuclease mediated homologous recombination. The Zinc Finger Nuclease was designed to cleave the ETIP locus at a defined Zinc Finger binding sequence, thereby creating a double strand break within the genome. Next, the donor plasmid was integrated within the genome of *Brassica napus* protoplast cells via homology directed repair. The intron-1 and intron-2 regions of the donor plasmid share homology with the corresponding intron-1 and intron-2 regions of the ETIP locus. As a result of the integration of the donor plasmid, the partial *DS-red* transgene was repaired to a full length, highly expressing *DS-red* transgene. The expression of the fully operational *DS-red* transgene was used to sort protoplast cells with the above described FACS method. As such, the ETIP locus serves as a site-specific locus for targeted integration of a donor polynucleotide sequence. Finally, the isolated protoplasts can be sorted and regenerated into mature plants. The integration of the donor plasmid can be confirmed within the ETIP-targeted plants using molecular confirmation methods.

The donor plasmid DNA and ZFN plasmid DNA were mixed at various concentrations and used to transfect the canola protoplast cells containing Event pDAS000036 – 88, and the transgenic protoplast cells were sorted using the FACS transfection that was previously described. Table 22 describes the various transfection experiments and the DNA concentrations which were used for the transfection of the canola protoplasts containing Event pDAS000036 – 88. The ZFN and donor plasmid DNA was isolated and prepared for the transfections via the previously described methods.

Table 22: Donor plasmids and Zinc Finger Nuclease constructs used for the ETIP targeting experiments. The DNA concentrations were used at the indicated ratio of donor to Zinc Finger Nuclease, for a total concentration of 30 micrograms of plasmid DNA per transfection.

REACTIONS	PLASMIDS	DONOR PLASMID DNA (µg)	ZFN PLASMID DNA (µg)	TOTAL (µg)
1	pDAS000074	-	30	30
2	pDAS000075	-	30	30
3	pDAS000064 + pDAS000074	26	4	30
4	pDAS000064 + pDAS000075	26	4	30
5	pDAS000068 + pDAS000074	28	2	30
6	pDAS000068 + pDAS000075	28	2	30
7	pDAS000070 + pDAS000074	28	2	30
8	pDAS000070 + pDAS000075	28	2	30
9	pDAS000072 + pDAS000074	28	2	30
10	pDAS000072 + pDAS000075	28	2	30
11	pDAS000064	30	-	30
12	pDAS000068	30	-	30
13	pDAS000070	30	-	30
14	pDAS000072	30	-	30

After the transfection experiments were completed the protoplasts were incubated at room temperature for 48 hours and sorted using the above described FACS protocol. Each experiment was sorted independently and Zinc Finger-mediated introgression of a transgene was confirmed via identification of individual events which expressed the *DS-red* transgene. FIGS. 21 – 24 show the results of the FACS sorting. As the results depicted in the graphs indicate, multiple events were produced which contained an intact fully integrated *DS-red* transgene. These multiple *Ds-Red* events were the result of Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus. This site-specific integration resulted in a highly expressing, complete copy of the *Ds-Red* transgene. The frequency of the *Ds-Red* transgene expression ranged from about 0.03 – 0.07% of the total canola protoplast cells (~50,000). However, the frequency of transfection efficiency for the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus was much higher and ranged from about 0.07 – 0.64%.

FIG. 21 shows the results of the transfections in which the donor plasmid and ZFN plasmid were co-transformed. The top graph, wherein donor, pDAS000064, and the Zinc Finger Nuclease, pDAS000074, were co-transformed at a ratio of 26 μg to 4 μg of plasmid DNA resulted in the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus at a recombination frequency of about 0.03% of the ~50,000 canola protoplast cells. In actuality, the recombination frequency is much higher. Of the ~50,000 canola protoplast cells which were provided during the transfection experiment, only about 10 – 30% of these canola protoplast cells are actually transformed. As such, the actual Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus transfection efficiency ranges from about 0.22 – 0.07%. Similarly the bottom graph, wherein donor, pDAS000064, and the Zinc Finger Nuclease, pDAS000075, were co-transformed at a ratio of 26 μg to 4 μg of plasmid DNA resulted in the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus at a recombination frequency of about 0.03% of the ~50,000 canola protoplast cells. In actuality, the recombination frequency is much higher. Of the ~50,000 canola protoplast cells which were provided during the transfection experiment, only about 10 – 30% of these cells are actually transfected. As such, the actual Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus transfection efficiency ranges from about

0.26 – 0.08%. The results of the zinc finger mediated homology directed repair are significantly greater than the negative control experiments, wherein only one protoplast of ~50,000 was identified to have red fluorescence, thereby resulting in a recombination frequency of 0.00%, as shown in FIG. 20.

FIG. 22 shows the results of the transfections in which the donor plasmid and ZFN plasmid were co-transformed. The top graph, wherein donor, pDAS000068, and the Zinc Finger Nuclease, pDAS000074, were co-transformed at a ratio of 28 μg to 2 μg of plasmid DNA resulted in the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus at a recombination frequency of about 0.03% of the ~50,000 canola protoplast cells. In actuality, the recombination frequency is much higher. Of the ~50,000 canola protoplast cells which were provided during the transfection experiment, only about 10 – 30% of these canola protoplast cells are actually transformed. As such, the actual Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus transfection efficiency ranges from about 0.22 – 0.07%. Similarly the bottom graph, wherein donor, pDAS000068, and the Zinc Finger Nuclease, pDAS000075, were co-transformed at a ratio of 28 μg to 2 μg of plasmid DNA resulted in the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus at a recombination frequency of about 0.04% of the ~50,000 canola protoplast cells. In actuality, the recombination frequency is much higher. Of the ~50,000 canola protoplast cells which were provided during the transfection experiment, only about 10 – 30% of these cells are actually transfected. As such, the actual Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus transfection efficiency ranges from about 0.38 – 0.12%. The results of the zinc finger mediated homology directed repair are significantly greater than the negative control experiments, wherein only one protoplast of ~50,000 was identified to have red fluorescence, thereby resulting in a recombination frequency of 0.00%, as shown in FIG. 20.

FIG. 23 shows the results of the transfections in which the donor plasmid and ZFN plasmid were co-transformed. The top graph, wherein donor, pDAS000070, and the Zinc Finger Nuclease, pDAS000074, were co-transformed at a ratio of 28 μg to 2 μg of plasmid DNA resulted in the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus at a recombination frequency of about 0.07% of the ~50,000 canola protoplast cells. In actuality, the recombination frequency

is much higher. Of the ~50,000 canola protoplast cells which were provided during the transfection experiment, only about 10 – 30% of these canola protoplast cells are actually transformed. As such, the actual Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus transfection efficiency ranges from about 0.64 – 0.21%. Similarly the bottom graph, wherein donor, pDAS000070, and the Zinc Finger Nuclease, pDAS000075, were co-transformed at a ratio of 28 μg to 2 μg of plasmid DNA resulted in the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus at a recombination frequency of about 0.04% of the ~50,000 canola protoplast cells. In actuality, the recombination frequency is much higher. Of the ~50,000 canola protoplast cells which were provided during the transfection experiment, only about 10 – 30% of these cells are actually transfected. As such, the actual Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus transfection efficiency ranges from about 0.34 – 0.11%. The results of the zinc finger mediated homology directed repair are significantly greater than the negative control experiments, wherein only one protoplast of ~50,000 was identified to have red fluorescence, thereby resulting in a recombination frequency of 0.00%, as shown in FIG. 20.

FIG. 24 shows the results of the transfections in which the donor plasmid and ZFN plasmid were co-transformed. The top graph, wherein donor, pDAS000072, and the Zinc Finger Nuclease, pDAS000074, were co-transformed at a ratio of 28 μg to 2 μg of plasmid DNA resulted in the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus at a recombination frequency of about 0.07% of the ~50,000 canola protoplast cells. In actuality, the recombination frequency is much higher. Of the ~50,000 canola protoplast cells which were provided during the transfection experiment, only about 10 – 30% of these canola protoplast cells are actually transformed. As such, the actual Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus transfection efficiency ranges from about 0.62 – 0.20%. Similarly the bottom graph, wherein donor, pDAS000072, and the Zinc Finger Nuclease, pDAS000075, were co-transformed at a ratio of 28 μg to 2 μg of plasmid DNA resulted in the Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus at a recombination frequency of about 0.05% of the ~50,000 canola protoplast cells. In actuality, the recombination frequency is much higher. Of the ~50,000 canola protoplast cells which were provided

during the transfection experiment, only about 10 – 30% of these cells are actually transfected. As such, the actual Zinc Finger Nuclease mediated integration of the donor DNA construct within the ETIP genomic locus transfection efficiency ranges from about 0.44 – 0.14%. The results of the zinc finger mediated homology directed repair are significantly greater than the negative control experiments, wherein only one protoplast of ~50,000 was identified to have red fluorescence, thereby resulting in a recombination frequency of 0.00%, as shown in FIG. 20.

Selected explants were transferred and cultured upon regeneration media containing phosphothrinocin. After the culturing period the surviving explants were transferred to elongation medium and root induction medium for culturing and plant development. Whole plants that consisted of developed root and shoot structures were transferred into soil and further propagated in the greenhouse. The tissue culture process utilized media and culture conditions as previously described above. The results of plants produced from the tissue culturing process are shown in Table 23 below.

Table 23: Results of tissue culturing process.

Construct	No. of explants transferred to regeneration media: B2-2 PPT	No. of explants surviving in regeneration media: B2-2 PPT	No. of shoots surviving in shoot elongation media: SEM- 2 PPT	No. of shoots surviving in RIM - 2 PPT	No. of rooted plants transferred to soil
pDAS000064+ pDAS000074-I	**4021**	**36**	**74**	**1**	--
pDAS000064+ pDAS000074-II	**1300**	**90**	**13**	**1**	1
pDAS000064+ pDAS000074-III	**1760**	**15**	**36**	**2**	--
pDAS000068+ pDAS000074-I	**1700**	**100**	**4**	**8**	2
pDAS000068+ pDAS000074-II	**1630**	--	**29**	**15**	--
pDAS000068+ pDAS000074-III	**2523**	**30**	**11**	**1**	--
pDAS000070+ pDAS000074-I	**2084**	**10**	**34**	**1**	--

Construct	No. of explants transferred to regeneration media: B2-2 PPT	No. of explants surviving in regeneration media: B2-2 PPT	No. of shoots surviving in shoot elongation media: SEM- 2 PPT	No. of shoots surviving in RIM - 2 PPT	No. of rooted plants transferred to soil
pDAS000070+ pDAS000074-II	4151	--	88	7	--
pDAS000070+ pDAS000074-III	1480	415	14	0	--
pDAS000072+ pDAS000074-I	1980	7	19	16	--
pDAS000072+ pDAS000074-II	1050	0	0	0	--
pDAS000072+ pDAS000074-III	1200	--	2	0	--
pDAS000064+ pDAS000074-I	556	--	8	1	--
pDAS000064+ pDAS000074-II	581	13	7	--	--
pDAS000064+ pDAS000074-III	1160	90	17	1	--
pDAS000068+ pDAS000074-I	516	0	13	--	--
pDAS000068+ pDAS000074-II	1725	55	19	3	--
pDAS000068+ pDAS000074-III	930	57	0	--	--
pDAS000070+ pDAS000074-I	600	8	3	--	--
pDAS000070+ pDAS000074-II	4410	1410	360	3	--
pDAS000070+ pDAS000074-III	2350	108	51	8	--
pDAS000072+ pDAS000074-I	1660	10	19	3	1

Construct	No. of explants transferred to regeneration media: B2-2 PPT	No. of explants surviving in regeneration media: B2-2 PPT	No. of shoots surviving in shoot elongation media: SEM- 2 PPT	No. of shoots surviving in RIM - 2 PPT	No. of rooted plants transferred to soil
pDAS000072+ pDAS000074-II	**175**	--	**13**	--	--
pDAS000072+ pDAS000074-III	**250**	**9**	**2**	--	--

CONSTRUCTS	No. of protoplasts recovered	No. of explants transferred to regeneration media: B2-2 PPT	No. of explants surviving in regeneration media: B2-2 PPT	No. of shoots recovered on shoot elongation media: SEM- 2 PPT	No. of shoots transferred to RIM - 2 PPT	No. of rooted plants transferred to soil
pDAS000064 + pDAS000074	$3X10^5$	--	--	--	--	--
pDAS000068 + pDAS000074	$1X10^5$	**114**	**12**	**1**	--	--
pDAS000070 + pDAS000074	$3X10^5$	**478**	**391**	--	--	--
pDAS000072 + pDAS000074	$3X10^5$	**81**	**12**	--	--	--
pDAS000064 + pDAS000074	$3X10^5$	**38**	**7**	--	--	--
pDAS000068 + pDAS000074	$3X10^5$	--	--	--	--	--
pDAS000070 + pDAS000074	$1X10^5$	**80**	**7**	**1**	--	--
pDAS000072 + pDAS000074	$3X10^5$	**7**	**3**	--	--	--

The FACS sorting method is directly applicable to screen any fluorescent transgene sequence and is used to isolate a proportion of any protoplast, herein *Brassica napus* protoplast cells that are targeted with a fluorescent transgene via homology mediated repair within a specific site in the ETIP region within a genomic locus.

While certain exemplary embodiments have been described herein, those of ordinary skill in the art will recognize and appreciate that many additions, deletions, and modifications to the exemplary embodiments may be made without departing from the

scope of the following claims. In addition, features from one embodiment may be combined with features of another embodiment.

5

CLAIMS

What may be claimed is:

1. A method for generating a plant from a population of plant cells comprising isolating a plant protoplast comprising a polynucleotide of interest, the method comprising:

providing a population of plant protoplasts having at least one protoplast comprising a polynucleotide of interest and a fluorescent marker, wherein the population is substantially free of plant protoplasts comprising the fluorescent marker and not comprising the polynucleotide of interest; wherein the plant protoplast is encapsulated by sodium alginate;

separating the at least one protoplast comprising the polynucleotide of interest and the fluorescent marker from the remaining plant protoplasts in the population, thereby isolating a plant protoplast comprising the polynucleotide of interest;

regenerating a plant from said isolated plant protoplast; and

culturing said plant.

2. The method according to claim 1, wherein separating the at least one protoplast comprises utilizing flow cytometry.

3. The method according to claim 1, wherein separating the at least one protoplast comprises utilizing fluorescence-activated cell sorting (FACS).

4. The method according to claim 1, wherein the fluorescent marker is a fluorescent polypeptide that is expressed from a polynucleotide in the plant protoplast.

5. The method according to claim 1, wherein the polynucleotide of interest encodes a polypeptide of interest.

6. The method according to claim 5, wherein the polypeptide of interest is a zinc-finger nuclease.

7. The method according to claim 1, wherein the population of plant protoplasts is obtained from a plant tissue.

8. The method according to claim 1, comprising separating a plurality of protoplasts comprising the polynucleotide of interest and the fluorescent marker.

9. The method according to claim 1, wherein the plant is a monocot or dicot.

10. A plant regenerated by isolating a plant protoplast comprising a polynucleotide of interest integrated into the genome of the plant protoplast, the method comprising:

providing a population of plant protoplasts having at least one protoplast comprising a polynucleotide of interest and a fluorescent marker; wherein the plant protoplast is encapsulated by sodium alginate;

recovering microcalli from the population of protoplasts comprising the polynucleotide of interest and the fluorescent marker wherein the at least one protoplast comprises the polynucleotide of interest and the fluorescent marker has been transformed with the polynucleotide of interest and a polynucleotide encoding the fluorescent marker;

regenerating a plant from said microcalli; and

culturing said plant.

11. The method according to claim 9, wherein the polynucleotide of interest and the polynucleotide encoding the fluorescent marker were both present in a nucleic acid molecule used to transform the at least one protoplast comprising the polynucleotide of interest and the fluorescent marker.

12. The method according to claim 9, wherein the polynucleotide of interest and the polynucleotide encoding the fluorescent marker are integrated in the genome of the at least one protoplast comprising the polynucleotide of interest and the fluorescent marker.

13. The method according to claim 12, wherein the polynucleotide of interest and the polynucleotide encoding the fluorescent marker are integrated in a site-specific manner in the genome of the at least one protoplast.

14. The method according to claim 13, wherein the polynucleotide of interest and the polynucleotide encoding the fluorescent marker are integrated in a site-specific manner by utilizing a zinc-finger nuclease.

15. A method for producing a transgenic plant, the method comprising:

providing a population of plant protoplasts having at least one protoplast comprising a polynucleotide of interest and a fluorescent marker, wherein the at least one protoplast comprises a site-specific nuclease, such that the polynucleotide of interest is capable of being integrated in the genome of the at least one plant protoplast by homologous recombination at a recognition site of the site-specific nuclease and wherein the plant protoplast is encapsulated by sodium alginate;

separating the at least one protoplast comprising the polynucleotide of interest and the fluorescent marker from the remaining plant protoplasts in the population;

regenerating the transgenic plant from the at least one protoplast; and

culturing said transgenic plant.

16. The plant produced by the method according to claim 15, wherein the plant produces a polypeptide of interest that is encoded by the polynucleotide of interest.

17. The plant produced by the method according to claim 15, wherein the plant comprises a value-added trait conferred to the plant by the polynucleotide of interest.

18. A method of producing seed comprising FACS sorting, culturing/optimizing and regeneration, culture plant, recover seed.

FIG 1A

```
                                    1                                                 40
FAD2-3 (SEQ ID NO:8)          (1)   ATGGGTGCAGGTGGAAGAATGCAAGTGTCTCCTCCCTCCA
 FAD2A (SEQ ID NO:5)          (1)   ATGGGTGCAGGTGGAAGAATGCAAGTGTCTCCTCCCTCCA
FAD2-2 (SEQ ID NO:7)          (1)   ATGGGCGCAGGTGGAAGAATGCAAGTCTCTCCTCCCTCCA
FAD2-1 (SEQ ID NO:6)          (1)   ATGGGTGCAGGTGGAAGAATGCAAGTCTCTCCTCCCTCCA

                                    41                                                80
FAD2-3 (SEQ ID NO:8)         (41)   AGAAGTCTGAAACCGACACCATCAAGCGCGTACCCTGCGA
 FAD2A (SEQ ID NO:5)         (41)   AAAAGTCTGAAACCGACAACATCAAGCGCGTACCCTGCGA
FAD2-2 (SEQ ID NO:7)         (41)   GCTGCCCCGAAACCAAAACCGTCAAACGCGTGCCCTGCGA
FAD2-1 (SEQ ID NO:6)         (41)   GCTGCCCCGGAACCAACAGCGTCAAACGCGTCCCCTGCGA

                                    81                                               120
FAD2-3 (SEQ ID NO:8)         (81)   GACACCGCCCTTCACTGTCGGAGAACTCAAGAAAGCAATC
 FAD2A (SEQ ID NO:5)         (81)   GACACCGCCCTTCACTGTCGGAGAACTCAAGAAAGCAATC
FAD2-2 (SEQ ID NO:7)         (81)   GACACCACCCTTCACTCTCGGAGACCTCAAGAAAGCAATC
FAD2-1 (SEQ ID NO:6)         (81)   GACACCACCATTCACTCTCGGAGACCTCAAGAAAGCAATC

                                   121                                               160
FAD2-3 (SEQ ID NO:8)        (121)   CCACCGCACTGTTTCAAACGCTCGATCCCTCGCTCTTTCT
 FAD2A (SEQ ID NO:5)        (121)   CCACCGCACTGTTTCAAACGCTCGATCCCTCGCTCTTTCT
FAD2-2 (SEQ ID NO:7)        (121)   CCACCTCACTGCTTCAAACGCTCCATCCCTCGCTCCTTCT
FAD2-1 (SEQ ID NO:6)        (121)   CCACCTCACTGCTTCAAACGCTCCATCCCACGCTCCTTCT

                                   161                                               200
FAD2-3 (SEQ ID NO:8)        (161)   CCTACCTCATCTGGGACAT--CATCATAGCCTCCTGCTTC
 FAD2A (SEQ ID NO:5)        (161)   CCTACCTCATCTGGGACAT--CATCATAGCCTCCTGCTTC
FAD2-2 (SEQ ID NO:7)        (161)   CCTACCTCCTCTTCGACAT--CCTCGTCTCCTCCTCCCTC
FAD2-1 (SEQ ID NO:6)        (161)   CCT-CTTCGACATCATCATCTCCTCCTGGCTCCTCCCTC

                                   201                                               240
FAD2-3 (SEQ ID NO:8)        (199)   TACTACGTCGCCACCACTTACTTCCCTCTCCTCCCTCACG
 FAD2A (SEQ ID NO:5)        (199)   TACTACGTCGCCACCACTTACTTCCCTCTCCTCCCTCACG
FAD2-2 (SEQ ID NO:7)        (199)   TACCACCTCTCCACACGCCTACTTCCCTCTCCTCCCCCACC
FAD2-1 (SEQ ID NO:6)        (200)   TACCACCTCTCCACACGCCTACTTCCCTCTCC---------
```

FIG. 1A

FIG 1B

```
                                  241                                 280
FAD2-3 (SEQ ID NO:8)    (239) CTCTCTCCTACTTCGCCTGGCCTCTCTACTGGGCCTGCCA
 FAD2A (SEQ ID NO:5)    (239) CTCTCTCCTACTTCGCCTGGCCTCTCTACTGGGCCTGCCA
FAD2-2 (SEQ ID NO:7)    (239) CTCTCCCTTACCTCGCCTGGCCCCTCTACTGGGCCTGCCA
FAD2-1 (SEQ ID NO:6)    (231) ------CTTACCTCGCCTGACCCCTCTACTGGGCCTGCCA

                                  281                                 320
FAD2-3 (SEQ ID NO:8)    (279) AGGGTGCGTCCTAACCGGCGTCTGGGTCATAGCCCACGAG
 FAD2A (SEQ ID NO:5)    (279) GGGCTGCGTCCTAACCGGCGTCTGGGTCATAGCCCACGAG
FAD2-2 (SEQ ID NO:7)    (279) AGGCTGCGTCCTAACGGGCCTCTGGGTCATCGCCCACGAA
FAD2-1 (SEQ ID NO:6)    (265) AGGCTGCGTCCTAACGGGCCTCTGGGTCATAGCCCACGAG

                                  321                                 360
FAD2-3 (SEQ ID NO:8)    (319) TGCGGCCACCACGCCTTCAGCGACTACCAGTGGCTTGACG
 FAD2A (SEQ ID NO:5)    (319) TGCGGCCACCACGCCTTCAGCGACTACCAGTGGCTGGACG
FAD2-2 (SEQ ID NO:7)    (319) TGCGGCCACCACGCCTTCAGCGACCACCAGTGGCTGGACG
FAD2-1 (SEQ ID NO:6)    (305) TGCGGCCACCACGCCTTCAGCGACCACCAGTGGCTGGACG

                                  361                                 400
FAD2-3 (SEQ ID NO:8)    (359) ACACCGTCGGTCTCATCTTCCACTCCTTCCTCCTCGTCCC
 FAD2A (SEQ ID NO:5)    (359) ACACCGTGGGCCTCATCTTCCACTCCTTCCTCCTCGTCCC
FAD2-2 (SEQ ID NO:7)    (359) ACGCCGTGGGCCTCGTCTTCCACTCCTTCCTCCTCGTCCC
FAD2-1 (SEQ ID NO:6)    (345) ACGCCGCGGGCCTCGTCTTCCACTCCTTCCTCCTCGTCCC

                                  401                                 440
FAD2-3 (SEQ ID NO:8)    (399) TTACTTCTCCTGGAAGTACAGTCATCGACGCCACCATTCC
 FAD2A (SEQ ID NO:5)    (399) TTACTTCTCCTGGAAGTACAGTCATCGACGCCACCATTCC
FAD2-2 (SEQ ID NO:7)    (399) TTACTTCTCCTGGAAGTACAGCCATCGACGCCACCATTCC
FAD2-1 (SEQ ID NO:6)    (385) GTACTTCTCCTGGAAGTACATCCAT-GACGCCACCATTCC

                                  441                                 480
FAD2-3 (SEQ ID NO:8)    (439) AACACTGGCTCCCTCGAGAGAGACGAAGTGTTTGTCCCCA
 FAD2A (SEQ ID NO:5)    (439) AACACTGGCTCCCTCGAGAGAGACGAAGTGTTTGTCCCCA
FAD2-2 (SEQ ID NO:7)    (439) AACACCGGATCCCTCGAGAGGGATGAAGTGTTCGTCCCCA
FAD2-1 (SEQ ID NO:6)    (424) AACACCGGATCCCTCGATAGGGACGAAGTGTTCGTCCCCA
```

FIG. 1B

FIG 1C

```
                                  481                                 520
FAD2-3 (SEQ ID NO:8)    (479) AGAAGAAGTCAGACATCAAGTGGTACGGCAAGTACCTCAA
 FAD2A (SEQ ID NO:5)    (479) AGAAGAAGTCAGACATCAAGTGGTACGGCAAGTACCTCAA
FAD2-2 (SEQ ID NO:7)    (479) AGAAGAAATCCGACATCAAGTGGTACGGAAAGTACCTCAA
FAD2-1 (SEQ ID NO:6)    (464) AGAAGAAATCCGACATCAAGTGGTACGGCAAGTACCTCAA

                                  521                                 560
FAD2-3 (SEQ ID NO:8)    (519) CAACCCTTTGGGACGCACCGTGATGTTAACGGTTCAGTTC
 FAD2A (SEQ ID NO:5)    (519) CAACCCTTTGGGACGCACCGTGATGTTAACGGTTCAGTTC
FAD2-2 (SEQ ID NO:7)    (519) CAACCCGCTAGGACGCACGGTGATGCTAACCGTCCAGTTC
FAD2-1 (SEQ ID NO:6)    (504) CAACCCGCTAGGACGCACGGTGATGCTAACCGTCCAGTTC

                                  561                                 600
FAD2-3 (SEQ ID NO:8)    (559) ACTCTCGGCTGGCCGTTGTACTTAGCCTTCAACGTCTCGG
 FAD2A (SEQ ID NO:5)    (559) ACTCTCGGCTGGCCTTTGTACTTAGCCTTCAACGTCTCGG
FAD2-2 (SEQ ID NO:7)    (559) ACGCTCGGCTGGCCGTTGTACTTACCCTTCAACGTCTCTG
FAD2-1 (SEQ ID NO:6)    (544) AAGCTCGGCTGGCCGTTGTACTTAGCCTTCAACGTCTCGG

                                  601                                 640
FAD2-3 (SEQ ID NO:8)    (599) GAAGACCTTACGACGGCGGCTTCGCTTGCCATTTCCACCC
 FAD2A (SEQ ID NO:5)    (599) GGAGACCTTACGACGGCGGCTTCGCTTGCCATTTCCACCC
FAD2-2 (SEQ ID NO:7)    (599) GAAGACCTTACAGCGACGCTTTCGCTTGCCATTTCCACCC
FAD2-1 (SEQ ID NO:6)    (584) GAAGACCTTACAGCGACGGTTTCGCTTGCCATTTCCACCC

                                  641                                 680
FAD2-3 (SEQ ID NO:8)    (639) CAACGCTCCCATCTACAACGACCGCGAGCGTCTCCAGATA
 FAD2A (SEQ ID NO:5)    (639) CAACGCTCCCATCTACAAGGACCGTGAGCGTCTCCAGATA
FAD2-2 (SEQ ID NO:7)    (639) GAACGCTCCCATCTACAACGACCCCGAGCGTCTCCAGATA
FAD2-1 (SEQ ID NO:6)    (624) GAACGCTCCCATCTACAACGACCGCGAGCGTCTCCAGATA

                                  681                                 720
FAD2-3 (SEQ ID NO:8)    (679) TACATCTCCGACGCTGGCATCCTCGCCGTCTGCTACGGTC
 FAD2A (SEQ ID NO:5)    (679) TACATCTCCGACGCTGGCATCCTCGCCGTCTGCTACGGTC
FAD2-2 (SEQ ID NO:7)    (679) TACATCTCTGACGCTGGCGTCCTCTCCGTATGTTACGGTC
FAD2-1 (SEQ ID NO:6)    (664) TACATCTCTGACGCTGGCGTCCTCTCCGTATGTTACGGTC
```

FIG. 1C

FIG 1D

		721	760
FAD2-3 (SEQ ID NO:8)	(719)	TCTTCCGTTACGCCGCCGCGCAGGGAGTGGCCTCGATGGT	
FAD2A (SEQ ID NO:5)	(719)	TCTACCGCTACGCTGCTGTCCAAGGAGTTGCCTCGATGGT	
FAD2-2 (SEQ ID NO:7)	(719)	TCTACCGCTACGCTGGTTCGCGAGGAGTGGCCTCGATGGT	
FAD2-1 (SEQ ID NO:6)	(704)	TCTACCGTTACGCTGCTTCGCGAGGAGTAGCCTCTGTGGT	
		761	800
FAD2-3 (SEQ ID NO:8)	(759)	CTGCTTCTACGGAGTCCCGCTTGTGATTGTCAATGGTTTC	
FAD2A (SEQ ID NO:5)	(759)	CTGCTTCTACGGAGTTCCTCTTCTGATTGTCAACGGGTTC	
FAD2-2 (SEQ ID NO:7)	(759)	CTGTGTCTACGGAGTTCCGCTTATGATTGTCAACTGTTTC	
FAD2-1 (SEQ ID NO:6)	(744)	CTGTGTCTACGGAGTTCCGCTTCTAATTGTCAACTGTTTC	
		801	840
FAD2-3 (SEQ ID NO:8)	(799)	CTCGTGTTGATCACTTACTTGCAGCACACGCATCCTTCCC	
FAD2A (SEQ ID NO:5)	(799)	TTAGTTTTGATCACTTACTTGCAGCACACGCATCCTTCCC	
FAD2-2 (SEQ ID NO:7)	(799)	CTCGTCTTGATCACTTACTTGCAGCACACGCACCCTTCGC	
FAD2-1 (SEQ ID NO:6)	(784)	CTCGTCTTGATCACTTACTTGCAGCACACGCACCCTTCGC	
		841	880
FAD2-3 (SEQ ID NO:8)	(839)	TGCCTCACTACGATTCGTCCGAGTGGGATTGGTTGAGGGG	
FAD2A (SEQ ID NO:5)	(839)	TGCCTCACTATGACTCGTCTGAGTGGGATTGGTTGAGGGG	
FAD2-2 (SEQ ID NO:7)	(839)	TGCCTCACTATGATTCTTCGGAGTGGGATTGGTTGAGACG	
FAD2-1 (SEQ ID NO:6)	(824)	TGCCTCACTATGATTCTTCCGAGTGGGATTGGTTGAGAGG	
		881	920
FAD2-3 (SEQ ID NO:8)	(879)	AGCTTTGGCTACCGTTGACAGAGACTACGGAATCTTGAAC	
FAD2A (SEQ ID NO:5)	(879)	AGCTTTGGCCACCGTTGACAGAGACTACGGAATCTTGAAC	
FAD2-2 (SEQ ID NO:7)	(879)	AGCTTTGGCTACTGTGGATAGAGACTATGGAATCTTGAAC	
FAD2-1 (SEQ ID NO:6)	(864)	AGCTTTGGCTACTGTGGATAGAGACTATGGAATCTTGAAC	
		921	960
FAD2-3 (SEQ ID NO:8)	(919)	AAGGTCTTCCACAATATTACCGACACGCACGTGGCGCATC	
FAD2A (SEQ ID NO:5)	(919)	AAGGTCTTCCACAATATCACGGACACGCACGTGGCGCATC	
FAD2-2 (SEQ ID NO:7)	(919)	AAGGTGTTTCATAACATCACGGACACGCACGTGGCGCATC	
FAD2-1 (SEQ ID NO:6)	(904)	AAGGTGTTCCATAACATCACGGACACGCACGTGGCGCATC	

FIG. 1D

```
FIG1E                          961                                  1000
FAD2-3 (SEQ ID NO:8)    (959) ATCTGTTCTCCACGATGCCGCATTATCACGCGATGGAAGC
 FAD2A (SEQ ID NO:5)    (959) ACCTGTTCTCGACCATGCCGCATTATCACGCGATGGAAGC
FAD2-2 (SEQ ID NO:7)    (959) ATCTGTTCTCGACGATGCCGCATTATAACGCGATGGAAGC
FAD2-1 (SEQ ID NO:6)    (944) ATCTGTTCTCGACGATGCCGCATTATAACGCGATGGAAGC
                               1001                                 1040
FAD2-3 (SEQ ID NO:8)    (999) TACCAAGGCGATAAAGCCGATACTG-GGAGAGTATTATCA
 FAD2A (SEQ ID NO:5)    (999) TACGAAGGCGATAAAGCCGATACTG-GGAGAGTATTATCA
FAD2-2 (SEQ ID NO:7)    (999) GACCAAGGCGATAAAGCCGATACTT-GGAGAGTATTACCA
FAD2-1 (SEQ ID NO:6)    (984) GACCAAGGCGATAAAGCCGATACTTTGGAGAGTATTACCA
                               1041                                 1080
FAD2-3 (SEQ ID NO:8)   (1038) GTTCGATGGGACGCCGGTGGTTAAGGCGATGTGGAGGGAG
 FAD2A (SEQ ID NO:5)   (1038) GTTCGATGGGACGCCGGTGGTTAAGGCGATGTGGAGGGAG
FAD2-2 (SEQ ID NO:7)   (1038) GTTTGATGGAACGCCGGTGGTTAAGGCGATGTGGAGGGAG
FAD2-1 (SEQ ID NO:6)   (1024) GTTTGATGGAACGCCGGCGGTTAAGGCGATGTGGAGGGAG
                               1081                                 1120
FAD2-3 (SEQ ID NO:8)   (1078) GCGAAGGAGTGTATCTATGTGGAACCGGACAGGCAAGGTG
 FAD2A (SEQ ID NO:5)   (1078) GCGAAGGAGTGTATCTATGTGGAACCGGACAGGCAAGGTG
FAD2-2 (SEQ ID NO:7)   (1078) GCGAAGGAGTGTATCTATGTTGAACCGGATAGGCAAGGTG
FAD2-1 (SEQ ID NO:6)   (1064) GCGAAGGAGTGTATCTATGTTGAACCGGATAGGCAAGGTG
                               1121                                 1160
FAD2-3 (SEQ ID NO:8)   (1118) AGAAGAAAGGTGTGTTCTGG--------------------
 FAD2A (SEQ ID NO:5)   (1118) ACAAGAAAGGTGTGTTCTGGTACAACAATAAGTTATCTTG
FAD2-2 (SEQ ID NO:7)   (1118) AGAAGAAAGGTGTGTTCTGGTACAACAATAAGTTATGAGG
FAD2-1 (SEQ ID NO:6)   (1104) AGAAGAAAGGTGTGTTCTGGTACAACAATAA---------
                               1161
FAD2-3 (SEQ ID NO:8)   (1138) ----
 FAD2A (SEQ ID NO:5)   (1158) CTAA
FAD2-2 (SEQ ID NO:7)   (1158) ATGA
FAD2-1 (SEQ ID NO:6)   (1135) ----
```

FIG. 1E

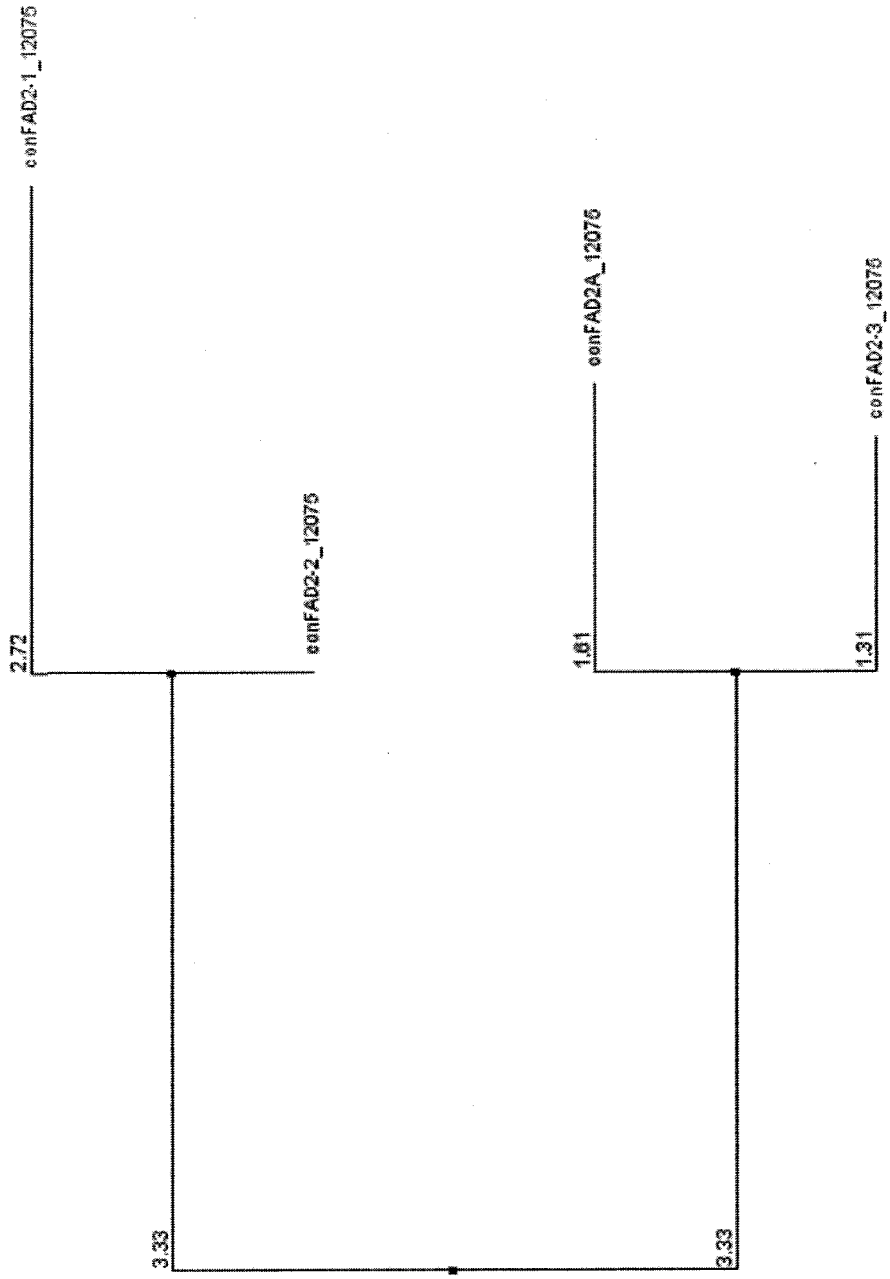
conFAD2-1_12075
conFAD2-2_12075
conFAD2A_12075
conFAD2-3_12075
2.72
1.61
1.31
3.33
3.33

FIG. 2

```
FIG 3A                                  1                                   40
  FAD3A (SEQ ID NO:15)      (1) CATCAGACCCTTTCTTCACCACATTTCACTCAGAGCCCAC
 FAD3A' (SEQ ID NO:16)      (1) CATCGAACCCTTTCTTCACCACATTCCACTTCCCACACTC
 FAD3C' (SEQ ID NO:20)      (1) CATCGAACCCTTTCTTCACCACATTCCAGTTCCCACACTT
FAD3A'' (SEQ ID NO:17)      (1) CATCAAAC-CTTTCTTCACCACATTTCACTGAAAGGCCAC
FAD3C'' (SEQ ID NO:19)      (1) CATCAAAC-CTTTATTCACCACATTTCACTGAAAGGCCAC
  FAD3C (SEQ ID NO:18)      (1) CATCAAA--CTCTCTCCACCACATTTCACTCAGAGCCCAC

                                41                                  80
  FAD3A (SEQ ID NO:15)     (41) ACAGTTTTTAG------AGAGAGAGAGAAACATCCCTCAAA
 FAD3A' (SEQ ID NO:16)     (41) TCTTTTTTTTTGAATTATAGAGAGAGAATCCTCCTCCAAA
 FAD3C' (SEQ ID NO:20)     (41) TCTTTTTTTT-GAATTATAGAGAGAGAATCTTCCTCCAAA
FAD3A'' (SEQ ID NO:17)     (40) ACATCT----------AGAGAGAGA--AACTTCGTCCAAA
FAD3C'' (SEQ ID NO:19)     (40) ACATCT----------AGAGAGAGA--AACTTCGTCCAAA
  FAD3C (SEQ ID NO:18)     (39) ACAGTTTTTAG------AGAGAGAGA--AACATCCCTCAAA

                               81                                  120
  FAD3A (SEQ ID NO:15)     (75) GCTCTCTCTCTTTCTCCGGCGATGGTTGTCGCTATGGACC
 FAD3A' (SEQ ID NO:16)     (81) TCTCTCTCTCTC----CCAGGATGGTTGTTGCTATGGACC
 FAD3C' (SEQ ID NO:20)     (80) TCTCTCTCTCTCTCTCCCAGGATGGTTGTTGCTATGGACC
FAD3A'' (SEQ ID NO:17)     (68) TCTCTCTC------TCCAGCAATGGTTGTTGCTATGGACC
FAD3C'' (SEQ ID NO:19)     (68) TCTCTCTC------TCCAGCGATGGTTGTTGCTATGGACC
  FAD3C (SEQ ID NO:18)     (71) GCTCTCTC--TTTCTCCGGCGATGGTTGTCGCTATGGACC

                               121                                 160
  FAD3A (SEQ ID NO:15)    (115) AGCGTAGCAATGCGAACGGAGA----------------
 FAD3A' (SEQ ID NO:16)    (117) AACGGACCAATGTGAACGGAGATGCCGCTGCCCGGAAGGA
 FAD3C' (SEQ ID NO:20)    (120) AACGCACCAATGTGAACGAAGATGCCGGTGCCCGGAAGGA
FAD3A'' (SEQ ID NO:17)    (102) AGCGCAGCAATGTTAACGGAGATTCCGGTGCCCGGAAGGA
FAD3C'' (SEQ ID NO:19)    (102) AGCGCAGCAATGTTAACGGAGATTCCGGTGCCCGGAAGGA
  FAD3C (SEQ ID NO:18)    (109) AGCGTAGCAATGTGAACGGAGATTCC---------AAGGA
```

FIG. 3A

FIG 3B

```
                                  161                                 200
FAD3A (SEQ ID NO:15)    (137) CGAAAGGTTTGATCCGAGCGCACAACCACCGTTCAAGATC
FAD3A' (SEQ ID NO:16)   (157) AGAAGGGTTTGATCCGAGCGCACAACCGCCGTTTAAGATC
FAD3C' (SEQ ID NO:20)   (160) AGAAGGGTTTGATCCGAGCGCACAACCGCCGTTTAAGATC
FAD3A'' (SEQ ID NO:17)  (142) AGAAGGGTTTGATCCAAGCGAACAACCACCGTTTAAGATC
FAD3C'' (SEQ ID NO:19)  (142) AGAAGGGTTTGATCCAAGCGCACAACCACCGTTTAAGATC
FAD3C (SEQ ID NO:18)    (140) CGAAAGGTTTGATCCGAGCGCACAACCACCGTTTAAGATC
                                  201                                 240
FAD3A (SEQ ID NO:15)    (177) GGAGATATAAGGGCGGCCATTCCTAAGCATTGTTGGGTAA
FAD3A' (SEQ ID NO:16)   (197) GGGGACATAAGGGCTGCGATTCCTAAGCATTGTTGGGTGA
FAD3C' (SEQ ID NO:20)   (200) GGGGACATAAGGGCTGCGATTCCTAAGCATTGTTGGGTGA
FAD3A'' (SEQ ID NO:17)  (182) GGAGATATCAGGGCGGCGATTCCTAAGCATTGTTGGGTGA
FAD3C'' (SEQ ID NO:19)  (182) GGAGATATAAGGGCGGCGATTCCTAAGCATTGCTGGGTGA
FAD3C (SEQ ID NO:18)    (180) GGAGATATAAGGGCTGCGATTCCTAAGCATTGTTGGGTCA
                                  241                                 280
FAD3A (SEQ ID NO:15)    (217) AGAGTCCTTTGAGATCCATGAGCTATGTCGCCAGAGACAT
FAD3A' (SEQ ID NO:16)   (237) AAAGTCCTTTGAGATCTATGAGCTACGTAGCCAGAGACAT
FAD3C' (SEQ ID NO:20)   (240) AAAGTCCTTTGAGATCTATGAGCTACGTAGCCAGAGACAT
FAD3A'' (SEQ ID NO:17)  (222) AGAGTCCTTTGAGATCTATGAGCTACGTCGCCAGAGACAT
FAD3C'' (SEQ ID NO:19)  (222) AGAGTCCTTTGAGATCTATGAGCTACGTCGCCAGAGACAT
FAD3C (SEQ ID NO:18)    (220) AGAGTCCTTTGAGATCCATGAGCTACGTCGCGAGAGACAT
                                  281                                 320
FAD3A (SEQ ID NO:15)    (257) TTTCGCCGTCGTGGCTCTTGCCGTCGCCGCCGTGTATTTT
FAD3A' (SEQ ID NO:16)   (277) TTGTGCCGTCGCGGCTTTGGCCATTGCCGCCGTGTATTTT
FAD3C' (SEQ ID NO:20)   (280) TTGTGCCGTCGCTGCTTTGGCCATTGCCGCCGTGTATTTT
FAD3A'' (SEQ ID NO:17)  (262) TTTCGCCGTCGCGGCTCTGGCCATGGCCGCCGTGTATTTT
FAD3C'' (SEQ ID NO:19)  (262) TTTCGCCGTCGCGGCTCTGGCCATGGCCGCCGTGTATTTT
FAD3C (SEQ ID NO:18)    (260) TTTCTCCGTCGTGGCTCTGGCCGTCGCCGCCGTGTATTTT
```

FIG. 3B

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FIG 3C                                  321                                 360

  FAD3A (SEQ ID NO:15)    (297) GATAGCTGGTTCTTTTGGCCTCTTTATTGGGCCGCCCAAG
 FAD3A' (SEQ ID NO:16)    (317) GATAGCTGGTTCCTCTGTCCTCTCTATTGGGTCGCCCAAG
 FAD3C' (SEQ ID NO:20)    (320) GATAGCTGGTTCCTCTGGCCTCTCTATTGGGTCGCCCAAG
FAD3A'' (SEQ ID NO:17)    (302) GATAGCTGGTTCCTCTGGCCACTCTACTGGGTTGCCCAAG
FAD3C'' (SEQ ID NO:19)    (302) GATAGCTGGTTCCTCTGGCCACTCTACTGGGTTGCCCAAG
  FAD3C (SEQ ID NO:18)    (300) GATAGCTGGTTCTTCTGGCCTCTTTATTGGGCCGCCCAAG
                                  361                                 400

  FAD3A (SEQ ID NO:15)    (337) GAACCCTGTTCTGGGCTATCTTCGTACTCGGCCACGACTG
 FAD3A' (SEQ ID NO:16)    (357) GAACCCTTTTCTGGGCCATCTTCGTCCTCGGCCACGACTG
 FAD3C' (SEQ ID NO:20)    (360) GAACCCTTTTCTGGGCCATCTTCGTCCTCGGCCACGACTG
FAD3A'' (SEQ ID NO:17)    (342) GAACCCTTTTCTGGGCCATCTTCGTTCTTGGCCACGACTG
FAD3C'' (SEQ ID NO:19)    (342) GAACCCTTTTCTGGGCCATCTTCGTTCTTGGCCACGACTG
  FAD3C (SEQ ID NO:18)    (340) GAACCCTTTTCTGGGCCATCTTCGTACTCGGCCACGACTG
                                  401                                 440

  FAD3A (SEQ ID NO:15)    (377) GTAATTTAATTTT----------TCTTTCAACTTCTTAA
 FAD3A' (SEQ ID NO:16)    (397) GTAA----AGTTT--------------------------
 FAD3C' (SEQ ID NO:20)    (400) GTAA----AGTTT--------------------------
FAD3A'' (SEQ ID NO:17)    (382) GTAAATTAAATTT----------------------TCTG
FAD3C'' (SEQ ID NO:19)    (382) GTAAATTAAATTT----------------------TCAG
  FAD3C (SEQ ID NO:18)    (380) GTAATTTAATTTTCAATTTATTTTTTCTTCAACTTCTTAA
                                  441                                 480

  FAD3A (SEQ ID NO:15)    (406) TTTTGATATGTTTATATGTTTTTTTCGTTTTTTGCATTGT
 FAD3A' (SEQ ID NO:16)    (406) CTTCCAT----------------------TTTGCATTGC
 FAD3C' (SEQ ID NO:20)    (409) CTTCCAT----------------------TTTGCATTGC
FAD3A'' (SEQ ID NO:17)    (399) TTTTAAT-------TATTTTGACT-CTTTTTGTTCAATTT
FAD3C'' (SEQ ID NO:19)    (399) TTTTAAT-------TATTTTGTCT-CTTTTTGTTCAATTT
  FAD3C (SEQ ID NO:18)    (420) TTTTGATATGTTTATATGTTTTTT-CGTTTTTTGCATCGT
```

FIG. 3C

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FIG 3D                                  481                                 520
  FAD3A (SEQ ID NO:15)    (446) CTTTGATTTCTTGACCGTACGTTCGATATGAGATTTTC--
 FAD3A' (SEQ ID NO:16)    (423) ATCG-ATTTATTGAATGCACGTTCTACGAGT-ATTGTTTG
 FAD3C' (SEQ ID NO:20)    (426) ATCG-ATTTATTGAATGCACGTTCTATGAGT-ATTGT---
FAD3A'' (SEQ ID NO:17)    (431) ATTA-ATTTGTTGAATGCACGTTCGATGAGT-ATCGTCGT
FAD3C'' (SEQ ID NO:19)    (431) ATTA-ATTTCTTGAATGCACGTTCGATGAGT-ATCGTC--
  FAD3C (SEQ ID NO:18)    (459) CTTTGATTTCTTGAACGCACGTTCGATATGAGATTTTC--
                                  521                                 560
  FAD3A (SEQ ID NO:15)    (484) --ACTGACTTCAAGATTTGATTCTCTTCAGGTTTACTTTT
 FAD3A' (SEQ ID NO:16)    (461) TCAGTTACTTCGTAAAATGATTCTTTGATGTTCATTTTT
 FAD3C' (SEQ ID NO:20)    (461) -CAGT-ACTTTATGAATTGATTCTTTGATGTTCATTTTT
FAD3A'' (SEQ ID NO:17)    (469) -CACTGACTTCAAGATTTAATTCTTTGAGGTT-ACCTTT
FAD3C'' (SEQ ID NO:19)    (467) --ACTGACTTCAAGATTTAATTCTTTGAGGTT-ACTTTT
  FAD3C (SEQ ID NO:18)    (497) --ACTGACTTCAAGATTTGATTCTCTTCAGGTTTACTTTT
                                  561                                 600
  FAD3A (SEQ ID NO:15)    (522) TTGAATTTTAATTATTATGTTCATCCAATTTGGCCTATTT
 FAD3A' (SEQ ID NO:16)    (501) TGAAGATCTAAG-ATTT--------------------TTT
 FAD3C' (SEQ ID NO:20)    (499) TGAAGATCTAAG-ATTT--------------------TTT
FAD3A'' (SEQ ID NO:17)    (507) T-CATGTTCAATTATTA---------AA--------AAAT
FAD3C'' (SEQ ID NO:19)    (504) T-CATGTTTAATTATTA---------AA--------AAAT
  FAD3C (SEQ ID NO:18)    (535) AAAAAAAAAAATTATTATGTTCACCCAAATTGGCCTATTT
                                  601                                 640
  FAD3A (SEQ ID NO:15)    (562) TAAAAGCAAAAGGGGATCTAAGATTTTTAATTCTTTGTT
 FAD3A' (SEQ ID NO:16)    (520) T---------------TTT-AGATTTTCT-TTTTAAATCA
 FAD3C' (SEQ ID NO:20)    (518) T---------------TTTTAGATTTTCT-TTTTAAATCA
FAD3A'' (SEQ ID NO:17)    (529) AAAATAAAATATAGGATCTAAGATTTTT--TTCTTCATCA
FAD3C'' (SEQ ID NO:19)    (526) AAAAGAAAATATAGGATCTAAGATTTTT--TTCTTCATCA
  FAD3C (SEQ ID NO:18)    (575) TAAAAGCAAAAGGGGATCTAAGATTTTTAATTCTTCTCTT
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FIG. 3D

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FIG 3E                                641                                680

  FAD3A (SEQ ID NO:15)   (602) TTTTTTTGGT---------------TCTTTTTCATCAG-T
 FAD3A' (SEQ ID NO:16)   (543) TTGTTCCACCACCA-------------CCTTTCATCGG-T
 FAD3C' (SEQ ID NO:20)   (542) TTGTTCCACCACC----------------TTTCATCGG-T
FAD3A'' (SEQ ID NO:17)   (567) --GTTCAAGCA-------------TCATCACTCATCAG-T
FAD3C'' (SEQ ID NO:19)   (564) ATGTTCAAGCA-------------TCGTCACTCATCAG-T
  FAD3C (SEQ ID NO:18)   (615) TTTCAGTCGTAACACTGCTAACTTTTTTTTTTGATCAAAT

                               681                                720

  FAD3A (SEQ ID NO:15)   (626) CGTAACACTC-------CTAACTAAACATCTTTTTCTTTC
 FAD3A' (SEQ ID NO:16)   (569) CGTACGACTC----GTTACAACACCACATCTT--TATTTT
 FAD3C' (SEQ ID NO:20)   (565) CGTACGACTC----GTTACAAAACCACATCTT--TATTTT
FAD3A'' (SEQ ID NO:17)   (591) CGTAAGACTC-------GTAACAAAATATCTT---GTTTT
FAD3C'' (SEQ ID NO:19)   (590) CGTCAGACTC-------GTAACAAAATATCTT---GTTTT
  FAD3C (SEQ ID NO:18)   (655) CGTAACACTCATAAGTCCTAACTAAACATCTTTTTGTTTC

                               721                                760

  FAD3A (SEQ ID NO:15)   (659) CTATAATTATTGTTGTTTCCGCGTTTTATGGATCTACGTT
 FAD3A' (SEQ ID NO:16)   (603) CTATAATTACTACTGCTTCCGCATTTTATGGATCTCTCAA
 FAD3C' (SEQ ID NO:20)   (599) CTATAATTACGACTGCTTCCGCATTTTATGGATCTCTCAA
FAD3A'' (SEQ ID NO:17)   (621) CTATAATTAATATTATTTCCGCATTTAATGGATCTACGTT
FAD3C'' (SEQ ID NO:19)   (620) CTATAATTAATATTATTTCCGCATTTTATGGATCTACGTT
  FAD3C (SEQ ID NO:18)   (695) CTATAATTATTGTTGTTCCGCATTTTATGGATCTACGTT

                               761                                800

  FAD3A (SEQ ID NO:15)   (699) T-GAAATTTTCAA------------------TAAAAC---
 FAD3A' (SEQ ID NO:16)   (643) CTTATAATTAAAG------------------TATAATATC
 FAD3C' (SEQ ID NO:20)   (639) CTTATAATTAAAG------------------TATAAAATC
FAD3A'' (SEQ ID NO:17)   (661) TTGATGTTCTCAAATTTTGTTTCTCTTTCTCTAGATCCCC
FAD3C'' (SEQ ID NO:19)   (660) TTGATGTTCTCAATTTTTGTTTCTCTTTCTCTAGATCCCC
  FAD3C (SEQ ID NO:18)   (735) T-GAAAGTTTCAA------------------TAAAAC---
```

FIG. 3E

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FIG 3F                                  801                                 840
  FAD3A (SEQ ID NO:15)    (717) ---ACATTTTATTGTT-TTCT-GTA----ACAATTT----
 FAD3A' (SEQ ID NO:16)    (665) AAGAATATCTATTATTTTTCTTAAACAAGA-AAGAT----
 FAD3C' (SEQ ID NO:20)    (661) AAGAATATCTATTGTTTTTCTAAAACAAGA-AAGAT----
FAD3A'' (SEQ ID NO:17)    (701) GGAACTTTTAATTATAATTATAGTATAGTATAATATCAAG
FAD3C'' (SEQ ID NO:19)    (700) GGAACTTTTAATTATAATTATAGTATAGTATAATATCAAG
  FAD3C (SEQ ID NO:18)    (753) ---ACATTTTATTGTT-TGAAAGTA----ACAATAT----
                                  841                                 880
  FAD3A (SEQ ID NO:15)    (744) --AAT-TACTGTTTATTGGTTC------------------
 FAD3A' (SEQ ID NO:16)    (700) --AAT--ATTGTTTCTTTGTTA------------------
 FAD3C' (SEQ ID NO:20)    (696) --AAT--ATTGTTTCTTTGTTA------------------
FAD3A'' (SEQ ID NO:17)    (741) AAAATATACTGTTTATTTTTTTTGGCAACAAATATATTAC
FAD3C'' (SEQ ID NO:19)    (740) AAAATATACTGTTTATTTTTTT-GGCAACAAATATATT--
  FAD3C (SEQ ID NO:18)    (781) --AAT-TACTGTATATTGATTC------------------
                                  881                                 920
  FAD3A (SEQ ID NO:15)    (763) ----TTTT----------------A-----------ATTA
 FAD3A' (SEQ ID NO:16)    (718) ---------------------------------------TTT
 FAD3C' (SEQ ID NO:20)    (714) ---------------------------------------TTT
FAD3A'' (SEQ ID NO:17)    (781) TCTTGTTTCTTTGACAAGAAAAAAATATATTGTTTTTTTC
FAD3C'' (SEQ ID NO:19)    (777) ----GTTT-TTTGACAAGAAAAA--TATATTGTTTTTTTC
  FAD3C (SEQ ID NO:18)    (800) ----TTTT----------------A-----------ATTA
                                  921                                 960
  FAD3A (SEQ ID NO:15)    (772) TTGTGTGT-TGTTCCAATCTATTTTCGAAATATAGTCATG
 FAD3A' (SEQ ID NO:16)    (721) TGGTGTAT---TTCCAATCTA-TTTCGAGATTTAGAAATG
 FAD3C' (SEQ ID NO:20)    (717) TGGTGTAT---T-CCAATCTA-TTTCGAGATTTAGAAATG
FAD3A'' (SEQ ID NO:17)    (821) TTCTTTTTGTGTTCCAATCTATTTTCGAGATTTAGACAAG
FAD3C'' (SEQ ID NO:19)    (810) TTCTTTTTGTGTTCCAATCTATTTT-GTGATTTAGACAAG
  FAD3C (SEQ ID NO:18)    (809) TTGTGTGT-TGTTCCAATCTACTTTCGAAATATAGTCATG
```

FIG. 3F

FIG 3G

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                                  961                                1000
  FAD3A (SEQ ID NO:15)    (811) TGACACGTCATATTGTATTTTGTTACCTTGTTGAAACGT
  FAD3A' (SEQ ID NO:16)   (757) TGACACGTCAT------------TACCTTGTTGAAGTGT
  FAD3C' (SEQ ID NO:20)   (752) TGTCACGTCAT------------TACCTTGTTGAAGCTT
 FAD3A'' (SEQ ID NO:17)   (861) TGACACGTCATATACCGGATTTGTTACCTTGTTAAAGAGT
 FAD3C'' (SEQ ID NO:19)   (849) TGACACGTCATATACCGGATTTGTTACCTTGTTAAAGAGT
  FAD3C (SEQ ID NO:18)    (848) TGACACGTCATATTCTATTTTTGTTACCTTGTTGGAACGT
                                  1001                               1040
  FAD3A (SEQ ID NO:15)    (851) TTG----------AATTGAGGAAAGTTCAGTTAACATTGT
  FAD3A' (SEQ ID NO:16)   (784) TTA------AAACAAACATGGAAAGTTTAAATAA-ATAGT
  FAD3C' (SEQ ID NO:20)   (779) TTA------AAACAAACATGGAAAGTTTAAATAA-ATAGT
 FAD3A'' (SEQ ID NO:17)   (901) TTGGGTTAAAACAAATGTAGAAAAGTTAAAATAA-ATTGT
 FAD3C'' (SEQ ID NO:19)   (889) TTGAGTTAAAACAAATGTAGAAAAGTTAAAATAA-ATTGT
  FAD3C (SEQ ID NO:18)    (888) TTG----------AATTGAGTAAAGTTTAATTAACATTGT
                                  1041                               1080
  FAD3A (SEQ ID NO:15)    (881) GCAATAAATGATAAA-TGTGTTT------------ATGAT
  FAD3A' (SEQ ID NO:16)   (817) GCAATAAATGATATA-TATGTAT--ATGATGAATAATGAT
  FAD3C' (SEQ ID NO:20)   (812) GCAATAAATGATATACTATATTT--ACGATGAATAATGAT
 FAD3A'' (SEQ ID NO:17)   (940) GCAATAAATGATAAA-TACGTTTTTATGTTAAACAATGAT
 FAD3C'' (SEQ ID NO:19)   (928) GCACTAAATGATAAA-TACGTTTTTATGTTAAATAATGAT
  FAD3C (SEQ ID NO:18)    (918) GCAATAAATGATAAA-CATGTTT------------ATGAT
                                  1081                               1120
  FAD3A (SEQ ID NO:15)    (908) GTAAAATTTCATTTGAATAATA-CAGTGGACATGGGAGCT
  FAD3A' (SEQ ID NO:16)   (854) GTGAAA-TATAATTGAATAATGGCAGTGGACATGGGAGTT
  FAD3C' (SEQ ID NO:20)   (850) GTGAAA-TATAATTGAATAATGGCAGTGGACATGTGAGTT
 FAD3A'' (SEQ ID NO:17)   (979) GTGAAAATAAAATTGAATAATGGCAGTGGACATGGGAGTT
 FAD3C'' (SEQ ID NO:19)   (967) GTGAAAATAAAATTGAATAATGGCAGTGGACATGGGAGTT
  FAD3C (SEQ ID NO:18)    (945) GTAAAATTCAATTTGAATAATA-CAGTGGACATGGGAGCT
```

FIG. 3G

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FIG 3H                                1121                                1160
  FAD3A (SEQ ID NO:15)    (947) TCTCAGACATTCCTCTTCTGAATACTGCGGTTGGTCATAT
 FAD3A' (SEQ ID NO:16)    (893) TCTCAGACATTCCTCTGCTGAATAGTGTGGTTGGCCATAT
 FAD3C' (SEQ ID NO:20)    (889) TCTCAGACATTCCTCTGCTGAATAGCGTGGTTGGCCATAT
FAD3A'' (SEQ ID NO:17)   (1019) TTTCAGACATTCCTCTGCTGAACAGTGTGGTTGGTCACAT
FAD3C'' (SEQ ID NO:19)   (1007) TCTCAGACATTCCTCTGCTGAACAGTGTGGTTGGTCACAT
  FAD3C (SEQ ID NO:18)    (984) TCTCAGACATTCCTCTTCTGAATACTGCGGTTGGTCATAT
                                1161                                1200
  FAD3A (SEQ ID NO:15)    (987) TCTTCATTCGTTCATTCTCGTTCCATACCATGGTTGGTAA
 FAD3A' (SEQ ID NO:16)    (933) TCTTCATTCGTTCATCCTCGTTCCTTACCATGGTTGGTAA
 FAD3C' (SEQ ID NO:20)    (929) TCTTCATTCGTTCATCCTCGTTCCTTACCATGGTTGGTAA
FAD3A'' (SEQ ID NO:17)   (1059) TCTTCATTCATTCATCCTCGTTCCTTACCATGGTTGGTAA
FAD3C'' (SEQ ID NO:19)   (1047) TCTTCATTCATTCATCCTCGTTCCTTACCATGGTTGGTAA
  FAD3C (SEQ ID NO:18)   (1024) TCTTCATTCGTTCATTCTCGTTCCATACCATGGTTGGTAA
                                1201                                1240
  FAD3A (SEQ ID NO:15)   (1027) GTCAT-TTATTTTAACTTCTTTTTTCATGCAAA---TTTA
 FAD3A' (SEQ ID NO:16)    (973) GTCAGCTTATC--AACC-CTTTTT--ACTAT-ATTATTAA
 FAD3C' (SEQ ID NO:20)    (969) GTCAACTTATT--AACC-GTTTTT--ATTATTATTATTAA
FAD3A'' (SEQ ID NO:17)   (1099) GTCAT-TTATT--AAC---TATTTCCATGTAAACTATTAG
FAD3C'' (SEQ ID NO:19)   (1087) GTCAT-TTATT--AAC---TATTTCCATGTAAATTATTAG
  FAD3C (SEQ ID NO:18)   (1064) GTCAT-TTATTTAAACATCTTTTT-CATGCAAA---TTTA
                                1241                                1280
  FAD3A (SEQ ID NO:15)   (1063) TTCTTGTTTTCGTATTTCTTACATTTTCCTT-GTCATTCT
 FAD3A' (SEQ ID NO:16)   (1007) TTATTAAACTTGCATTTGT-ATACTT-----GGTGCAAGT
 FAD3C' (SEQ ID NO:20)   (1004) TTATTAAACTTTCATTTGTTATACTTTTTTTGGTTTAAAT
FAD3A'' (SEQ ID NO:17)   (1133) TACTTGTTTTCGTATTTCTTACATTTTCGTTTGTCATTCT
FAD3C'' (SEQ ID NO:19)   (1121) TACTTGTTTTCGTATTTCTTACATTTTCGTTTGTTATTCT
  FAD3C (SEQ ID NO:18)   (1099) TTCTTGTTTTCGTATTTCTTACATTTTCCTT-GTCATTCT
```

FIG. 3H

FIG 3I

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                                  1281                                1320
  FAD3A (SEQ ID NO:15)  (1102) T----GGTGCA-TGTTAGCAAACAGTAATCTGA--TAACT
 FAD3A' (SEQ ID NO:16)  (1041) TGGTAAATGTAATCTGATAACTGAA-AATCTAT--TCATT
 FAD3C' (SEQ ID NO:20)  (1044) -GTTAAATGAATTACTTGGTGCAAG-AATCTAT--TCATT
FAD3A'' (SEQ ID NO:17)  (1173) TCTTGGGTGCA-TGCTAGCAAACTGTAATCAGTATTAACT
FAD3C'' (SEQ ID NO:19)  (1161) T---GGGTGCAATGCTAGGAAACTGTAATCAGTATTAACT
  FAD3C (SEQ ID NO:18)  (1138) T----GGTGCA-TGTTAGCAAACTGTAATCTGA--TAACT
                                  1321                                1360
  FAD3A (SEQ ID NO:15)  (1135) GAAAA----------TATATTAATT-------------TT
 FAD3A' (SEQ ID NO:16)  (1078) GCTCGTTCTA-----TTTTTTTTTTGGCT-AGAGACAATT
 FAD3C' (SEQ ID NO:20)  (1080) GCTCGTTCT------TTTTTTTTTGGCT-AGAGCCAATT
FAD3A'' (SEQ ID NO:17)  (1212) GGGAACTACCAACTGTTTTTTTTTGCTAGAGTAGCAATT
FAD3C'' (SEQ ID NO:19)  (1198) GGAAGCTACCAACT-TTTTTTTGTTGCTAGAGTAGCAATT
  FAD3C (SEQ ID NO:18)  (1171) GAAAA----------TATATTAATT-------------TT
                                  1361                                1400
  FAD3A (SEQ ID NO:15)  (1152) TCATAGTAAAATAA------------------TGCATGTG
 FAD3A' (SEQ ID NO:16)  (1112) TTATAATTAAATAATGCATGTGAGAATATGACTATTTATG
 FAD3C' (SEQ ID NO:20)  (1113) TTATAATTAAATAATGCATGTGAAAGTATGACTATATATG
FAD3A'' (SEQ ID NO:17)  (1252) TTATAATTAAATAAGAATCCTATTA--AACAATGCATGTG
FAD3C'' (SEQ ID NO:19)  (1237) TTATAATTAAATAAGAATCCTATTA--AACAATGCATGTG
  FAD3C (SEQ ID NO:18)  (1188) CCATAGTAAAATAA------------------TGCATGTG
                                  1401                                1440
  FAD3A (SEQ ID NO:15)  (1174) ACTAAAAGCA--------------TCAAAA--------TC
 FAD3A' (SEQ ID NO:16)  (1152) TGAGGTAGCTTTTCTTATTCCTGTCGAAAAGCATCAAATC
 FAD3C' (SEQ ID NO:20)  (1153) TGAGGTAGCTTTTCTTATTCTTGACGAAAAGCATCGAATC
FAD3A'' (SEQ ID NO:17)  (1290) ACAATATGAGGTTGCTTTT-CTGTTCAAAA----CAAATC
FAD3C'' (SEQ ID NO:19)  (1275) ACTATATGAGGTTGCTTTTTCTGTTCAAAAGCATCAAATC
  FAD3C (SEQ ID NO:18)  (1210) ACTAAAAGCA--------------TCAAAA--------TC
```

FIG. 3I

FIG 3J

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                                  1441                                1480
  FAD3A (SEQ ID NO:15)  (1192) TTTAGCATCGAAGAAAAAAGAA-CCAAACTTTTATTT--A
 FAD3A' (SEQ ID NO:16)  (1192) TTTAGCAACGAAGGAAAAAGGAATCAAATTTTTATT-AA
 FAD3C' (SEQ ID NO:20)  (1193) TTTAGCAACGAAGGAAAAAGGAATCAAAACTTTTATT-AA
FAD3A'' (SEQ ID NO:17)  (1325) TTTAGAAGCCAATGAAAAAGAATCCAAAACTTTTTTTTAA
FAD3C'' (SEQ ID NO:19)  (1315) TTTAGCAGCCAATGAAAAAGAATCCAAACCTTTTCTT-AA
  FAD3C (SEQ ID NO:18)  (1228) TTTAGCATCGAAGAAAAAAGAA-CCAAACTTTTATTT--A
                                  1481                                1520
  FAD3A (SEQ ID NO:15)  (1229) ATGCTATGGGCCTATTTATGG--------TCCA-------
 FAD3A' (SEQ ID NO:16)  (1231) ATGCAATGGGTCTATGTCTTGC-------TCATTAGTTTT
 FAD3C' (SEQ ID NO:20)  (1232) ATGCAATGGGCCTATATCT-CC-------TCATTAGTATT
FAD3A'' (SEQ ID NO:17)  (1365) ATGATATGCGCCTATCTATTGGTCCTGACTCCTGAGTTTT
FAD3C'' (SEQ ID NO:19)  (1354) ATGATATGCGCCTATCTATGG--------TCCTGAGTTTT
  FAD3C (SEQ ID NO:18)  (1265) ATGCTATGGGCCTATTTATGG--------TCCA-------
                                  1521                                1560
  FAD3A (SEQ ID NO:15)  (1254) --------A--TTAGCTATTATCATATGAC-ATGTCCTTG
 FAD3A' (SEQ ID NO:16)  (1264) TTGCATATAATTTATTTATATTTTTTCTTAACAGCAGCT
 FAD3C' (SEQ ID NO:20)  (1264) TTGAATATAATTTATTTATAATTTTTTTGAACAACAGCT
FAD3A'' (SEQ ID NO:17)  (1405) CTTACTTTC--TTAAGTATAATTAGATTTTGATTTTTTTT
FAD3C'' (SEQ ID NO:19)  (1386) CTTAGTTCA--TTAAGTATAATTAGATTTTGATTTTTTTT
  FAD3C (SEQ ID NO:18)  (1290) --------A--TTAGCTATTATCATATGAC-ATGTCCTTG
                                  1561                                1600
  FAD3A (SEQ ID NO:15)  (1283) AA--------TAAATTAATGT-A----------TAAGTTT
 FAD3A' (SEQ ID NO:16)  (1304) AATTTAATTATAATTAAATATTCATTTTATAAATAATATT
 FAD3C' (SEQ ID NO:20)  (1304) AATTTATTTATAATTAAATATTCATTTTATAAATAATATT
FAD3A'' (SEQ ID NO:17)  (1443) TATAGGTTT-TCACT-ATTGTTATTTGTTTACATCAGCTT
FAD3C'' (SEQ ID NO:19)  (1424) TA--GGTTT-TCACTTATTGTTATTTGTTTACATCAGCTT
  FAD3C (SEQ ID NO:18)  (1319) AA--------TAAATTAATGT-AGCTTCATATGTGAGTTT
```

FIG. 3J

FIG 3K

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                                      1601                                                1640
FAD3A (SEQ ID NO:15)  (1304) AATAT----------------------AATATTTAT--A
FAD3A' (SEQ ID NO:16)  (1344) AGACCAATTATTAAAGGTTAGATATTTTAAGAATTATTCA
FAD3C' (SEQ ID NO:20)  (1344) AAACCAATTATTAAAGGTTAGATATTTGAAGAATTATTCA
FAD3A'' (SEQ ID NO:17)  (1481) CAGATATCTTCGAAA-------------AAGATTTAC--A
FAD3C'' (SEQ ID NO:19)  (1461) CAAACATCTTCGAAA-------------AAGACTTAC--A
FAD3C (SEQ ID NO:18)  (1350) AAT-------------------------AATATTTAT--A
                                      1641                                                1680
FAD3A (SEQ ID NO:15)  (1319) TATATTTGTTT---------TAATGGCTTAT---TTTA-T
FAD3A' (SEQ ID NO:16)  (1384) TGACTTTGTTTATTGGAA-----CTCGTTTTATCTTTTAA
FAD3C' (SEQ ID NO:20)  (1384) TGACTTTGTTTATTGCGAAATTACTCGTTTTATCTTTTAT
FAD3A'' (SEQ ID NO:17)  (1506) TGCATCAATTTCATGAGGATTTATAGTTTTTCT-TTTACT
FAD3C'' (SEQ ID NO:19)  (1486) TGCATCAATTTCCTGAGGATTTATAGTTTTT---TTTACT
FAD3C (SEQ ID NO:18)  (1363) TATTTTTGTTT---------TAATGGCTTAT---TTTA-T
                                      1681                                                1720
FAD3A (SEQ ID NO:15)  (1346) TGTTA-------AATGGATAC-----ATCAGCTTGAAATA
FAD3A' (SEQ ID NO:16)  (1419) TCTTTT---GTATTTCTCCATTTTTAATAATGAGAAACTG
FAD3C' (SEQ ID NO:20)  (1424) TCTTTT---GTATTTCTCTATTTTTAATATTGAGAAACTG
FAD3A'' (SEQ ID NO:17)  (1545) TATTTCCGAGACAATGTTTAGTAGTAAAAAGCATTAAATG
FAD3C'' (SEQ ID NO:19)  (1523) TATTTCTG-CACAATGTTTATTAGTAAAAAGCATCAAATG
FAD3C (SEQ ID NO:18)  (1390) TGTTA-------AATGGATAC-----ATCAGCTTGAAATG
                                      1721                                                1760
FAD3A (SEQ ID NO:15)  (1374) TCT----------ACGAACAT-GCATCATTTTCCTAGAT
FAD3A' (SEQ ID NO:16)  (1456) ACTTCAAATCTCCAATAAAGATGGTCTTATGTAGTAACAG
FAD3C' (SEQ ID NO:20)  (1461) ACTTCAAACCTCCAATAAAAATGGTTTCCTGTAGTAACAT
FAD3A'' (SEQ ID NO:17)  (1585) TTTTTTTG-CTCAAAAAAAAA-GAATGGGATTGTTAGAG
FAD3C'' (SEQ ID NO:19)  (1562) TTTTTTTG-CTCAAAAAAA---GAATGGGATTGTTAGAG
FAD3C (SEQ ID NO:18)  (1418) TCT----------ACGAACAT-GCATCATTTTCCTAGAT
```

FIG. 3K

```
FIG 3L                                1761                                1800
  FAD3A (SEQ ID NO:15)  (1402) A---CATTTGTTTGTTGCTCAAAAAATGAATAACGTAGTT
 FAD3A' (SEQ ID NO:16)  (1496) TA-TAATTTTTTGTTTGGTAAATGTAACATCATCTTCAAA
 FAD3C' (SEQ ID NO:20)  (1501) CA-TAATTTTTTGTTTGGTAAATGTAACATCATCTTCAAA
FAD3A'' (SEQ ID NO:17)  (1623) CACTCTATTGTTAGTTGTTCAATAAATATACCAACTAAAA
FAD3C'' (SEQ ID NO:19)  (1598) CACTCTATTGTTAGTTGTTCAATAAATATATCAACTAAAA
  FAD3C (SEQ ID NO:18)  (1446) A---CACTTGTTTGTTGCTCAAAAA-TGAATAACTTAGTT
                                1801                                1840
  FAD3A (SEQ ID NO:15)  (1439) AAAC-------------------GAGTGAGA---------
 FAD3A' (SEQ ID NO:16)  (1535) TATCTTTGAAAATAGACTTACATGCATTATTTTGCTGCGA
 FAD3C' (SEQ ID NO:20)  (1540) TATCTTTGAAAATAGACTTACATGCATTATTTTGCTGCGA
FAD3A'' (SEQ ID NO:17)  (1663) AAACAAAATAAATATA---AAATGAGTGAGATTGTTAAAT
FAD3C'' (SEQ ID NO:19)  (1638) AAACAAAATAAATATA---AAATGAGTGAGATTGTTAAAT
  FAD3C (SEQ ID NO:18)  (1482) AAAC-------------------GAGTGAGCATGTTCTAT
                                1841                                1880
  FAD3A (SEQ ID NO:15)  (1451) ---------------------TTCTTAG------------
 FAD3A' (SEQ ID NO:16)  (1575) CATTATTGTCACTTATTCCTGGCAATAAAT-TAGTTTATT
 FAD3C' (SEQ ID NO:20)  (1580) CATTATTGTAACTTATTCCTGGCAATAAAAATAATTTATT
FAD3A'' (SEQ ID NO:17)  (1700) CATTATAGAGAGCAATTTCATTTTCACAAAAATAAATAAAT
FAD3C'' (SEQ ID NO:19)  (1675) CATTATAGAGACAATTTCATTTTCACAAAAATAAATAAAT
  FAD3C (SEQ ID NO:18)  (1503) GGGG----------------TTTCTTAGAGCATGATTATT
                                1881                                1920
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (1614) ACTG-AACTTTTTTTGGTCAATTTATTACTAGTAACTTT
 FAD3C' (SEQ ID NO:20)  (1620) ACTGGAAACTATTTTTGGTCAATTTATTACTAGTAACTTA
FAD3A'' (SEQ ID NO:17)  (1740) ACAT--AACTTTTTATAATTGGGGTTTGCAGGAGAATAAG
FAD3C'' (SEQ ID NO:19)  (1715) ACAT--AACTTTTG-TAATTGGGGTTTGCAGGAGAATAAG
  FAD3C (SEQ ID NO:18)  (1527) GAGA--AGTTCCTA-GAGTGAGGTTCTTACCGGAATATAA
```

FIG. 3L

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FIG 3M                                  1921                                1960

  FAD3A (SEQ ID NO:15)  (1458) ------------------------------------------
 FAD3A' (SEQ ID NO:16)  (1653) AAACTTAAAAGAGTGAGATTGTTTGATCAAAAAAAAT---
 FAD3C' (SEQ ID NO:20)  (1660) AAACTTAAAAGAGTGAGATTGTTTGATCAAAAAAAAAGAG
FAD3A'' (SEQ ID NO:17)  (1778) CCATCGGACACACCACCAGAACCATGGCCATGTTGAAAAC
FAD3C'' (SEQ ID NO:19)  (1752) CCATCGGACACACCACCAGAACCATGGCCATGTTGAAAAC
  FAD3C (SEQ ID NO:18)  (1564) GAATCTATCTCTTAACTTTTAACTAAAAAAATTAAGAACC

                                        1961                                2000

  FAD3A (SEQ ID NO:15)  (1458) ------------------------------------------
 FAD3A' (SEQ ID NO:16)  (1690) ---AAAAATACAGTGAGATAGTTAGAATCTGCCATGAAAG
 FAD3C' (SEQ ID NO:20)  (1700) AAAAAAAATACAGTGAGATTGTTAGAATCTGCCATGAAAG
FAD3A'' (SEQ ID NO:17)  (1818) GACGAGTCTTGGCTTCCGGTAATCTTTCCTACTCTCGTAG
FAD3C'' (SEQ ID NO:19)  (1792) GACGAGTCTTGGCTTCCGGTAATCTTTCCTACTCTCATTG
  FAD3C (SEQ ID NO:18)  (1604) GGCTTTTAAAACTCGTATTTAAGAACCGTTTTTTAGTTTT

                                        2001                                2040

  FAD3A (SEQ ID NO:15)  (1458) ------------------------------------------
 FAD3A' (SEQ ID NO:16)  (1727) CAACACTATATAG--------------------------
 FAD3C' (SEQ ID NO:20)  (1740) CAACACTATATAGGTGATGATTGGTTCGACTGTGGCCGTA
FAD3A'' (SEQ ID NO:17)  (1858) TTTGTCTTGTCTTTTATTTATTTGTTTGTTTTTCGGAATT
FAD3C'' (SEQ ID NO:19)  (1832) TTTCTCTTGTCTTTTATTTATTTGTTCTTTTTGGGAATT
  FAD3C (SEQ ID NO:18)  (1644) TTTAGTTAAAAATCAAGAGACGACTTCTTATATTCCGCTA

                                        2041                                2080

  FAD3A (SEQ ID NO:15)  (1458) ------------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ------------------------------------------
 FAD3C' (SEQ ID NO:20)  (1780) GAATTTTAGCTGTAGATAAATTGGTTGTAGTTGTAAAGTT
FAD3A'' (SEQ ID NO:17)  (1898) TATTCTTA--TGTC--TATGTTCTTAGGATTCTATATGTT
FAD3C'' (SEQ ID NO:19)  (1872) CATTCTTA--TGTC--TAAGTTCTTATGATTATTGAAGTT
  FAD3C (SEQ ID NO:18)  (1684) AGAACTCC--ACCC--TGAGAACTTCTCAATAATTCATGCT
```

FIG. 3M

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FIG 3N                                2081                                2120
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (1820) GTTACTGTT-GATTATTTTTGCAGAGACTTTTGCTGTAGT
FAD3A'' (SEQ ID NO:17)  (1934) TATTTTATTAGTTTATGTTTTCAGTCTGAGGTCA-GACCG
FAD3C'' (SEQ ID NO:19)  (1908) CTTAAGGTGGGGTTCTTAACGGAATATGAGAACCTGTCTC
  FAD3C (SEQ ID NO:18)  (1720) CTTAGTGCTCTAAGAAGGGTCCTTAACAAAATAT------
                                2121                                2160
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (1859) TAAATTTGTTGTAGCTGTAAGCTATAGGGTGCAGATATTT
FAD3A'' (SEQ ID NO:17)  (1973) ACCACTTGTCAG-------ATCTGTTTTCTAGCTGT--AG
FAD3C'' (SEQ ID NO:19)  (1948) TTAACTTTTAACTAAAA-AAGCTAAGAACCAGGTTTTAAA
  FAD3C (SEQ ID NO:18)  (1754) --TAATAATAAG-------ATATAGTGTGGGCCCAA----
                                2161                                2200
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (1899) TAAAATAAAATATGTAAAATATGTGATGCATGTATATATA
FAD3A'' (SEQ ID NO:17)  (2004) TAAAA--------AACAA-TTTGCAAGTGTAATAGTTCAG
FAD3C'' (SEQ ID NO:19)  (1987) TAAGAGTTTTATGAACACGTTCTTAATTTTTTTAGTTAAA
  FAD3C (SEQ ID NO:18)  (1781) -AAAA--------AACAAAAAACCGGTTACAAAAGTCGCG
                                2201                                2240
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (1939) AAATAATTATTATTTTATCACTTAAAAT-AATTTATATT
FAD3A'' (SEQ ID NO:17)  (2035) CATAATTGATCTTGTT--------------AGAGCAT-TT
FAD3C'' (SEQ ID NO:19)  (2027) AGTTAAGAAACGGGTTCTTATATTCCGCTAAGAACCTCTT
  FAD3C (SEQ ID NO:18)  (1812) AAAGAAGGATCGATTT--------------TGGTCTTTTA
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FIG. 3N

```
FIG 3O                                  2241                                2280

  FAD3A (SEQ ID NO:15)  (1458) ------------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ------------------------------------------
 FAD3C' (SEQ ID NO:20)  (1978) AATATTTTTTAAAATTATCAAAGTTTACTGTTATTTAAAA
FAD3A'' (SEQ ID NO:17)  (2060) CCAAAA-----CAA--------------------------
FAD3C'' (SEQ ID NO:19)  (2067) CCTAAAAACCCCAATAATCATACTC--CTAGGATTCTATA
  FAD3C (SEQ ID NO:18)  (1838) CTTGTA-------------------------------------

                                2281                                2320

  FAD3A (SEQ ID NO:15)  (1458) ------------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ------------------------------------------
 FAD3C' (SEQ ID NO:20)  (2018) TGTGATATGTAAATAATCTATATTATTTAAAATATTTCAA
FAD3A'' (SEQ ID NO:17)  (2069) ------ACTTTATAATTTTAAATATACAGT-TT-------
FAD3C'' (SEQ ID NO:19)  (2105) TGTT-TATTTTATTAGTTTATGTTTTCAGTCTGAGGTCAG
  FAD3C (SEQ ID NO:18)  (1844) ------CTGTTTGTGGATCCCACTGGTGGT----------

                                2321                                2360

  FAD3A (SEQ ID NO:15)  (1458) ------------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ------------------------------------------
 FAD3C' (SEQ ID NO:20)  (2058) TAATTTAAAAGCACCCAAAATTAGAGTAAAATATTTATAG
FAD3A'' (SEQ ID NO:17)  (2095) ---------TT--------TGTTCTCT---------AAAA
FAD3C'' (SEQ ID NO:19)  (2144) ACCGGCCACTTGTCAGATCTGTTTTCTAGCTGTAGTAAAA
  FAD3C (SEQ ID NO:18)  (1868) -------------------GGTCCGGG---------ATTG

                                2361                                2400

  FAD3A (SEQ ID NO:15)  (1458) ------------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ------------------------------------------
 FAD3C' (SEQ ID NO:20)  (2098) ATGTTTTTTTATTATTATGATTATCTTATT--TATTTAATATT
FAD3A'' (SEQ ID NO:17)  (2109) AAGAATTT--------AAAAATT---------TTAAAGTT
FAD3C'' (SEQ ID NO:19)  (2184) AACAATTTGCAAGTGTAATAGTTCAGCGGTAATTAATGTT
  FAD3C (SEQ ID NO:18)  (1880) GTTTCTTT--------TTTAATT------TAATTTATTTT
```

FIG. 3O

```
FIG 3P                                  2401                                2440
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (2136) ATAGATATTTTTTGTTCTTACAGTTTCTACAGCTTATAAA
FAD3A'' (SEQ ID NO:17)  (2132) TGAGGGACGA------------------AACTTCAAATT
FAD3C'' (SEQ ID NO:19)  (2224) CTCGGATCTATCTCAAAAAAAAATTTTATAACTTCAAATA
  FAD3C (SEQ ID NO:18)  (1906) TTTAATCGGA------------------GAAAAAAATTA
                                  2441                                2480
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (2176) TGAAAGATGTAAGTTGTTTAACTAAAATACATAAGAA---
FAD3A'' (SEQ ID NO:17)  (2153) TGAAC----------TTTCACTACTCAACTTC-AAATTT
FAD3C'' (SEQ ID NO:19)  (2264) TAAAGATTTTTTTGTTTTTCAAAAATGAACTTCGAAACTT
  FAD3C (SEQ ID NO:18)  (1927) AGAAA---------C----CAAAAAACAGTTTT-----AA
                                  2481                                2520
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (2213) -AAATGTTTGGTTTTTTTTTTGCTGTAGCTTTATTTTTAA
FAD3A'' (SEQ ID NO:17)  (2181) GAAATTTCATCTTTTTTATTTACATTTTGATCATTATAAT
FAD3C'' (SEQ ID NO:19)  (2304) CAAATTTGAAGTTTTTTTTTTGCATTTTGATCATTATAAT
  FAD3C (SEQ ID NO:18)  (1949) TCATGGCCTCATGTTGGGGTTGAGTTTTATATTCTGATAA
                                  2521                                2560
  FAD3A (SEQ ID NO:15)  (1458) ----------------------------------------CA
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (2252) -ACTTAAAGCATG-ATTGGTAAAAATTAATAGAAATTTGA
FAD3A'' (SEQ ID NO:17)  (2221) TAATTATACATTACATTTATGATTCTTAAGTATTTTCTCA
FAD3C'' (SEQ ID NO:19)  (2344) TAATTACACGTTACATTTATAATTCTTAAGTATTTTTTCA
  FAD3C (SEQ ID NO:18)  (1989) GAATCCCATCTTAAAAACCCCGTTAAACATGCTCTTACCA
```

FIG. 3P

```
FIG 3Q                                  2561                                2600
  FAD3A (SEQ ID NO:15)  (1460) TCTGCC--------TCGAAAACG----ATATGTTATTGAC
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (2290) TGTAGACTTTAATTTTGAAAAGT----AAACGTAAAGCAT
FAD3A'' (SEQ ID NO:17)  (2261) TTTATTGTTTTAATTCTTAAATTTTTTATACATCATAAAT
FAD3C'' (SEQ ID NO:19)  (2384) TTTATCGTTTTAATTGTTAAATTTTTTATATATTATAAAT
  FAD3C (SEQ ID NO:18)  (2029) TCTGCT--------TCGAAAATG----ATATGTTATTGAC
                                  2601                                2640
  FAD3A (SEQ ID NO:15)  (1488) AATTCCAA---TTTCAT--TTT-----------------
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (2326) GATTGGTAAAGTTTAATGATTTAGAAA--AAAATAAAGCT
FAD3A'' (SEQ ID NO:17)  (2301) ATTTCCAA---TTTGTT--TTTATAAATTCAAATTTTACA
FAD3C'' (SEQ ID NO:19)  (2424) ATTTCCAA---TTTGTT--TTTATAAATTCAAATTTTATA
  FAD3C (SEQ ID NO:18)  (2057) AATTCCAA---TTTCAT--TTT-----------------
                                  2641                                2680
  FAD3A (SEQ ID NO:15)  (1505) --------TATGAAAA---TAA--AAT-----------AA
 FAD3A' (SEQ ID NO:16)  (1740) ----------------------------------------AC
 FAD3C' (SEQ ID NO:20)  (2364) AAAGTAGCTAGATAAAACCCAACCAATCACCTCCATGGAC
FAD3A'' (SEQ ID NO:17)  (2336) CAAAAAAGTAATAAAAATTTTA--AAT-----------AA
FAD3C'' (SEQ ID NO:19)  (2459) CATAAAACTAATAAAAATGTTA--AAT-----------AA
  FAD3C (SEQ ID NO:18)  (2074) --------TATGAAAA---TAA--AAT-----------AA
                                  2681                                2720
  FAD3A (SEQ ID NO:15)  (1521) TAGTT----TATTT-------TATAATTGGGGGTGG----
 FAD3A' (SEQ ID NO:16)  (1742) AATTTAATTTTTATGAAAACACAT--TTAATAATTTGAG-
 FAD3C' (SEQ ID NO:20)  (2404) AATTTAATTTTTATGTAAACACATATTTAATAATTTGAG-
FAD3A'' (SEQ ID NO:17)  (2363) GATTTTATAATATTTTAAAAC-TATAATTAGGCAAAAAAAA
FAD3C'' (SEQ ID NO:19)  (2486) GATTTTATAATATTT-AAGAC-TATAATTAGTCAACAAAA-
  FAD3C (SEQ ID NO:18)  (2090) TAGTT----TATTT-------TATAACTGAGGGTGG----
```

FIG. 3Q

```
FIG 3R                                  2721                                2760
  FAD3A (SEQ ID NO:15)  (1546) --TTGCAGGA------GAATAAG----------CCATCGG
 FAD3A' (SEQ ID NO:16)  (1779) -GCTGCAGGA------GAATAAG----------CCATCGG
 FAD3C' (SEQ ID NO:20)  (2443) -GCTGCAGGA------GAATAAG----------CCATCGG
FAD3A'' (SEQ ID NO:17)  (2402) TATTACAAAA-AAATGTAATAA---AAACTTTAAAATAAG
FAD3C'' (SEQ ID NO:19)  (2523) TATTACAAAAGAAATGTAATAATAAAAAATTTAAAATAAG
  FAD3C (SEQ ID NO:18)  (2115) --TTGCAGGA------GAATAAG----------CCATCGG
                                  2761                                2800
  FAD3A (SEQ ID NO:15)  (1568) ACACACCAC--CAGAACCATGGCCATGTTGAAA----ACG
 FAD3A' (SEQ ID NO:16)  (1802) ACACACCAC--CAGAACCATGGCCATGTTGAAA----ACG
 FAD3C' (SEQ ID NO:20)  (2466) ACACACCAC--CAGAACCATGGCCATGTTGAAA----ACG
FAD3A'' (SEQ ID NO:17)  (2438) ATATATCAAGACATAATTATTAGAAATTTTAAATATTATA
FAD3C'' (SEQ ID NO:19)  (2563) ATACATGAAGACATAACTATTAGAAAATTTAAATATTATA
  FAD3C (SEQ ID NO:18)  (2137) ACACACCAC--CAGAACCATGGCCATGTTGAAA----ACG
                                  2801                                2840
  FAD3A (SEQ ID NO:15)  (1602) ACGAGTCTTGGGTTCCGGTAA------TC-----CCCCTC
 FAD3A' (SEQ ID NO:16)  (1836) ACGAGTCTTGGGTTCCGGTAACATT--TC-----CCTCTT
 FAD3C' (SEQ ID NO:20)  (2500) ACGAGTCTTGGGTTCCGGTAACATT--TC-----CCTCTT
FAD3A'' (SEQ ID NO:17)  (2478) ACAATATTAATAATCTGGTAAATTTGCTCCAAAACCTCAA
FAD3C'' (SEQ ID NO:19)  (2603) ACAATACTAATAATCTGGTAAATTTGCTCTGGAACCTCTA
  FAD3C (SEQ ID NO:18)  (2171) ACGAGTCTTGGGTTCCGGTAA------TCTTTC-CCTCTC
                                  2841                                2880
  FAD3A (SEQ ID NO:15)  (1631) TCATT--------------------ATTTTTTTT-----
 FAD3A' (SEQ ID NO:16)  (1869) TAATA---------ATT------TCTATTTTTCT------
 FAD3C' (SEQ ID NO:20)  (2533) TAATA---------ATT------TCTATTTTTCTT--T--
FAD3A'' (SEQ ID NO:17)  (2518) AAATTTCTAAATTATTGTCCAAACAAATTT-GTTTAACCG
FAD3C'' (SEQ ID NO:19)  (2643) AAATT--------ATTGTCTAAACAAATTTTGTGTAACCG
  FAD3C (SEQ ID NO:18)  (2204) TCAT--------------------ATTTTTTTT-----
```

FIG. 3R

```
FIG 3S                                  2881                                2920
  FAD3A (SEQ ID NO:15)  (1645) --------------------TCTTTTTTTGAAAC------
 FAD3A' (SEQ ID NO:16)  (1888) ---------GTCAAAATAATTAGTTTTTCGAAATTTGAGG
 FAD3C' (SEQ ID NO:20)  (2554) ---------GTCAAAATAATTTGTTTTTCGAAATTTGAGG
FAD3A'' (SEQ ID NO:17)  (2557) AATATGGAGCATTACAAAAATAATTTTATGGAATAGTGTG
FAD3C'' (SEQ ID NO:19)  (2675) AAGATGGAGCATTACGAAAATAATTTTATGAAATAATATG
  FAD3C (SEQ ID NO:18)  (2217) --------------------CTTTTTTTTGAAAT------
                                2921                                2960
  FAD3A (SEQ ID NO:15)  (1659) -------------------T--CTTTCATTTTAATTTTCT-
 FAD3A' (SEQ ID NO:16)  (1919) CCAGAACGACCACTTGTCAA-ATTTGATT-TTTAGCTGTA
 FAD3C' (SEQ ID NO:20)  (2585) CCAGAACGACCACTTGTCAG-ATTTGATT-TCTAGCTGTA
FAD3A'' (SEQ ID NO:17)  (2597) GTATTTTGCTTGTAGTT-AATATTTAATTATGTATTTCTA
FAD3C'' (SEQ ID NO:19)  (2715) GTATTTTGCTTCTAGTTTAATATTTAATTATATATTTCTA
  FAD3C (SEQ ID NO:18)  (2231) -------------------T--CTTTCATTTTAATTTTCT-
                                2961                                3000
  FAD3A (SEQ ID NO:15)  (1678) --TAGAATTCTATGTATTTA-----------TTTTAATCA
 FAD3A' (SEQ ID NO:16)  (1957) GTAAAAACAGTTTGCTAGTGTCACAGTTAACCGGTAATTG
 FAD3C' (SEQ ID NO:20)  (2623) GTAAAAACAGTTTGCTAGTGTCACAGTTAACCGGTAATTG
FAD3A'' (SEQ ID NO:17)  (2636) TTTATAATTTTATATATTTAATGTAAGATTTTTTTAATTA
FAD3C'' (SEQ ID NO:19)  (2755) TTTATAATTTTATATATTTAATGTAAATTTTTATTAATTA
  FAD3C (SEQ ID NO:18)  (2250) --TAGGATTCTATGTATTTA-----------TTTTAATCA
                                3001                                3040
  FAD3A (SEQ ID NO:15)  (1705) ATCCT-----------------------------------
 FAD3A' (SEQ ID NO:16)  (1997) ATTCTTTTTAACGATTTATAGAAGTAACATTTTTGTAAAA
 FAD3C' (SEQ ID NO:20)  (2663) ATTCTTTTTAGCGATTTATAGAAGTAACATTTTTGTAAAA
FAD3A'' (SEQ ID NO:17)  (2676) ATATTACTGTAATATTTTTATATATGTACTAGTTATTTAT
FAD3C'' (SEQ ID NO:19)  (2795) ATATTACTGTAATATTTTTATATATGTGCTAGTTATTTAT
  FAD3C (SEQ ID NO:18)  (2277) ATCCT-----------------------------------
```

FIG. 3S

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FIG 3T                                3041                                3080
  FAD3A (SEQ ID NO:15)  (1710) -----TTTT-------------------------------
 FAD3A' (SEQ ID NO:16)  (2037) TAAAATATACATTATGGTATGTGACAACGGACCACGCTTA
 FAD3C' (SEQ ID NO:20)  (2703) TAAAATATACATAATAGTATGTGACAACGGACCACGCCTA
FAD3A'' (SEQ ID NO:17)  (2716) AAAAGTTTT-ATAGATTTGTATTAGTTATAACAAAAATAA
FAD3C'' (SEQ ID NO:19)  (2835) AATTTTTTTTATGGATTTATATTAG----ACCATGATTAA
  FAD3C (SEQ ID NO:18)  (2282) -----TTTT-------------------------------
                                3081                                3120
  FAD3A (SEQ ID NO:15)  (1714) ----------------------------C-------CAGTG
 FAD3A' (SEQ ID NO:16)  (2077) TTTGTATTGGTGAATCTTTTAATTAC-TC--CCT-CCAAT
 FAD3C' (SEQ ID NO:20)  (2743) TTTGTATCGGTGAATCTTCTAATTAC-TT--CCT-CCGAT
FAD3A'' (SEQ ID NO:17)  (2755) GGATCATTGTGTAAAATACAAATAATTTTGAAATTACGTT
FAD3C'' (SEQ ID NO:19)  (2871) CCCGGAGTTCTTAGAGTG-----------GAGTTTTAGTT
  FAD3C (SEQ ID NO:18)  (2286) ----------------------------C-------CAGTT
                                3121                                3160
  FAD3A (SEQ ID NO:15)  (1720) TGAGGCTTG-------------------------------
 FAD3A' (SEQ ID NO:16)  (2113) TTATTTTAGTTGCAGATTTAGATTTATGCACATAGATTAA
 FAD3C' (SEQ ID NO:20)  (2779) TTATTTTAGTTACAGTTTTAGATTTATACACATAGATTAC
FAD3A'' (SEQ ID NO:17)  (2795) TAAAGTTTTGGTTATGAAAAAAATACTTTGAAACTTTAAA
FAD3C'' (SEQ ID NO:19)  (2900) AAACGTT----------AAGAAACAGTTTCTTAACTTCCG
  FAD3C (SEQ ID NO:18)  (2292) TGAGGCTAG-------------------------------
                                3161                                3200
  FAD3A (SEQ ID NO:15)  (1729) -----------G---ACGACCACTTGTCAGATTTGTCG--
 FAD3A' (SEQ ID NO:16)  (2153) TAAAAATA-----TTTTGCACATTTTCAAAATAAAAACAC
 FAD3C' (SEQ ID NO:20)  (2819) AAAAAATAAAATATTTTGTCCATTTTTAAAATAAAAACAT
FAD3A'' (SEQ ID NO:17)  (2835) TTTAGAGTTTTGCAAACTTTAAAATGTTAGATAGATAGTT
FAD3C'' (SEQ ID NO:19)  (2930) GTAAGAACC---CCATCCTAAGAATCCCAGGTTAATC---
  FAD3C (SEQ ID NO:18)  (2301) -----------G---ACGACCACTTGTCAGATTTGTCG--
```

FIG. 3T

```
FIG 3U                                3201                                3240
  FAD3A (SEQ ID NO:15)  (1753) -------T-------TTAGCTGTAG----------------
 FAD3A' (SEQ ID NO:16)  (2188) CATTAC-TTATACAACTAACCATATTTCAACCAATAAAAA
 FAD3C' (SEQ ID NO:20)  (2859) CACTAA-TTATACACCTAACAATATTTTAACCAATAAAAA
FAD3A'' (SEQ ID NO:17)  (2875) TTTTTGGAGATGCATTTAGTGGTTATGGTAGTAACTCAGA
FAD3C'' (SEQ ID NO:19)  (2964) ---------ATGCTCTTAGTTATAA-----------CAAA
  FAD3C (SEQ ID NO:18)  (2325) -------T-------TTAGCTGTAG----------------
                                3241                                3280
  FAD3A (SEQ ID NO:15)  (1764) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (2227) --TAAATTAGAAAATATTATTTATAAATTTTGTATTGAAA
 FAD3C' (SEQ ID NO:20)  (2898) A-TAAACTAGAAAATATTATTCATAATTTTTACATTGAAA
FAD3A'' (SEQ ID NO:17)  (2915) AAATGAAAAATCTATACTTTTATACTCCCTCCGTTTTTTA
FAD3C'' (SEQ ID NO:19)  (2984) TAAGGATCATTGTGTAA----------------------A
  FAD3C (SEQ ID NO:18)  (2336) ----------------------------------------
                                3281                                3320
  FAD3A (SEQ ID NO:15)  (1764) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (2265) TTATAAAATAATACTTATTTTAAAACGAAATT------AA
 FAD3C' (SEQ ID NO:20)  (2937) TTATAAAACGATACTTATTTTAAAACAAAATTTT----AA
FAD3A'' (SEQ ID NO:17)  (2955) ATATAAGTCGTTTTACAGTTATACACGTAGATTAAGAAAA
FAD3C'' (SEQ ID NO:19)  (3002) ATACAAATAATTTTGAAGTTATGTTTGAAGTTTG------
  FAD3C (SEQ ID NO:18)  (2336) ----------------------------------------
                                3321                                3360
  FAD3A (SEQ ID NO:15)  (1764) ----------------------T----AAACAACTG---
 FAD3A' (SEQ ID NO:16)  (2299) TTTACAACGACAATTAAACTGAAACGGAAAGAAATTATTA
 FAD3C' (SEQ ID NO:20)  (2973) TTTACAACGACAATTAAATTGAAACGGAAGAAGTTTATTA
FAD3A'' (SEQ ID NO:17)  (2995) CCATTAATTTCTTATATTTTCTAGACAAAAACATCATTAA
FAD3C'' (SEQ ID NO:19)  (3036) ----------------TTTTC--GAAGAAAACCACTTTGA
  FAD3C (SEQ ID NO:18)  (2336) ----------------------T----AAACAACTG---
```

FIG. 3U

```
FIG 3V                              3361                                3400

  FAD3A (SEQ ID NO:15)  (1774) --ATTTA----------------------------------

 FAD3A' (SEQ ID NO:16)  (2339) ATACTTAATTAAAGAGTTTTT-------AGAAAAATTGAA

 FAD3C' (SEQ ID NO:20)  (3013) TTACTTAATTAAAGAGTTTTTT-----TAAAAAAAATGAA

FAD3A'' (SEQ ID NO:17)  (3035) TTATTTACCTAACCACAATTCAACCAATATAAAAATAGAA

FAD3C'' (SEQ ID NO:19)  (3058) AACTTTA-----------------AATTTAGAGT---AA

  FAD3C (SEQ ID NO:18)  (2346) --ATTTA----------------------------------

                              3401                                3440

  FAD3A (SEQ ID NO:15)  (1779) ----------------------------AATTGTTTATGG

 FAD3A' (SEQ ID NO:16)  (2372) AGACATGTTTATGCGAAACTCATGTGAAAGTCTTTGAAAT

 FAD3C' (SEQ ID NO:20)  (3048) AGACATGTTTATGCGAAACTCATGTGAAAGTCTTTCAAAT

FAD3A'' (SEQ ID NO:17)  (3075) GATATATTACCATTGGTCATACAACATTAATTATTAATAA

FAD3C'' (SEQ ID NO:19)  (3077) ACTCTATT-----------TAGAG----AGTTTTTTTTAG

  FAD3C (SEQ ID NO:18)  (2351) ----------------------------AATTGTTTATAG

                              3441                                3480

  FAD3A (SEQ ID NO:15)  (1791) ----TACT----------------------------------

 FAD3A' (SEQ ID NO:16)  (2412) AATAGATTTTGGTATAAATATTTCAAATTTTCTT------

 FAD3C' (SEQ ID NO:20)  (3088) AAAATATTTTGGTATAAATTTTTCAAATTTTCA-------

FAD3A'' (SEQ ID NO:17)  (3115) ATTTTACATAG-AAAACCGAAAAGGACATATAATTTGGAA

FAD3C'' (SEQ ID NO:19)  (3102) AGGTTACGCAGTAACTCAGAAAATGA--------------

  FAD3C (SEQ ID NO:18)  (2363) ----TACT----------------------------------

                              3481                                3520

  FAD3A (SEQ ID NO:15)  (1795) ----------------------------------------

 FAD3A' (SEQ ID NO:16)  (2446) -----AAAATAATAATTATATATTAATATAAT--------

 FAD3C' (SEQ ID NO:20)  (3121) -----AAAATAATAATTATAAATTAATATAATATAAT---

FAD3A'' (SEQ ID NO:17)  (3154) CAAAAAAATTTCTCTAAAACGACTTATATTAAAAAACGGA

FAD3C'' (SEQ ID NO:19)  (3128) ----AAAATCTAT--------ACTTTTAT-----------

  FAD3C (SEQ ID NO:18)  (2367) ----------------------------------------
```

FIG. 3V

```
FIG 3W                                3521                                3560
  FAD3A (SEQ ID NO:15)  (1795) ----G---TAGTTAACTTTAACAACGGGCCACTTATATTC
 FAD3A' (SEQ ID NO:16)  (2473) ----TTGTGATAAAATCTCGTCAAAAACTCACTAATGCAA
 FAD3C' (SEQ ID NO:20)  (3153) ----TTGTGATAAAATCTCGTCAAAAACTCACTAATGCAA
FAD3A'' (SEQ ID NO:17)  (3194) GGGAGTAGTACCTAACTTTAACGATGGACCACTTATATTC
FAD3C'' (SEQ ID NO:19)  (3145) ------AGTACCTAACTTTATCGATGGACCACTTATATTC
  FAD3C (SEQ ID NO:18)  (2367) ----G---TAGTTAACTTTAACAACGGACCACTTATATTC
                                3561                                3600
  FAD3A (SEQ ID NO:15)  (1828) GAGCCATTGG-CATAAAATGATT-CTTCTCGAAATTCGTT
 FAD3A' (SEQ ID NO:16)  (2509) ATGCTTTTAT-TTTGAATTTCTTACTCCTCTAAATGCATT
 FAD3C' (SEQ ID NO:20)  (3189) ATGCTTTTATATTTGAGTTTCTTACTCCTCTAAATGCATT
FAD3A'' (SEQ ID NO:17)  (3234) GAGTCCTTAG-CATAAAATCATT-CTCCTCGAAATCCGTT
FAD3C'' (SEQ ID NO:19)  (3179) GAGTCCTTAG-CATAACATGATT-CTCCTCGAAATCCGTT
  FAD3C (SEQ ID NO:18)  (2400) GAGCCATTGG-CATAAAATGATT-CTTCTCGAAATTCGTT
                                3601                                3640
  FAD3A (SEQ ID NO:15)  (1866) TACTTTTCT--TAGTATT-TTT-------CAGTTTTGTAG
 FAD3A' (SEQ ID NO:16)  (2548) TACTTTTATACTAATATTATTTTCTTTCTCTAATTTGCCG
 FAD3C' (SEQ ID NO:20)  (3229) TACTTTTATACTATTATTATTTTCTTTCTCTAATTTGGTG
FAD3A'' (SEQ ID NO:17)  (3272) TACTTTCTT--CATTATT-TTTTCCTTTTCAGTTTTGGCG
FAD3C'' (SEQ ID NO:19)  (3217) TACTTTCTT--CGTTATT-TTTTCCTTTTCAGTTTTGGCG
  FAD3C (SEQ ID NO:18)  (2438) TACTTTTCT--TAGTATT-TTT-------CAATTTTGGAG
                                3641                                3680
  FAD3A (SEQ ID NO:15)  (1896) TTTACGTAGAACTAAT------AA-----AAAG-------
 FAD3A' (SEQ ID NO:16)  (2588) TTT-CGTAATAGTTTG--TCTGTATTTTGAAAACTA----
 FAD3C' (SEQ ID NO:20)  (3269) TTTTCGTAATAGTTTG--CCTGTGTTTTGAAAACTA----
FAD3A'' (SEQ ID NO:17)  (3309) TTTTCGTAATACTTTTGTCTTCAATCTTGAAAGCTATTAG
FAD3C'' (SEQ ID NO:19)  (3254) TTTTCGTAATACTTTTGTCTGCAATCTTGAAAGCTATTAG
  FAD3C (SEQ ID NO:18)  (2468) TTTACGTAGAACTAAT------AA-----AAAG-------
```

FIG. 3W

```
FIG 3X                              3681                                3720
  FAD3A (SEQ ID NO:15)  (1918) -AAAAAAACTTATAAACACACC------------------
 FAD3A' (SEQ ID NO:16)  (2621) -ACAAAAAATAATAAAAACAAA---------AGCTTATAA
 FAD3C' (SEQ ID NO:20)  (3303) -ACAAAAAATAATAAAAACAAA---------AGTTTATAA
FAD3A'' (SEQ ID NO:17)  (3349) TATAAAAACTTATAAACACATCACATGCAATGAATTAATA
FAD3C'' (SEQ ID NO:19)  (3294) TATAAAA-CTTATAAACACAT----------GAATTAATA
  FAD3C (SEQ ID NO:18)  (2490) -AAAA--ACTTATAAACACACC------------------
                              3721                                3760
  FAD3A (SEQ ID NO:15)  (1939) ----------------------ACATGCAATGAATA---
 FAD3A' (SEQ ID NO:16)  (2651) ---ACACAT------------A-GCATGCAATGAATATG-
 FAD3C' (SEQ ID NO:20)  (3333) ---ACACAT------------A-GCATGCAATGAAT----
FAD3A'' (SEQ ID NO:17)  (3389) CGAATACATAACCAGAATGACAAATTTTCAATGAATATTT
FAD3C'' (SEQ ID NO:19)  (3323) CGAATACATAACCAGAATGACAAATTTTCAATGAATATTT
  FAD3C (SEQ ID NO:18)  (2509) ----------------------ACATGCAATGAATA---
                              3761                                3800
  FAD3A (SEQ ID NO:15)  (1953) AATTCGAATATATAA----CCATACTGTTAAA--------
 FAD3A' (SEQ ID NO:16)  (2674) TACGAATATATATACCAATACATA-TCTAAGTACTATTTT
 FAD3C' (SEQ ID NO:20)  (3353) -------ATATATATCAATACATA-TCTAAGTACTATTTT
FAD3A'' (SEQ ID NO:17)  (3429) AATACCAGTAAGTACTACTCCGTAATAGTAATAGTAATAG
FAD3C'' (SEQ ID NO:19)  (3363) AATACTAGTAAGTACTACTCCGTAATAGTAAT-----TAG
  FAD3C (SEQ ID NO:18)  (2523) AATTCGAATATATAA----CCATACTGTTAAA--------
                              3801                                3840
  FAD3A (SEQ ID NO:15)  (1981) ---TATTAAT--------------------T----AA---
 FAD3A' (SEQ ID NO:16)  (2713) TCCAAGTACT---T--------------AATCTTGATTAC
 FAD3C' (SEQ ID NO:20)  (3385) TGCAAGTACT---T--------------AATCTTGATTAC
FAD3A'' (SEQ ID NO:17)  (3469) TCATATTAATTTTTTTTGTCATCAAACAAACAGTAATAG
FAD3C'' (SEQ ID NO:19)  (3398) TAATAGTAAT----------------------AGTAATAG
  FAD3C (SEQ ID NO:18)  (2551) ---TATTAAT--------------------T----TA---
```

FIG. 3X

```
FIG 3Y                                  3841                                3880

  FAD3A (SEQ ID NO:15)  (1991) -CATTTTAATCTTAATTTTGCATTCCAGTTGCCAGAAAAA
 FAD3A' (SEQ ID NO:16)  (2736) TAAAATTCATTTTAATTGTTCCTTTCAGTTACCAGAAAGG
 FAD3C' (SEQ ID NO:20)  (3408) TAAAATTCATTTTAATTGTTCCTTTCAGTTACCAGAAAAG
FAD3A'' (SEQ ID NO:17)  (3509) TAATATTAATTATAATTATGTATTTCAGTTGCCAGAAAAG
FAD3C'' (SEQ ID NO:19)  (3416) TCATATTAATTATAATTATGTATTTCAGTTGCCAGAAAAG
  FAD3C (SEQ ID NO:18)  (2561) -CATTTTAATCTTAATTTTGCATTCCAGTTGCCAGAAAAA

                                  3881                                3920

  FAD3A (SEQ ID NO:15)  (2030) TTATACAAGAATTTGTCCCACAGTACACGGATGCTCAGAT
 FAD3A' (SEQ ID NO:16)  (2776) TTATACAAGAATTTACCCCACAGTACTCGGATGCTCAGAT
 FAD3C' (SEQ ID NO:20)  (3448) TTATACAAGATTTTACCCCACAGTACTCGGATGCTCAGAT
FAD3A'' (SEQ ID NO:17)  (3549) TTGTACAAGAACTTGCCCCATAGTACTCGGATGCTCAGAT
FAD3C'' (SEQ ID NO:19)  (3456) TTGTACAAGAACTTGCCCCATAGTACTCGGATGCTCAGAT
  FAD3C (SEQ ID NO:18)  (2600) TTATACAAGAATTTGTCCCACAGTACACGGATGCTCAGAT

                                  3921                                3960

  FAD3A (SEQ ID NO:15)  (2070) ACACTGTCCCTCTCCCCATGCTCGCTTACCCTCTCTATCT
 FAD3A' (SEQ ID NO:16)  (2816) ACACTGTCCCTCTGCCCATGCTCGCTTACCCGATCTATCT
 FAD3C' (SEQ ID NO:20)  (3488) ACACTGTCCCTCTGCCCATGCTCGCTTACCCGATCTATCT
FAD3A'' (SEQ ID NO:17)  (3589) ACACTGTTCCTCTGCCCATGCTCGCTTACCCGATCTATCT
FAD3C'' (SEQ ID NO:19)  (3496) ACACTGTCCCTCTGCCCATGCTCGCTTACCCGATCTATCT
  FAD3C (SEQ ID NO:18)  (2640) ACACTGTCCCTCTCCCCATGCTCGCTTACCCTCTCTATCT

                                  3961                                4000

  FAD3A (SEQ ID NO:15)  (2110) GGTAAATCCTAATTCCTCATTTTTCTTCCTGATTATAATT
 FAD3A' (SEQ ID NO:16)  (2856) GGTAT----------TTTTTAATTCCTAAAATTTACT
 FAD3C' (SEQ ID NO:20)  (3528) GGTAT----------TTTTTAATTCCTAAAACTTACC
FAD3A'' (SEQ ID NO:17)  (3629) GGTAAAAAAAAA-TACAATTTCAATTTTTTTCTTAAAATT
FAD3C'' (SEQ ID NO:19)  (3536) GGTAAAAAAAA--TACAATTTCTATTTTTT-CTTAAAATT
  FAD3C (SEQ ID NO:18)  (2680) GGTAAATCCTAATTCCTAATTTTTCTTCCTGATTATAATT
```

FIG. 3Y

FIG 3Z

```
                                    4001                                4040
FAD3A (SEQ ID NO:15)  (2150) ACAATTTTGAATTTTTAGATTTTGAGTATTAA--CTAAAT
FAD3A' (SEQ ID NO:16) (2883) ACAAGT----CATTTTAGAC--TGTGTTTTAA--AACAAT
FAD3C' (SEQ ID NO:20) (3555) ACAATT----CATTTTAGAT--TGTGTTTTAA--AACAAT
FAD3A'' (SEQ ID NO:17) (3668) ACAAAT----GGTTTTATATTTTGAGTTTTAAGCCAATAT
FAD3C'' (SEQ ID NO:19) (3573) ACAAAT----GATTTTATATTTTGAGTTTTAAGCCAATAT
FAD3C (SEQ ID NO:18)  (2720) ACAATTTTGAATTTTTAGATTTTGAGTATTAA--CTAAAT
                                    4041                                4080
FAD3A (SEQ ID NO:15)  (2188) ATAAATTAAATTTGTTTGGGGATGA-CTACAGTGGTACAG
FAD3A' (SEQ ID NO:16) (2915) ATAA-TTATTTTTG-TTTGGTTTTA-CTGCAGTGGTACAG
FAD3C' (SEQ ID NO:20) (3587) ATAAATTATTTTTTCTTTGGTTTTA-CTGCAGTGGTACAG
FAD3A'' (SEQ ID NO:17) (3704) ATAAATTAATTTTGATTGGATTTTAACTACAGTGGTACAG
FAD3C'' (SEQ ID NO:19) (3609) ATAAATTAATTTTGATTGGATTTTAACTACAGTGGTACAG
FAD3C (SEQ ID NO:18)  (2758) ATAAATTAAATTTGTTTGGGGATGA-CTACAGTGGTACAG
                                    4081                                4120
FAD3A (SEQ ID NO:15)  (2227) AAGTCCTGGTAAAGAAGGGTCACATTATAACCCATACAGT
FAD3A' (SEQ ID NO:16) (2952) AAGTCCTGGAAAAGAAGGGTCACATTTTAACCCATACAGT
FAD3C' (SEQ ID NO:20) (3626) AAGTCCTGGAAAAGAAGGGTCACATTTTAACCCATACAGT
FAD3A'' (SEQ ID NO:17) (3744) AAGTCCTGGAAAAGAAGGGTCACATTTTAACCCATACAGT
FAD3C'' (SEQ ID NO:19) (3649) AAGTCCTGGAAAAGAAGGGTCACATTTTAACCCATACAGT
FAD3C (SEQ ID NO:18)  (2797) AAGTCCTGGTAAAGAAGGGTCACATTATAACCCATACAGT
                                    4121                                4160
FAD3A (SEQ ID NO:15)  (2267) AGTTTATTTGCCCCAAGCGAGAGAAAGCTTATTGCAACTT
FAD3A' (SEQ ID NO:16) (2992) GGTTTATTTGCTCCAAGCGAGAGAAAGCTTATTGCAACTT
FAD3C' (SEQ ID NO:20) (3666) GGTTTATTTGCTCCAAGCGAGAGAAAGCTTATTGCAACTT
FAD3A'' (SEQ ID NO:17) (3784) AGTTTATTTGCTCCAAGCGAGAGGAAGCTTATTGCAACTT
FAD3C'' (SEQ ID NO:19) (3689) AGTTTATTTGCTCCAAGCGAGAGGAAGCTTATTGCAACTT
FAD3C (SEQ ID NO:18)  (2837) AGTTTATTTGCCCCAAGCGAGAGAAAGCTTATTGCAACTT
```

FIG. 3Z

FIG 3A'

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                                        4161                                    4200
  FAD3A (SEQ ID NO:15)  (2307) CAACTACTTGCTGGTCGATCATGTTGGCCACTCTTGTTTA
  FAD3A' (SEQ ID NO:16) (3032) CGACTACTTGCTGGTCCATAATGTTGGCAATTCTTATCTG
  FAD3C' (SEQ ID NO:20) (3706) CAACTACTTGCTGGTCCATAATGTTGGCCATTCTTATCTG
 FAD3A'' (SEQ ID NO:17) (3824) CAACAACTTGCTGGTCCATAATGTTGGCCACTCTTGTTTA
 FAD3C'' (SEQ ID NO:19) (3729) CAACTACTTGCTGGTCCATAATGTTGGCCACTCTTGTTTA
  FAD3C (SEQ ID NO:18)  (2877) CAACTACTTGCTGGTCGATCGTGTTGGCCACTCTTGTTTA
                                        4201                                    4240
  FAD3A (SEQ ID NO:15)  (2347) TCTATCATTCCTCGTTGGTCCAGTCACAGTTCTAAAAGTC
  FAD3A' (SEQ ID NO:16) (3072) TCTTTCCTTCCTCGTTGGTCCAGTCACAGTTCTCAAAGTA
  FAD3C' (SEQ ID NO:20) (3746) TCTTTCCTTCCTCGTTGGTCCAGTCACAGTTCTCAAAGTA
 FAD3A'' (SEQ ID NO:17) (3864) TCTATCGTTCCTCGTTGGTCCAGTCACAGTTCTCAAAGTC
 FAD3C'' (SEQ ID NO:19) (3769) TCTATCGTTCCTCGTTGATCCAGTCACAGTTCTCAAAGTC
  FAD3C (SEQ ID NO:18)  (2917) TCTATCATTCCTCGTTGGTCCAGTCACAGTTCTAAAAGTC
                                        4241                                    4280
  FAD3A (SEQ ID NO:15)  (2387) TATGGTGTTCCTTACATTGTAAGTTTCATA-TATTTC---
  FAD3A' (SEQ ID NO:16) (3112) TACGGTGTTCCTTACATTGTAAGTTTCTTAGTATATTCATA
  FAD3C' (SEQ ID NO:20) (3786) TACGGTGTTCCTTACATCGTAAGTTTCTTAGTATATTCATA
 FAD3A'' (SEQ ID NO:17) (3904) TATGGTGTTCCTTACATTGTAAGTTTCACA-TATTATTAC
 FAD3C'' (SEQ ID NO:19) (3809) TATGGCGTTCCTTACATTGTAAGTTTCACA-TATTATTAC
  FAD3C (SEQ ID NO:18)  (2957) TATGGTGTTCCTTACATTGTAAGTTTCATA-TATTTC---
                                        4281                                    4320
  FAD3A (SEQ ID NO:15)  (2423) ------ATTATTATATCATTGCTAATATA---------AT
  FAD3A' (SEQ ID NO:16) (3152) AAGGGTATATATTTATTATTCAATATATATACTATATGAT
  FAD3C' (SEQ ID NO:20) (3826) AAGGGTATATATTTATTATTCAATATATATACTATATGAT
 FAD3A'' (SEQ ID NO:17) (3943) AAGAG-ATTTATATATTATTAATAATAAA---------TT
 FAD3C'' (SEQ ID NO:19) (3848) AAGAA-ATTTATATATTATTAATAATAAA---------TT
  FAD3C (SEQ ID NO:18)  (2993) ------TTTATTATATCATTGCTAATATA---------AT
```

FIG. 3A'

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FIG 3B'                                  4321                                4360
  FAD3A (SEQ ID NO:15)  (2448) TTGTTTTTGACATAAA-GTTTTGGAAAAATTTCAGATCTT
 FAD3A' (SEQ ID NO:16)  (3192) TTGTTTTTGTCATATA-TTTTTG--AAATATTCAGATCTT
 FAD3C' (SEQ ID NO:20)  (3866) TTGTTTTTGTCATAAA-CTTTTG--AAAT--TCAGATCTT
FAD3A'' (SEQ ID NO:17)  (3973) TGTTTTTTGACATAAA-GTTTTGGAAAATTTTCAGATCTT
FAD3C'' (SEQ ID NO:19)  (3878) TGTTTTTTGACATAAG-GGTTTGGAAAATTTTCAGATCTT
  FAD3C (SEQ ID NO:18)  (3018) TTGTTTTTGACATAAAAGTTTTGGAAAAATTTCAGATCTT
                                 4361                                4400
  FAD3A (SEQ ID NO:15)  (2487) TGTAATGTGGTTGGACGCTGTCACGTACTTGCATCATCAT
 FAD3A' (SEQ ID NO:16)  (3229) TGTGATGTGGTTGGACGCTGTCACTTACTTGCATCACCAT
 FAD3C' (SEQ ID NO:20)  (3901) TGTGATGTGGTTGGACGCTGTCACTTACTTGCATCACCAT
FAD3A'' (SEQ ID NO:17)  (4012) TGTAATGTGGTTGGACGCTGTCACGTACTTGCATCATCAT
FAD3C'' (SEQ ID NO:19)  (3917) TGTGATGTGGTTGGACGCTGTCACGTACTTGCATCATCAT
  FAD3C (SEQ ID NO:18)  (3058) TGTAATGTGGTTGGACGCTGTCACGTACTTGCATCATCAT
                                 4401                                4440
  FAD3A (SEQ ID NO:15)  (2527) GGTCAGGATGATAAGTTGCCTTGGTACAGAGGCAAGGTAA
 FAD3A' (SEQ ID NO:16)  (3269) GGTCATGATGAGAAGTTGCCTTGGTACAGAGGCAAGGTAA
 FAD3C' (SEQ ID NO:20)  (3941) GGTCATGATGAGAAGTTGCCTTGGTACAGAGGCAAGGTAA
FAD3A'' (SEQ ID NO:17)  (4052) GGTCAGGATGAGAAGTTGCCTTGGTACAGAGGCAAGGTAA
FAD3C'' (SEQ ID NO:19)  (3957) GGTCAGGATGAGAAGTTGCCTTGGTACAGAGGCAAGGTAA
  FAD3C (SEQ ID NO:18)  (3098) GGTCAGGATGATAAGCTGCCTTGGTACAGAGGCAAGGTAA
                                 4441                                4480
  FAD3A (SEQ ID NO:15)  (2567) GTAGATCAACATT--------AATTTATAA---------G
 FAD3A' (SEQ ID NO:16)  (3309) TTAAATTAACTATTACAA--GTATTTTAC----------A
 FAD3C' (SEQ ID NO:20)  (3981) TTAAATTAACTCCTAGGT--GATTTTCCCGTGCTCATGTA
FAD3A'' (SEQ ID NO:17)  (4092) ATAAATCAATTTTTAAAAGAAATGTACAG---------A
FAD3C'' (SEQ ID NO:19)  (3997) TTAAATCAATTTTTAAAAGAAATGTACAG---------A
  FAD3C (SEQ ID NO:18)  (3138) GTAGATCAACATT--------A-TTTATAA---------G
```

FIG. 3B'

```
FIG 3C'                                  4481                                4520
  FAD3A (SEQ ID NO:15)  (2590) AAGCAACAATGATTAGTAT-TTGATTAATCTA-AATTATT
 FAD3A' (SEQ ID NO:16)  (3337) AAAAACTAATGATTAGTATATTTGATTAATCTTAATTCTT
 FAD3C' (SEQ ID NO:20)  (4019) CGGATATAAATATTTCTAAAGTAAATATACTATAATAATT
FAD3A'' (SEQ ID NO:17)  (4123) AAGCAATAATGGTTAGTA--TTGATTAATCTT-AATTTTT
FAD3C'' (SEQ ID NO:19)  (4028) AAGCAATAATGGTTAGTA--TTGATTAATCTT-AATTTTT
  FAD3C (SEQ ID NO:18)  (3160) AAGCAATAATGATTAGTAG-TTGAATAATCTG-AATTTTT
                                 4521                                4560
  FAD3A (SEQ ID NO:15)  (2628) GATGTTTTGTGTACAATAATAGGAATGGAGTTATTTACGT
 FAD3A' (SEQ ID NO:16)  (3377) GATGTTTTGTGATTAATAATAGGAATGGAGTTACTTACGT
 FAD3C' (SEQ ID NO:20)  (4059) AATTGTTATTTATTTTTAATTTTAAATTAGTTTATAATTT
FAD3A'' (SEQ ID NO:17)  (4160) GATGTTTTGCATACAATAATAGGAATGGAGTTATTTACGT
FAD3C'' (SEQ ID NO:19)  (4065) GATGTTTTGCATACAATAATAGGAATGGAGTTATTTACGT
  FAD3C (SEQ ID NO:18)  (3198) GATGTTTT-TGTACAATAATACGAATGGAGTTATTTACGT
                                 4561                                4600
  FAD3A (SEQ ID NO:15)  (2668) GGAGGATTAACAACTATTGATAGAG-----ATTACGG-GA
 FAD3A' (SEQ ID NO:16)  (3417) GGAGGATTAACAACTATTGATAGAG-----ATTACGG-AA
 FAD3C' (SEQ ID NO:20)  (4099) GTATGCATGATTTATATTAATAAAATTTATATTACTTTAA
FAD3A'' (SEQ ID NO:17)  (4200) GGAGGATTAACAACTATTGATAGAG-----ATTACGG-AA
FAD3C'' (SEQ ID NO:19)  (4105) GGAGGATTAACAACTATTGATAGAG-----ATTACGG-AA
  FAD3C (SEQ ID NO:18)  (3237) GGAGGATTAACAACTGTTGATAGAG-----ATTACGG-GA
                                 4601                                4640
  FAD3A (SEQ ID NO:15)  (2702) TCTTCAACAACATTCATCACGATATTCGAACTCACGTGAT
 FAD3A' (SEQ ID NO:16)  (3451) TTTTCAACAACATTCATCACGACATTGGAACTCACGTGAT
 FAD3C' (SEQ ID NO:20)  (4139) TTATAAATATGATTT-TATATATGTTATATCTAATCGGTT
FAD3A'' (SEQ ID NO:17)  (4234) TCTTCAACAACATCCATCACGACATTGGAACTCACGTGAT
FAD3C'' (SEQ ID NO:19)  (4139) TCTTCAACAACATCCATCACGACATTGGAACTCACGTGAT
  FAD3C (SEQ ID NO:18)  (3271) TCTTCAACAACATTCATCACGATATTGGAACTCACGTGAT
```

FIG. 3C'

```
FIG 3D'                                4641                                4680
  FAD3A (SEQ ID NO:15)  (2742) CCATCATCTTTTCCCACAAATCCCTCACTATCACTTGGTT
 FAD3A' (SEQ ID NO:16)  (3491) CCATCATCTTTTCCCACAAATCCCTCACTATCACTTGGTC
 FAD3C' (SEQ ID NO:20)  (4178) TTGTTGTTTTTACAGTCGATTTAGT---TATCATTTGGGT
FAD3A'' (SEQ ID NO:17)  (4274) CCATCATCTTTTCCCACAAATCCCTCACTATCACTTGGTC
FAD3C'' (SEQ ID NO:19)  (4179) CCATCATCTTTTCCCACAAATCCCTCACTATCACTTGGTC
  FAD3C (SEQ ID NO:18)  (3311) CCATCATCTTTTCCCACAAATCCCTCACTATCACTTGGTC
                                4681                                4720
  FAD3A (SEQ ID NO:15)  (2782) GATGCCGTGAGTGATCTCGCT----CTCTCTC---TAGTT
 FAD3A' (SEQ ID NO:16)  (3531) GATGCTGTGAGTCATCTCACTCTCTGGCTAC------TTT
 FAD3C' (SEQ ID NO:20)  (4215) -AAATTGGATTGCATCTCAGAATTCAACTGTAATATTTTT
FAD3A'' (SEQ ID NO:17)  (4314) GATGCGGTGAGTGATCTAGCTTTCTCTCTCTC---TAGTT
FAD3C'' (SEQ ID NO:19)  (4219) GATGCCGTGAGTGATCTAGCTTTCTCTCTCTC---TAGTT
  FAD3C (SEQ ID NO:18)  (3351) GATGCCGTGAGTGATCTCGCT----CTCTCTC---TAGTT
                                4721                                4760
  FAD3A (SEQ ID NO:15)  (2815) TCATTTGATTAAAA--TTAAAGGGTGATTAATTACTAAAT
 FAD3A' (SEQ ID NO:16)  (3565) CATCAAAACCATTTGATTAAAGGGTGATTAATTACTAATG
 FAD3C' (SEQ ID NO:20)  (4254) TATTTTAACTATAT--TAAAATTTTGATTAATTTCTTATT
FAD3A'' (SEQ ID NO:17)  (4351) TCATTTGATTAAA-------TG-GTGATTAATTACTAATT
FAD3C'' (SEQ ID NO:19)  (4256) TCATTTGATTAAA-------TG-GTGATTAATTACTAATT
  FAD3C (SEQ ID NO:18)  (3384) TCATTTGATTATA---TTAAAGGGTGATTAATTACTAAAT
                                4761                                4800
  FAD3A (SEQ ID NO:15)  (2853) TAGTGATCTTAATTAATGATATGCG-ACAGACGAAATCAG
 FAD3A' (SEQ ID NO:16)  (3605) TAGTCATTTTA-ACAAATGGAATGTGACAGACAAAAGCAG
 FAD3C' (SEQ ID NO:20)  (4292) T--TCATTT-----AGGTGGTTGTTGTCTTAGAACTT---
FAD3A'' (SEQ ID NO:17)  (4383) TA--------A-TTAATGAATTGTGGACAGACGAGAGCAG
FAD3C'' (SEQ ID NO:19)  (4288) TA--------A-TTAATGAATTGTGGACAGACGAGAGCAG
  FAD3C (SEQ ID NO:18)  (3421) TAGTGATCTTAATTAATGACATGCG-ACAGACGAAAGCAG
```

FIG. 3D'

```
FIG 3E'                                  4801                                4840
  FAD3A (SEQ ID NO:15)  (2892) CTAAACATGTGTTGGGAAGATACTACAGAGAACCAAAGAC
 FAD3A' (SEQ ID NO:16)  (3644) CTAAACATGTGTTGGGAAGATACTACAGAGAACCAAAGAC
 FAD3C' (SEQ ID NO:20)  (4322) -TAAATATATTTTATAAAGATTATGTATAACTTAATATAT
FAD3A'' (SEQ ID NO:17)  (4414) CTAAACATGTGTTAGGAAGATACTACAGAGAGCCGAAGAC
FAD3C'' (SEQ ID NO:19)  (4319) CTAAACATGTGTTAGGAAGATACTACAGAGAGCCGAAGAC
  FAD3C (SEQ ID NO:18)  (3460) CTAAACATGTGTTGGGAAGATACTACAGAGAACCAAAGAC
                                 4841                                4880
  FAD3A (SEQ ID NO:15)  (2932) GTCAGGAGC----AAT--ACCGATCCACTTGGTCGAAAGT
 FAD3A' (SEQ ID NO:16)  (3684) GTCAGGAGC----AAT--ACCGATCCACTTGGTCGAGAGT
 FAD3C' (SEQ ID NO:20)  (4361) ATATTGTGCTTAAAATGAAATAAAAAATAAAATAAAGTGT
FAD3A'' (SEQ ID NO:17)  (4454) GTCAGGAGC----AAT--ACCGATTCACTTGGTGGAGAGT
FAD3C'' (SEQ ID NO:19)  (4359) GTCAGGAGC----AAT--ACCGATTCACTTGGTGGAGAGT
  FAD3C (SEQ ID NO:18)  (3500) GTCAGGAGC----AAT--ACCGATCCACTTAGTGGAAAGT
                                 4881                                4920
  FAD3A (SEQ ID NO:15)  (2966) TTGGTGGCAAGTATTAAGAAAGATCATTACGTCAGTGACA
 FAD3A' (SEQ ID NO:16)  (3718) TTGGTAGCAAGTATTAAGAAAGATCATTACGTCAGTGACA
 FAD3C' (SEQ ID NO:20)  (4401) CTGATTCTAAATTACATAAATTAATATAACGATAAT-ATT
FAD3A'' (SEQ ID NO:17)  (4488) TTGGTCGCAAGTATTAAAAAAGATCATTACGTCAGTGACA
FAD3C'' (SEQ ID NO:19)  (4393) TTGGTCGGAAGTATTAAAAAAGATCATTACGTCAGTGACA
  FAD3C (SEQ ID NO:18)  (3534) TTGGTGGCAAGTATTAAGAAAGATCATTACGTCAGTGACA
                                 4921                                4960
  FAD3A (SEQ ID NO:15)  (3006) CTG--GTGATATTGTCTTCTACG---AGACAGATCCAGAT
 FAD3A' (SEQ ID NO:16)  (3758) CTG--GTGACATTGTCTTCTACG---AGACTGATCCAGAT
 FAD3C' (SEQ ID NO:20)  (4440) CTGAAGTCTCATGCATATATATATATAAATTTTACAAAAG
FAD3A'' (SEQ ID NO:17)  (4528) CTG--GTGATATTGTCTTCTACG---AGACAGATCCAGAT
FAD3C'' (SEQ ID NO:19)  (4433) CTG--GTGATATTGTCTTCTACG---AGACAGATCCAGAT
  FAD3C (SEQ ID NO:18)  (3574) CTG--GTGATATTGTCTTCTACG---AGACAGATCCAGAT
```

FIG. 3E'

```
FIG 3F'                                  4961                                5000
  FAD3A (SEQ ID NO:15)  (3041) CTCTACGTT-TATGCTTCTGACAA-ATCCAAAATCAACTA
 FAD3A' (SEQ ID NO:16)  (3793) CTCTACGTT-TATGCTTCTGTCAA-ATCGAAAATCAATTA
 FAD3C' (SEQ ID NO:20)  (4480) AACTAAATTGTAACATTTGGTTAATATTTTACAGTAATTA
FAD3A'' (SEQ ID NO:17)  (4563) CTCTACGTT-TATGCTTCGGACAA-ATCTAAAATCAATTA
FAD3C'' (SEQ ID NO:19)  (4468) CTCTACGTT-TATGCTTCTGACAA-ATCTAAAATCAATTA
  FAD3C (SEQ ID NO:18)  (3609) CTCTACGTT-TATGCTTCTGACAA-ATCCAAAATCAATTA
                                 5001                                5040
  FAD3A (SEQ ID NO:15)  (3079) ACCTTTCTTCCTAGCTCTATTTAG----------GAATAA
 FAD3A' (SEQ ID NO:16)  (3831) AACTTTCTTCCCCCTTTTTGTTTAGCACTATTATGAATAA
 FAD3C' (SEQ ID NO:20)  (4520) AAATATTTTATAAATTCTAAATA---ACT-TTATGTATTT
FAD3A'' (SEQ ID NO:17)  (4601) ACTTTTCTTCCTAGCTCTATT-AG----------GAATAA
FAD3C'' (SEQ ID NO:19)  (4506) ACTTTTCTTCCTAGCTCTATT-AG----------GAATAA
  FAD3C (SEQ ID NO:18)  (3647) ATCTTTCTTCCTAGCTCTATTTAG----------GAATAA
                                 5041                                5080
  FAD3A (SEQ ID NO:15)  (3109) AACAGTCCTTTGGTTTTTACTTATTTCTGGTTGTTTTTAA
 FAD3A' (SEQ ID NO:16)  (3871) A--CCAGTTTTTTTT---ACTTATATATTCTTGTTTTTAA
 FAD3C' (SEQ ID NO:20)  (4556) A--ATTTATTGAATGGAAACTGAAATTTATTTTAAATAAT
FAD3A'' (SEQ ID NO:17)  (4630) A-CACTCCTTCTCTTTT-ACTTATTTGTTTCTGCTTT-AA
FAD3C'' (SEQ ID NO:19)  (4535) A-CACTCCTTCTCTTTT-ACTTATTTGTTTCTGCTTT-AA
  FAD3C (SEQ ID NO:18)  (3677) AACACTCCTTTGGTTTT-ACTTATTTCTGGTTGTTTTTAA
                                 5081                                5120
  FAD3A (SEQ ID NO:15)  (3149) GTTAAA--TGTACTCGTGAAACTTTTTTTA-ATTAAATGT
 FAD3A' (SEQ ID NO:16)  (3906) GTTAAAAATGTACTCGTGAAACTCTTCTTAATTTAGATAT
 FAD3C' (SEQ ID NO:20)  (4594) CTTAAAAATGAAAACATATTTGCTTTGGTATTTTGCTTAT
FAD3A'' (SEQ ID NO:17)  (4667) GTTTAAAATGTACTCGTGAAACCTTTTT---TATTAATGT
FAD3C'' (SEQ ID NO:19)  (4572) GTTTAAAATGTACTCGTGAAACCTTTTTTT-TATTAATGT
  FAD3C (SEQ ID NO:18)  (3716) GTTAAAAATGTACTCGTGAAACTTTTTTTT-ATTAAATGT
```

FIG. 3F'

```
FIG 3G'                                5121                                5160
  FAD3A (SEQ ID NO:15)  (3186) ATTTACATT-------ACAAATC----AAGTTTTTGTTCG
 FAD3A' (SEQ ID NO:16)  (3946) TATTCCATT------TACA--CTGAAAAACATACAATTTC
 FAD3C' (SEQ ID NO:20)  (4634) GGTTCCATTAAGTTCTACAAACATAAAAACATAACATTTA
FAD3A'' (SEQ ID NO:17)  (4704) ATTTACGTT-------ACAAAAAGTGGAAGTTTT-GTTAT
FAD3C'' (SEQ ID NO:19)  (4611) ATTTACGTT-------ACAAAAAGTGGAAGTTTT-GTTAT
  FAD3C (SEQ ID NO:18)  (3755) ATTTACATT-------ACAAATCGTAAAAGTTTTTGTTCG
                                5161                                5200
  FAD3A (SEQ ID NO:15)  (3215) TTTTCTTTATGTTTTTAGTTACAA---TA---AATAAAG-
 FAD3A' (SEQ ID NO:16)  (3978) AAAGGT-TGAAAAGAAAGACAAAATTTTCT---AGAATGA
 FAD3C' (SEQ ID NO:20)  (4674) AAAACTGTGATTATTTTGTAACTATTTGATCAAACAATGA
FAD3A'' (SEQ ID NO:17)  (4736) CTTTTTCTCTAGTTGCAATCAAAAGG--------------
FAD3C'' (SEQ ID NO:19)  (4643) CTTTTTCTCTGGTTGCAATCAAAAGG--------------
  FAD3C (SEQ ID NO:18)  (3788) TTTTGTCTATGTTTTTAGTTACAAACTTAC--AATCAAAA
                                5201                                5240
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4014) C---------------------------------------
 FAD3C' (SEQ ID NO:20)  (4714) TTATTTTTTAATTTTAATTTTAGTTTTTTAATAACTCTTA
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3826) AG--------------------------------------
                                5241                                5280
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (4754) AAAATAAGCAGTGAACAAAAGTGAGATTGTATTTGAAATT
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
```

FIG. 3G'

```
FIG 3H'                          5281                                5320
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (4794) AATATTATACAAGTAAAATATAATTTTTTAAGTTTATAAA
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
                         5321                                5360
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (4834) AAAATTCCTTTTTATTATATGTATATGTTTTTTTGGAAAA
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
                         5361                                5400
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (4874) TTTTAAAAAGGAAACTAAATAAAAAAATAAATAATAGTAT
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
                         5401                                5440
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (4914) TTTAAATGTAATATTTTTAATTCATTAAGTGTATTAGTGT
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
```

FIG. 3H'

```
FIG 3I'                                  5441                                5480
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (4954) AATCAACTATCGTGAGAGTTAACGTGAGAGCGATACATAG
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
                                 5481                                5520
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (4994) AAAACCGACTTCTCAAATAATATTTTATAGAGATTACGAT
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
                                 5521                                5560
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (5034) GTTTCACAAAAAAAAATTATTAGTATTTGATTAATCTTAA
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
                                 5561                                5600
  FAD3A (SEQ ID NO:15)  (3248) ----------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ----------------------------------------
 FAD3C' (SEQ ID NO:20)  (5074) TTCTTGATGTTTTGTGATTAATAATAGGAATGGAGTTACT
FAD3A'' (SEQ ID NO:17)  (4762) ----------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ----------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ----------------------------------------
```

FIG. 3I'

FIG 3J'

		5601	5640
FAD3A (SEQ ID NO:15)	(3248)	----------	
FAD3A' (SEQ ID NO:16)	(4015)	----------	
FAD3C' (SEQ ID NO:20)	(5114)	TACGTGGAGGATTAACAACTATTGATAGAGATTACGGAAT	
FAD3A'' (SEQ ID NO:17)	(4762)	----------	
FAD3C'' (SEQ ID NO:19)	(4669)	----------	
FAD3C (SEQ ID NO:18)	(3828)	----------	
		5641	5680
FAD3A (SEQ ID NO:15)	(3248)	----------	
FAD3A' (SEQ ID NO:16)	(4015)	----------	
FAD3C' (SEQ ID NO:20)	(5154)	TTTCAACAACATTCATCACGACATTGGAACTCACGTGATC	
FAD3A'' (SEQ ID NO:17)	(4762)	----------	
FAD3C'' (SEQ ID NO:19)	(4669)	----------	
FAD3C (SEQ ID NO:18)	(3828)	----------	
		5681	5720
FAD3A (SEQ ID NO:15)	(3248)	----------	
FAD3A' (SEQ ID NO:16)	(4015)	----------	
FAD3C' (SEQ ID NO:20)	(5194)	CATCATCTTTTCCCACAAATCCCTCACTATCACTTGGTCG	
FAD3A'' (SEQ ID NO:17)	(4762)	----------	
FAD3C'' (SEQ ID NO:19)	(4669)	----------	
FAD3C (SEQ ID NO:18)	(3828)	----------	
		5721	5760
FAD3A (SEQ ID NO:15)	(3248)	----------	
FAD3A' (SEQ ID NO:16)	(4015)	----------	
FAD3C' (SEQ ID NO:20)	(5234)	ATGCTGTGAGTCATCTCACTCTCTCGCTACTTTCATCTAA	
FAD3A'' (SEQ ID NO:17)	(4762)	----------	
FAD3C'' (SEQ ID NO:19)	(4669)	----------	
FAD3C (SEQ ID NO:18)	(3828)	----------	

FIG. 3J'

```
FIG 3K'                                  5761                                5800
  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5274) ACCATTTCATTAAAGGGTGATTAATTACTAATGTACTGAT
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------
                                 5801                                5840
  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5314) TTTAACAAATGGAATGTGACAGACAAAAGCAGCTAAACAT
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------
                                 5841                                5880
  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5354) GCGTTGGGAAGATACTACAGAGAACCGAAGACGTCAGGAG
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------
                                 5881                                5920
  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5394) CAATACCGATCCACTTGGTGGAGAGTTTGGTAGCAAGTAT
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------
```

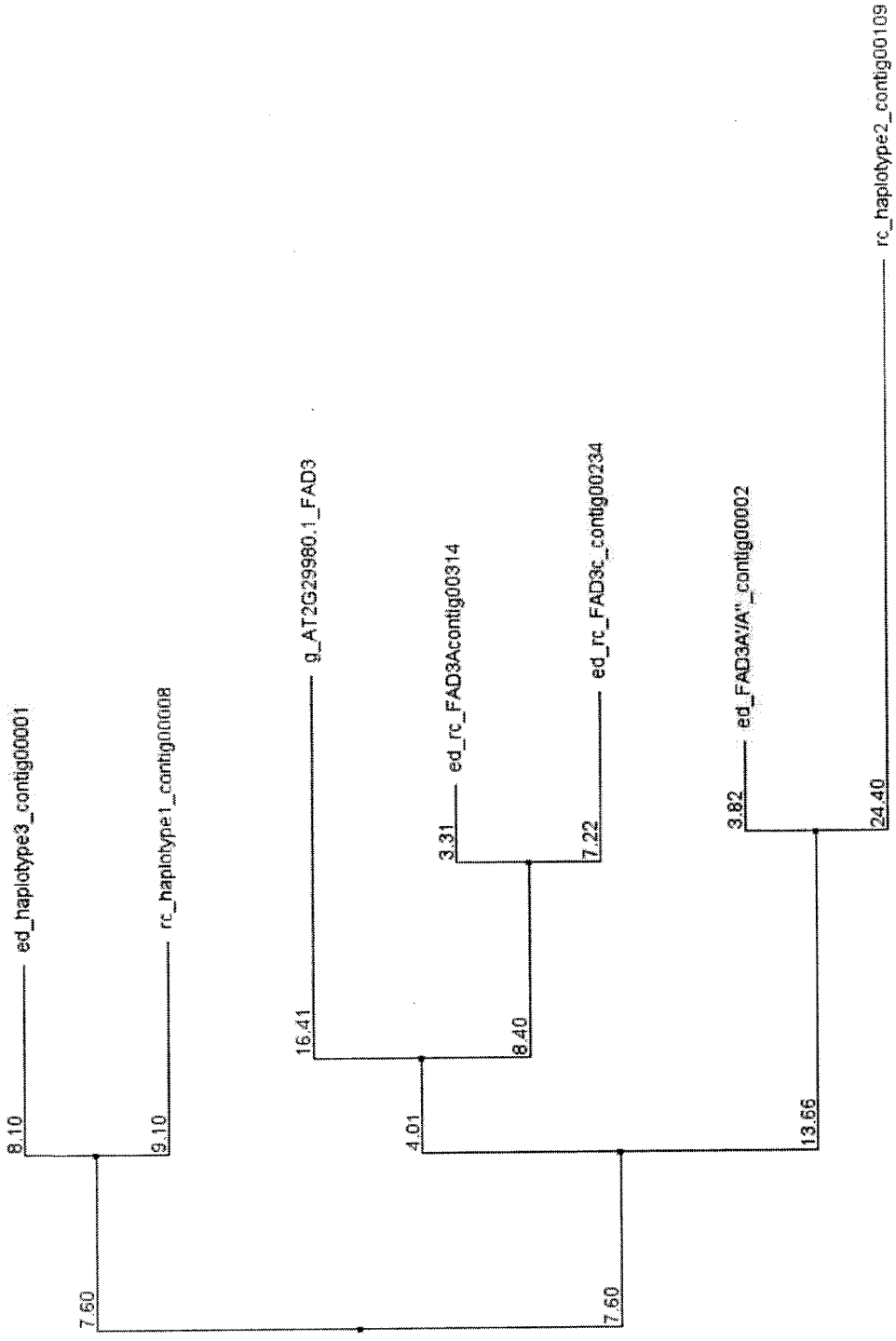
FIG. 3K'

```
FIG 3L'                                  5921                                5960

  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5434) TAAGAAAGATCATTACGTCAGTGACACCGGTGACATTGTC
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------

                                 5961                                6000

  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5474) TTCTACGAGACTGATCCAGATCTCTACGTTTATGCTTCTG
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------

                                 6001                                6040

  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5514) TCAAATCGAAAATCAATTAAACTTTCTTCCCCCTTTTTGT
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------

                                 6041                                6080

  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5554) TTAGCCCTATTATGAATAAACCAGTCTTTTTTCACTTATT
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------
```

FIG. 3L'

```
FIG 3M'                                  6081                                6120

  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5594) TATTGGTGTTTTTAAGTTAAAAATGTACTCGTGAAACTCT
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------

                                 6121                                6160

  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5634) TCTTTTATTATTAATCCATTTATACACTGAAAAACATACA
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------

                                 6161                                6200

  FAD3A (SEQ ID NO:15)  (3248) ------------------------------------
 FAD3A' (SEQ ID NO:16)  (4015) ------------------------------------
 FAD3C' (SEQ ID NO:20)  (5674) ATTTCAAAGGTTAAAAAGAAAAATAAATTTTCTAGACTGA
FAD3A'' (SEQ ID NO:17)  (4762) ------------------------------------
FAD3C'' (SEQ ID NO:19)  (4669) ------------------------------------
  FAD3C (SEQ ID NO:18)  (3828) ------------------------------------

                                 6201

  FAD3A (SEQ ID NO:15)  (3248) -
 FAD3A' (SEQ ID NO:16)  (4015) -
 FAD3C' (SEQ ID NO:20)  (5714) C
FAD3A'' (SEQ ID NO:17)  (4762) -
FAD3C'' (SEQ ID NO:19)  (4669) -
  FAD3C (SEQ ID NO:18)  (3828) -
```

FIG. 3M'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rc_haplotype1_contig00008
g_AT2G29980.1_FAD3
ed_rc_FAD3Acontig00314
ed_rc_FAD3c_contig00234
ed_FAD3A'/A''_contig00002
rc_haplotype2_contig00109
8.10
9.10
16.41
3.31
8.40
7.22
4.01
3.82
24.40
13.66
7.60
7.60

FIG. 4

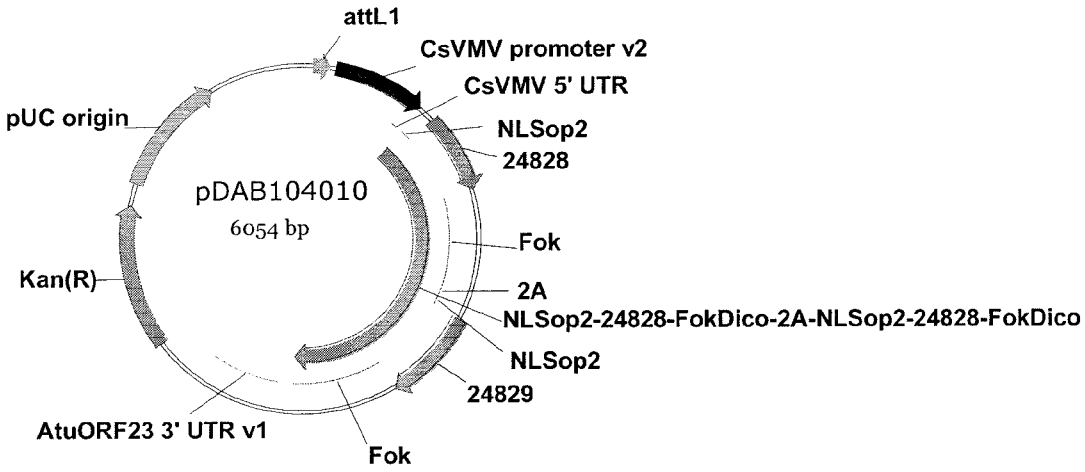

attL1
CsVMV promoter v2
CsVMV 5' UTR
pUC origin
NLSop2
24828
pDAB104010
6054 bp
Fok
Kan(R)
2A
NLSop2-24828-FokDico-2A-NLSop2-24828-FokDico
NLSop2
24829
AtuORF23 3' UTR v1
Fok

FIG. 5

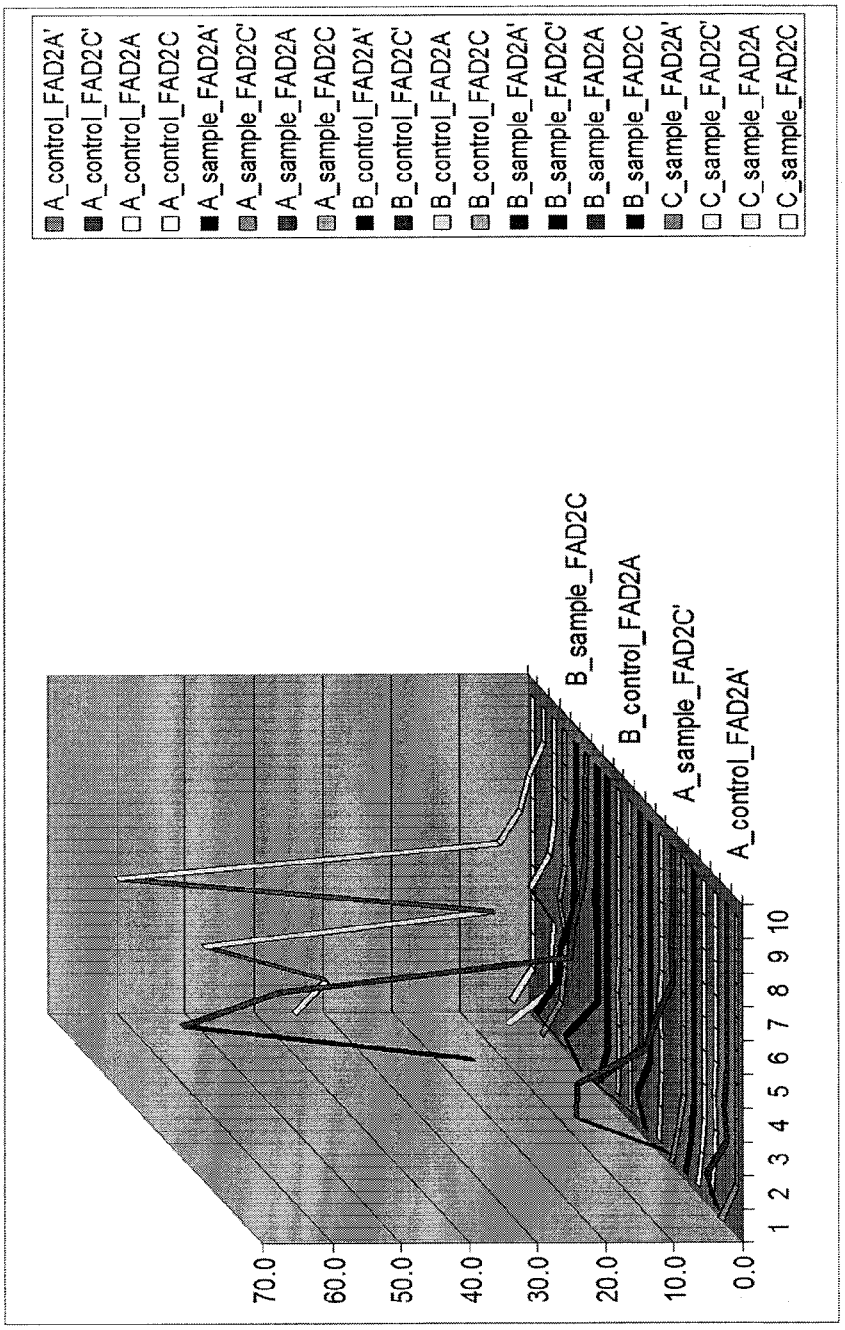

A_control_FAD2A'
A_control_FAD2C'
A_control_FAD2A
A_control_FAD2C
A_sample_FAD2A'
A_sample_FAD2C'
A_sample_FAD2A
A_sample_FAD2C
B_control_FAD2A'
B_control_FAD2C'
B_control_FAD2A
B_control_FAD2C
B_sample_FAD2A'
B_sample_FAD2C'
B_sample_FAD2A
B_sample_FAD2C
C_sample_FAD2A'
C_sample_FAD2C'
C_sample_FAD2A
C_sample_FAD2C
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
1 2 3 4 5 6 7 8 9 10
A_control_FAD2A'
A_sample_FAD2C'
B_control_FAD2A
B_sample_FAD2C

FIG. 6

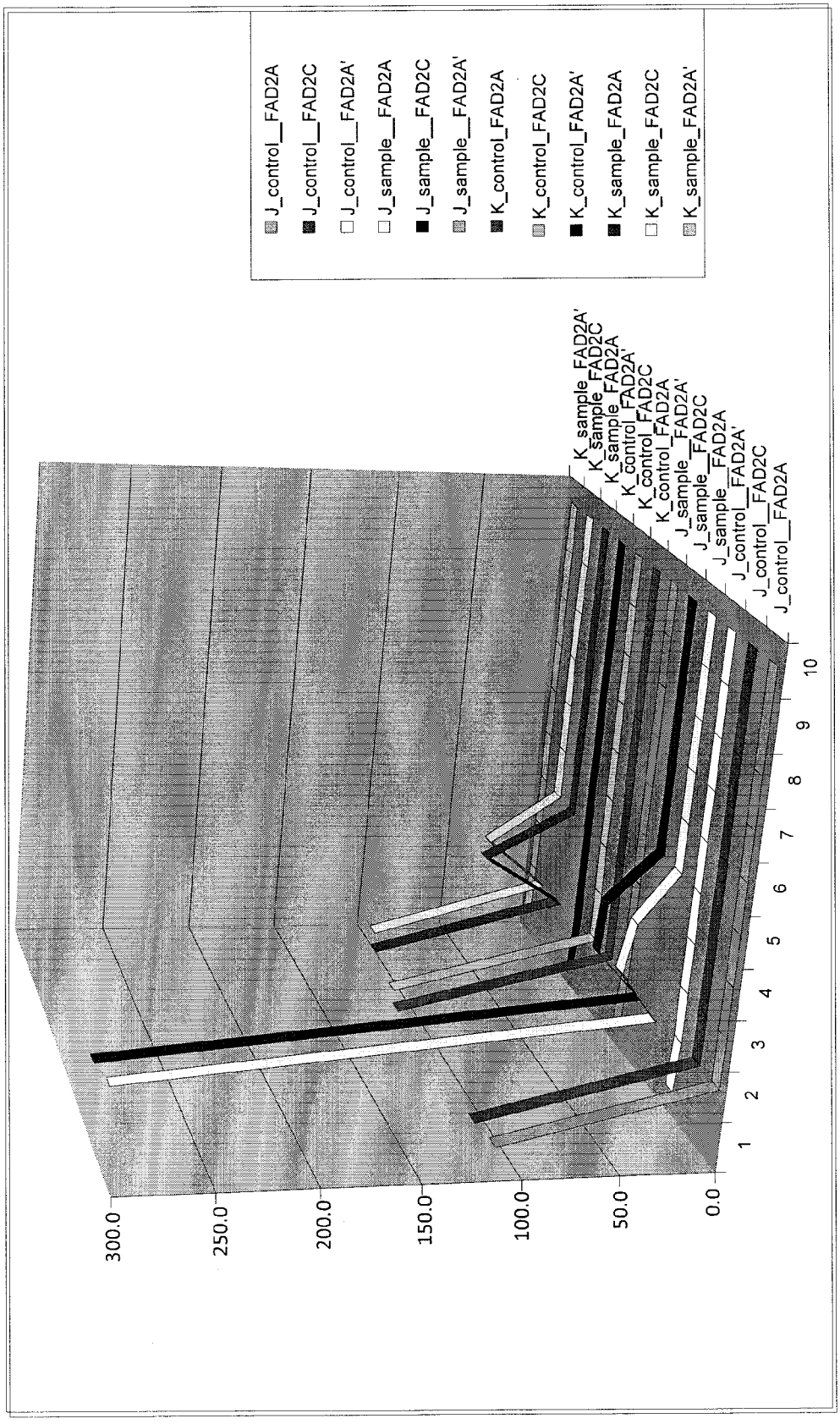

J_control__FAD2A
J_control__FAD2C
J_control__FAD2A'
J_sample__FAD2A
J_sample__FAD2C
J_sample__FAD2A'
K_control_FAD2A
K_control_FAD2C
K_control_FAD2A'
K_sample_FAD2A
K_sample_FAD2C
K_sample_FAD2A'
300.0
250.0
200.0
150.0
100.0
50.0
0.0
1
2
3
4
5
6
7
8
9
10
K_sample_FAD2A'
K_sample_FAD2C
K_sample_FAD2A
K_control_FAD2A'
K_control_FAD2C
K_control_FAD2A
J_sample__FAD2A'
J_sample__FAD2C
J_sample__FAD2A
J_control__FAD2A'
J_control__FAD2C
J_control__FAD2A

FIG. 7A

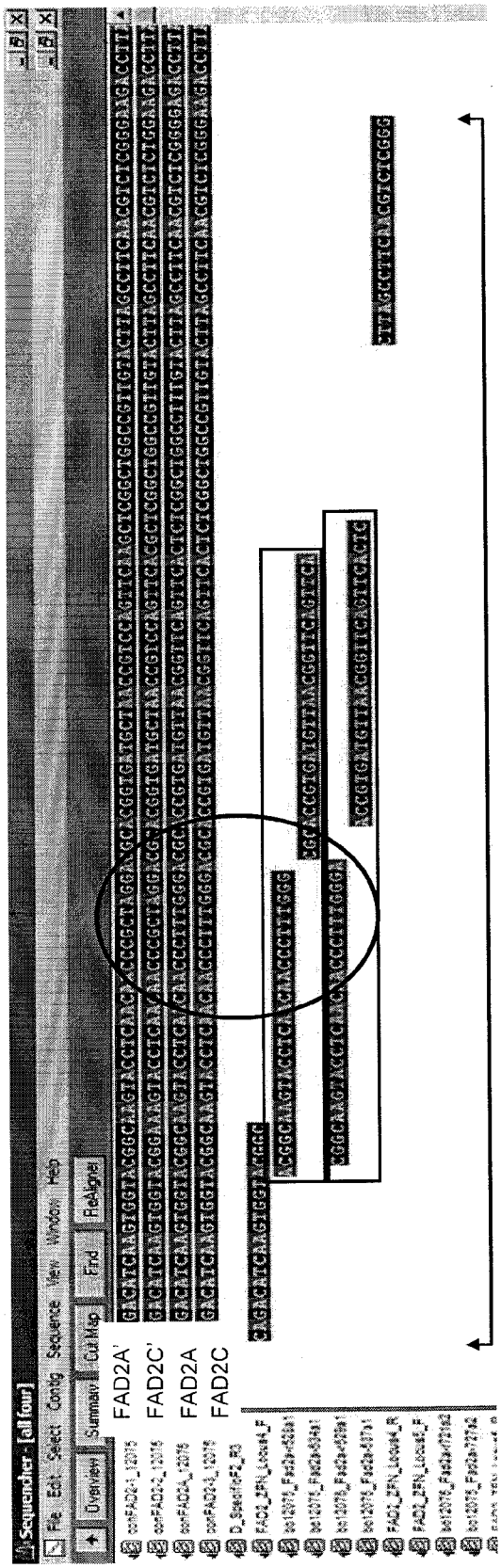

SeqMan - [all four]
File Edit Select Contig Sequence View Window Help
Overview
Summary
Col Map
Find
ReAlign
FAD2A'
FAD2C'
FAD2A
FAD2C

FIG. 7B

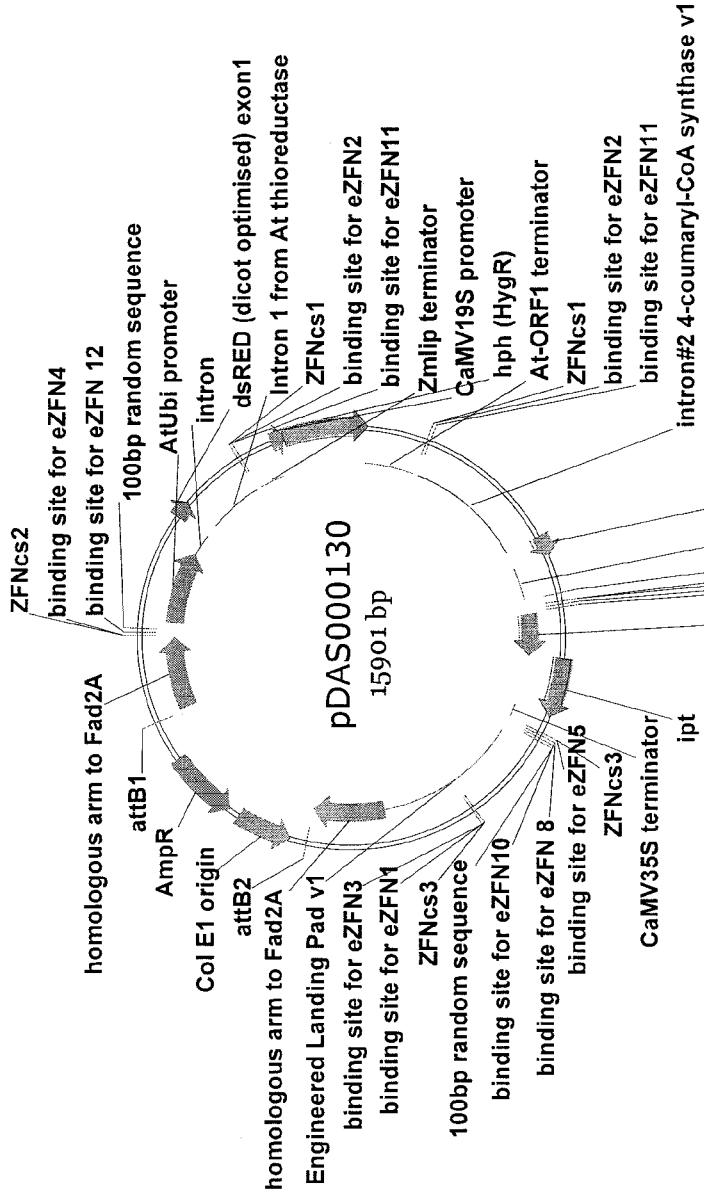

pDAS000130
15901 bp
ZFNcs2
binding site for eZFN4
binding site for eZFN 12
100bp random sequence
AtUbi promoter
intron
dsRED (dicot optimised) exon1
Intron 1 from At thioreductase
ZFNcs1
binding site for eZFN2
binding site for eZFN11
Zmlip terminator
CaMV19S promoter
hph (HygR)
At-ORF1 terminator
ZFNcs1
binding site for eZFN2
binding site for eZFN11
intron#2 4-coumaryl-CoA synthase v1
PAT (v6) 3' end
AtuORF23 terminator
100bp random sequence
eZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
AtMYB32(T) promoter
ipt
CaMV35S terminator
ZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
100bp random sequence
ZFNcs3
binding site for eZFN1
binding site for eZFN3
Engineered Landing Pad v1
homologous arm to Fad2A
attB2
Col E1 origin
AmpR
attB1
homologous arm to Fad2A

FIG. 8

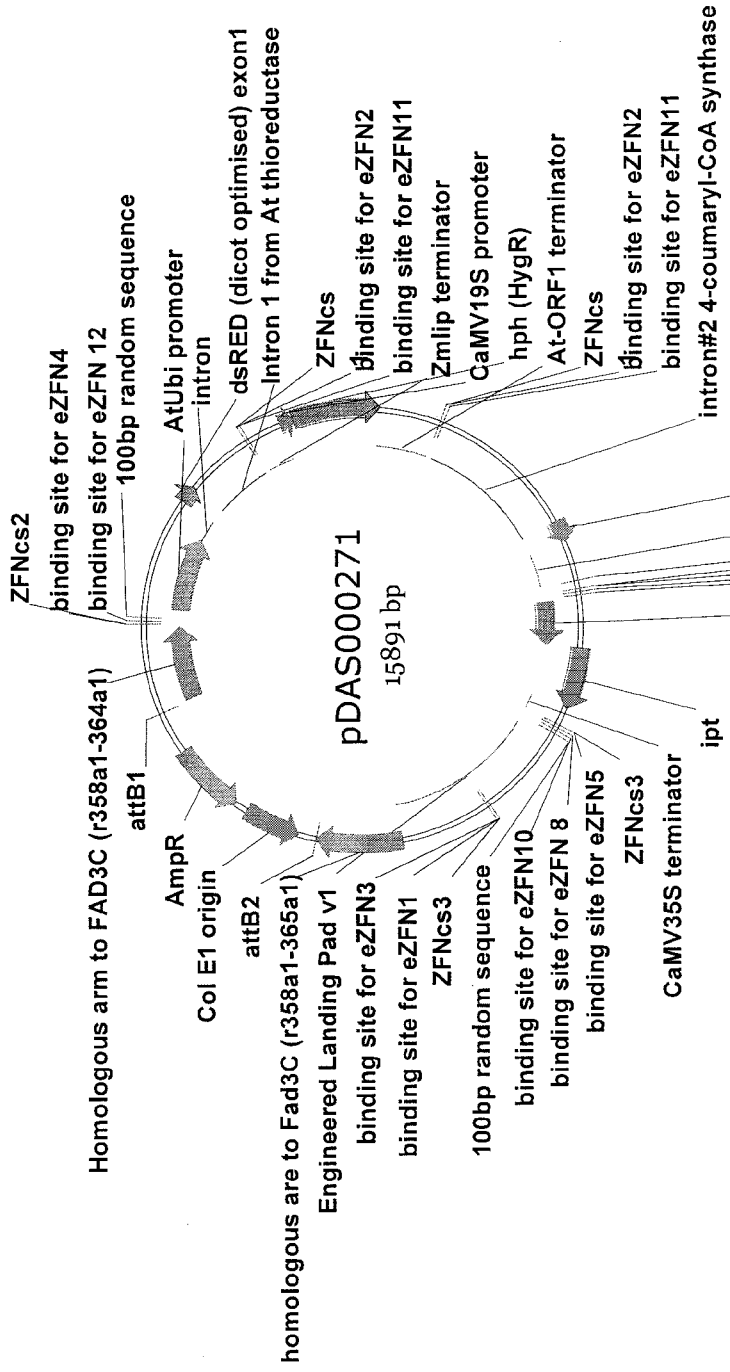

pDAS000271
15891 bp
ZFNcs2
binding site for eZFN4
binding site for eZFN 12
100bp random sequence
AtUbi promoter
intron
dsRED (dicot optimised) exon1
Intron 1 from At thioreductase
ZFNcs
binding site for eZFN2
binding site for eZFN11
Zmlip terminator
CaMV19S promoter
hph (HygR)
At-ORF1 terminator
ZFNcs
binding site for eZFN2
binding site for eZFN11
intron#2 4-coumaryl-CoA synthase
PAT (vf1) 3' end
AtuORF23 terminator
100bp random sequence
eZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
AtMYB32(T) promoter
ipt
CaMV35S terminator
ZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
100bp random sequence
ZFNcs3
binding site for eZFN1
binding site for eZFN3
Engineered Landing Pad v1
homologous are to Fad3C (r358a1-365a1)
attB2
Col E1 origin
AmpR
attB1
Homologous arm to FAD3C (r358a1-364a1)

FIG. 9

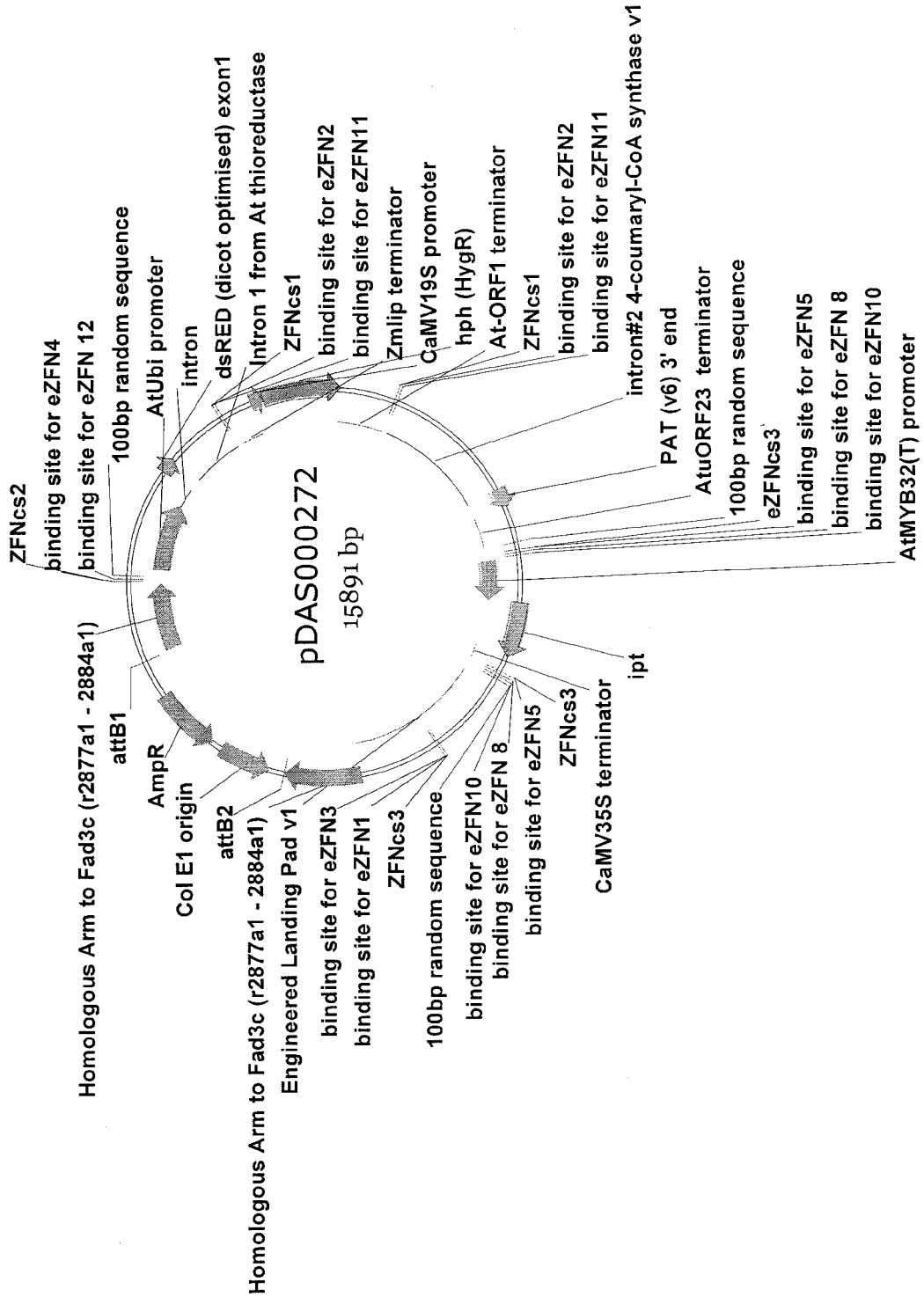

pDAS000272
15891 bp
ZFNcs2
binding site for eZFN4
binding site for eZFN 12
100bp random sequence
AtUbi promoter
intron
dsRED (dicot optimised) exon1
Intron 1 from At thioreductase
ZFNcs1
binding site for eZFN2
binding site for eZFN11
Zmlip terminator
CaMV19S promoter
hph (HygR)
At-ORF1 terminator
ZFNcs1
binding site for eZFN2
binding site for eZFN11
intron#2 4-coumaryl-CoA synthase v1
PAT (v6) 3' end
AtuORF23 terminator
100bp random sequence
eZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
AtMYB32(T) promoter
ipt
CaMV35S terminator
ZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
100bp random sequence
ZFNcs3
binding site for eZFN1
binding site for eZFN3
Engineered Landing Pad v1
Homologous Arm to Fad3c (r2877a1 - 2884a1)
attB2
Col E1 origin
AmpR
attB1
Homologous Arm to Fad3c (r2877a1 - 2884a1)

FIG. 10

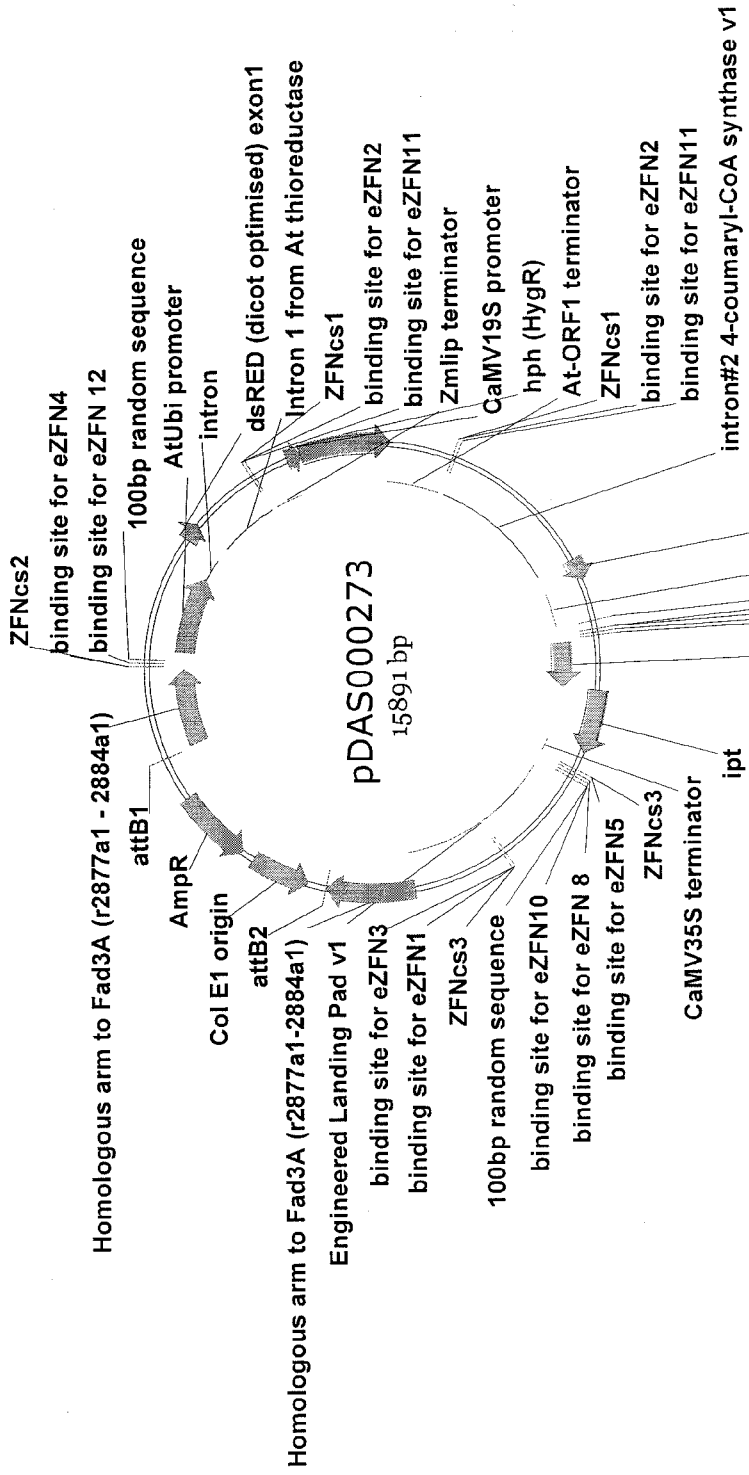

pDAS000273
15891 bp
ZFNcs2 binding site for eZFN4
binding site for eZFN 12
100bp random sequence
AtUbi promoter
intron
dsRED (dicot optimised) exon1
Intron 1 from At thioreductase
ZFNcs1
binding site for eZFN2
binding site for eZFN11
Zmlip terminator
CaMV19S promoter
hph (HygR)
At-ORF1 terminator
ZFNcs1
binding site for eZFN2
binding site for eZFN11
intron#2 4-coumaryl-CoA synthase v1
PAT (v6) 3' end
AtuORF23 terminator
100bp random sequence
eZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
AtMYB32(T) promoter
ipt
CaMV35S terminator
ZFNcs3
binding site for eZFN5
binding site for eZFN 8
100bp random sequence
binding site for eZFN10
ZFNcs3
binding site for eZFN1
binding site for eZFN3
Engineered Landing Pad v1
Homologous arm to Fad3A (r2877a1-2884a1)
attB2
Col E1 origin
AmpR
attB1
Homologous arm to Fad3A (r2877a1 - 2884a1)

FIG. 11

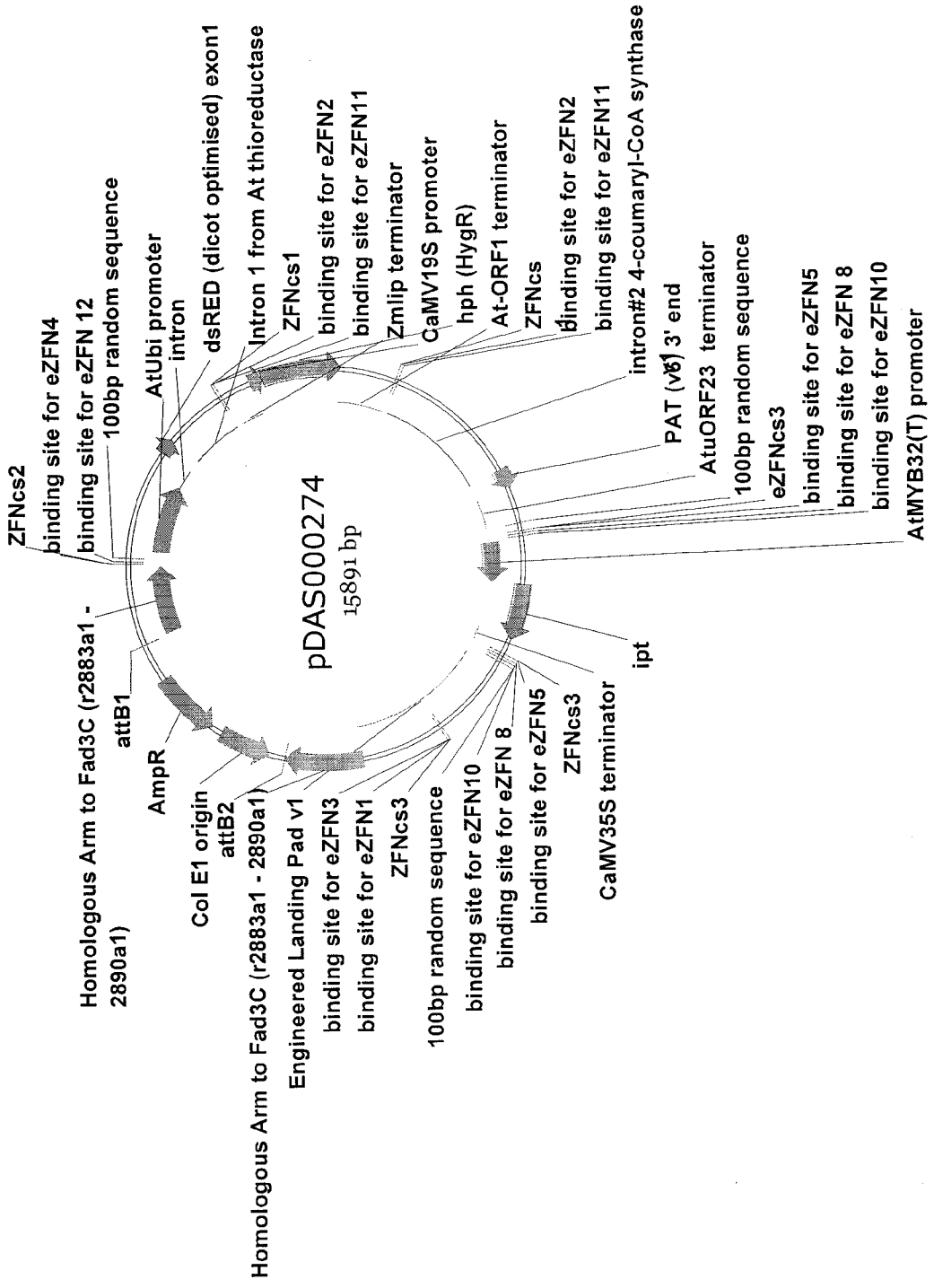

pDAS000274
15891 bp
ZFNcs2
binding site for eZFN4
binding site for eZFN 12
100bp random sequence
AtUbi promoter
intron
dsRED (dicot optimised) exon1
Intron 1 from At thioreductase
ZFNcs1
binding site for eZFN2
binding site for eZFN11
Zmlip terminator
CaMV19S promoter
hph (HygR)
At-ORF1 terminator
ZFNcs
binding site for eZFN2
binding site for eZFN11
intron#2 4-coumaryl-CoA synthase
PAT (v6) 3' end
AtuORF23 terminator
100bp random sequence
eZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
AtMYB32(T) promoter
ipt
CaMV35S terminator
ZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
100bp random sequence
ZFNcs3
binding site for eZFN1
binding site for eZFN3
Engineered Landing Pad v1
Homologous Arm to Fad3C (r2883a1 - 2890a1)
attB2
Col E1 origin
AmpR
attB1
Homologous Arm to Fad3C (r2883a1 - 2890a1)

FIG. 12

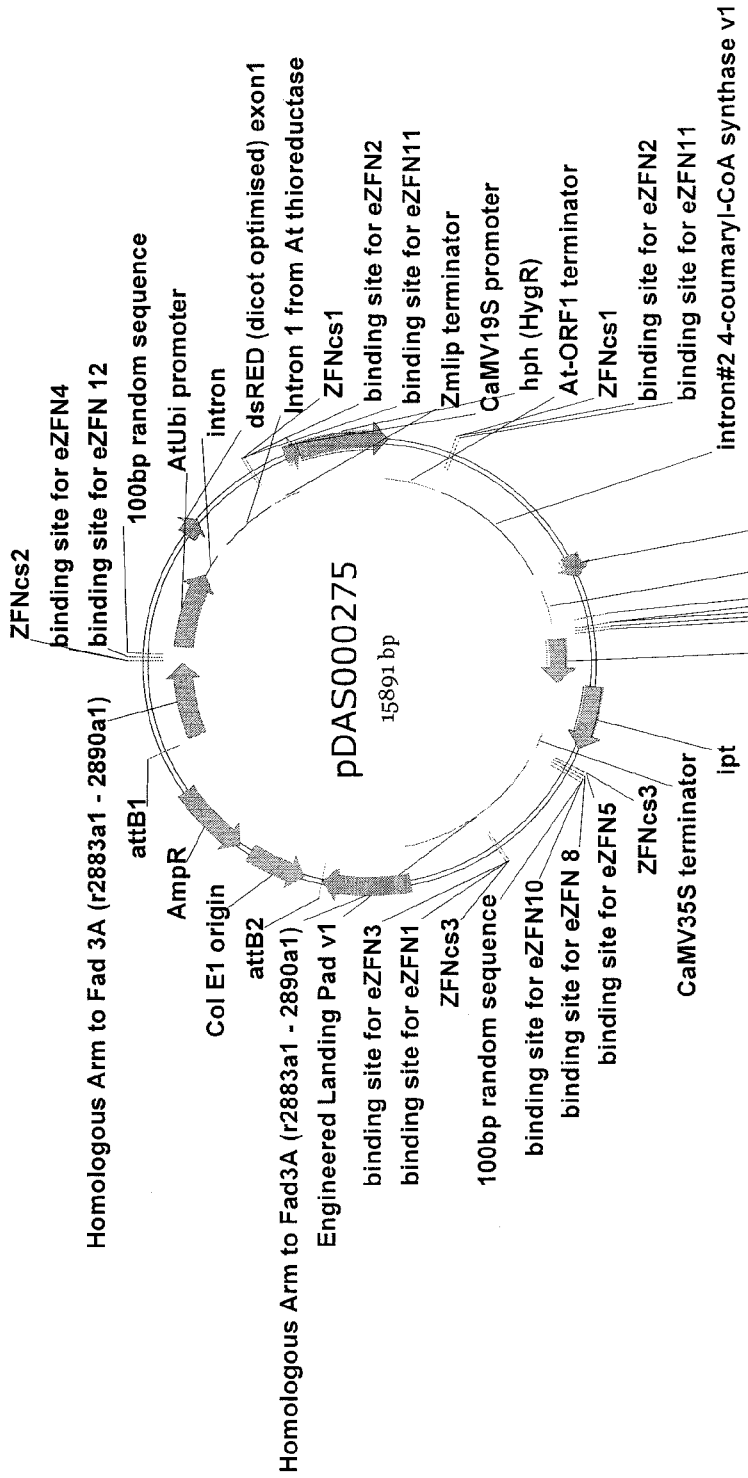

pDAS000275
15891 bp
ZFNcs2
binding site for eZFN4
binding site for eZFN 12
100bp random sequence
AtUbi promoter
intron
dsRED (dicot optimised) exon1
Intron 1 from At thioreductase
ZFNcs1
binding site for eZFN2
binding site for eZFN11
Zmlip terminator
CaMV19S promoter
hph (HygR)
At-ORF1 terminator
ZFNcs1
binding site for eZFN2
binding site for eZFN11
intron#2 4-coumaryl-CoA synthase v1
PAT (v6) 3' end
AtuORF23 terminator
100bp random sequence
eZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
AtMYB32(T) promoter
ipt
CaMV35S terminator
ZFNcs3
binding site for eZFN5
binding site for eZFN 8
binding site for eZFN10
100bp random sequence
ZFNcs3
binding site for eZFN1
binding site for eZFN3
Engineered Landing Pad v1
Homologous Arm to Fad3A (r2883a1 - 2890a1)
attB2
Col E1 origin
AmpR
attB1
Homologous Arm to Fad 3A (r2883a1 - 2890a1)

FIG. 13

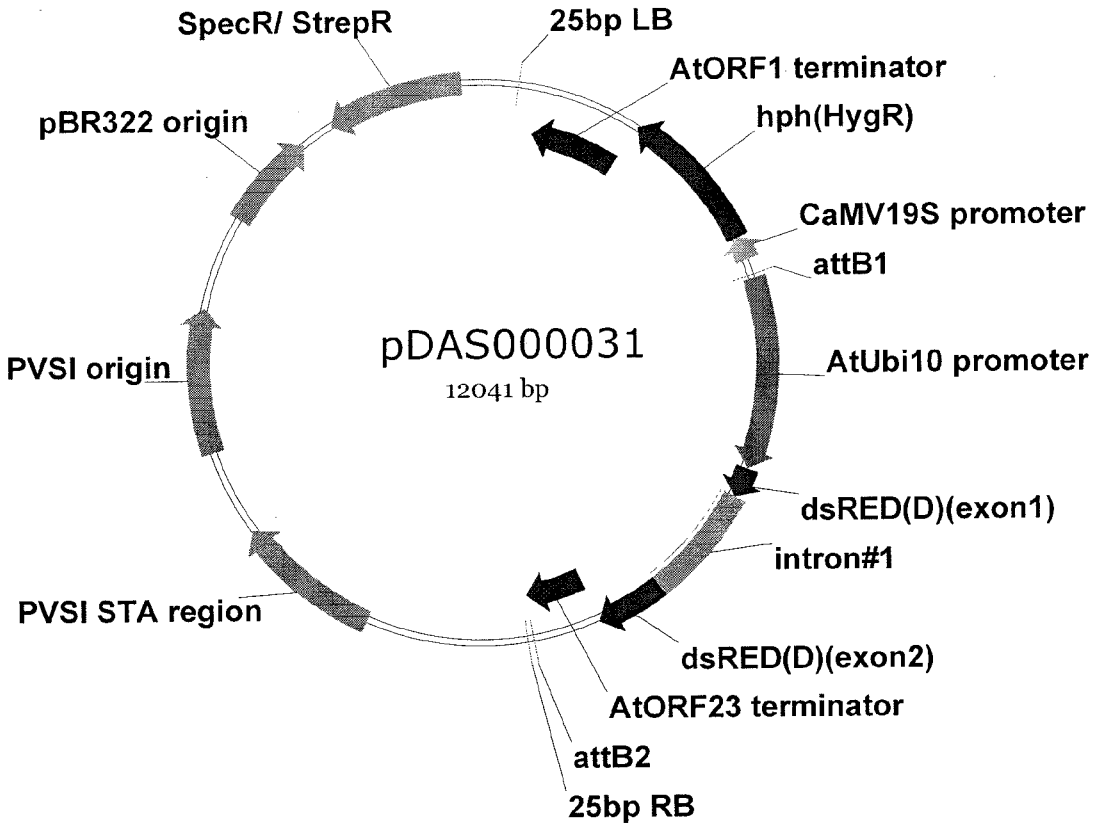

SpecR/ StrepR
25bp LB
AtORF1 terminator
hph(HygR)
pBR322 origin
CaMV19S promoter
attB1
pDAS000031
12041 bp
PVSI origin
AtUbi10 promoter
dsRED(D)(exon1)
intron#1
PVSI STA region
dsRED(D)(exon2)
AtORF23 terminator
attB2
25bp RB

FIG. 14

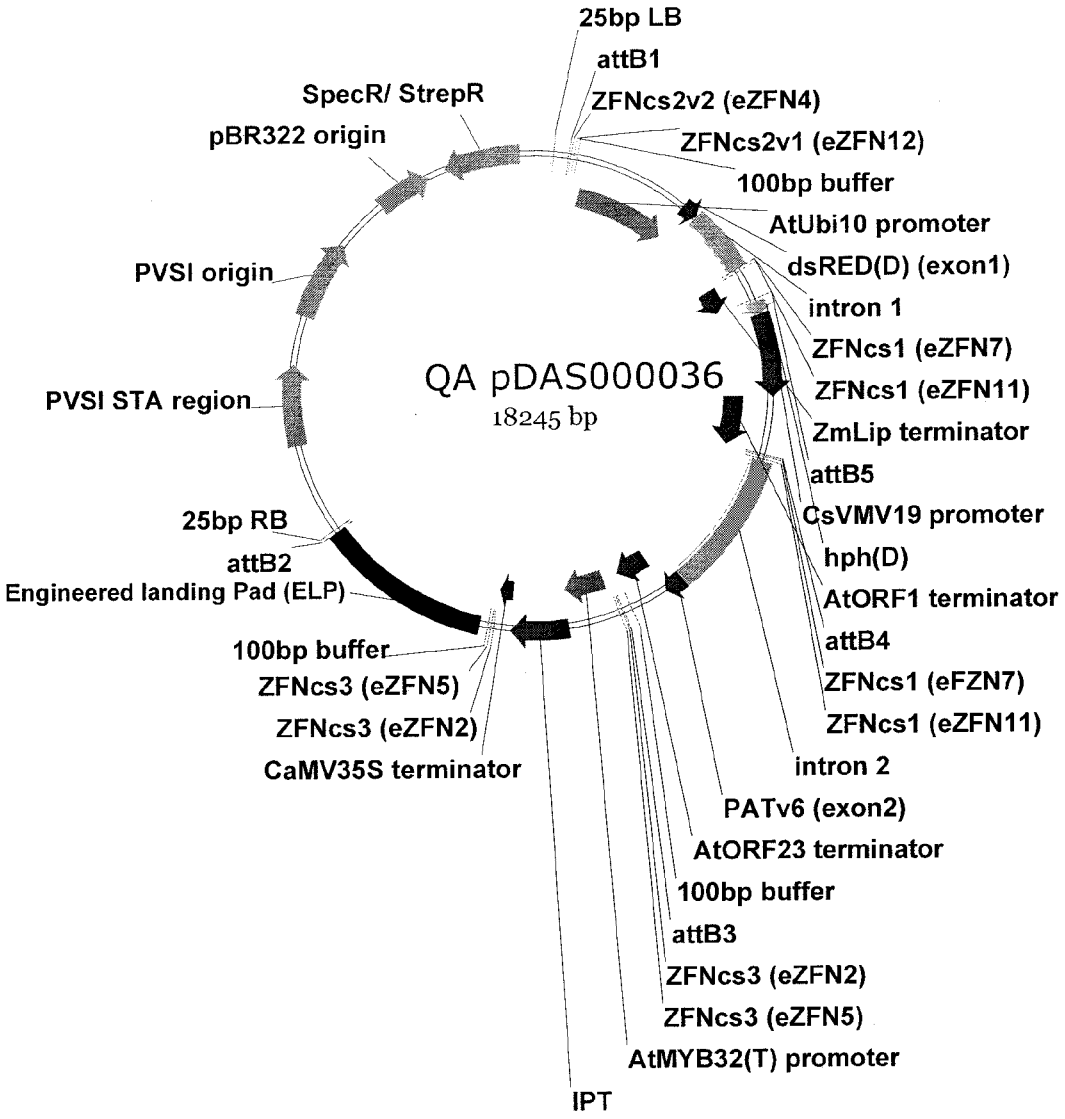

25bp LB
attB1
ZFNcs2v2 (eZFN4)
ZFNcs2v1 (eZFN12)
100bp buffer
AtUbi10 promoter
dsRED(D) (exon1)
intron 1
ZFNcs1 (eZFN7)
ZFNcs1 (eZFN11)
ZmLip terminator
attB5
CsVMV19 promoter
hph(D)
AtORF1 terminator
attB4
ZFNcs1 (eFZN7)
ZFNcs1 (eZFN11)
intron 2
PATv6 (exon2)
AtORF23 terminator
100bp buffer
attB3
ZFNcs3 (eZFN2)
ZFNcs3 (eZFN5)
AtMYB32(T) promoter
IPT
CaMV35S terminator
ZFNcs3 (eZFN2)
ZFNcs3 (eZFN5)
100bp buffer
Engineered landing Pad (ELP)
attB2
25bp RB
PVSI STA region
PVSI origin
pBR322 origin
SpecR/ StrepR
QA pDAS000036
18245 bp

FIG. 15

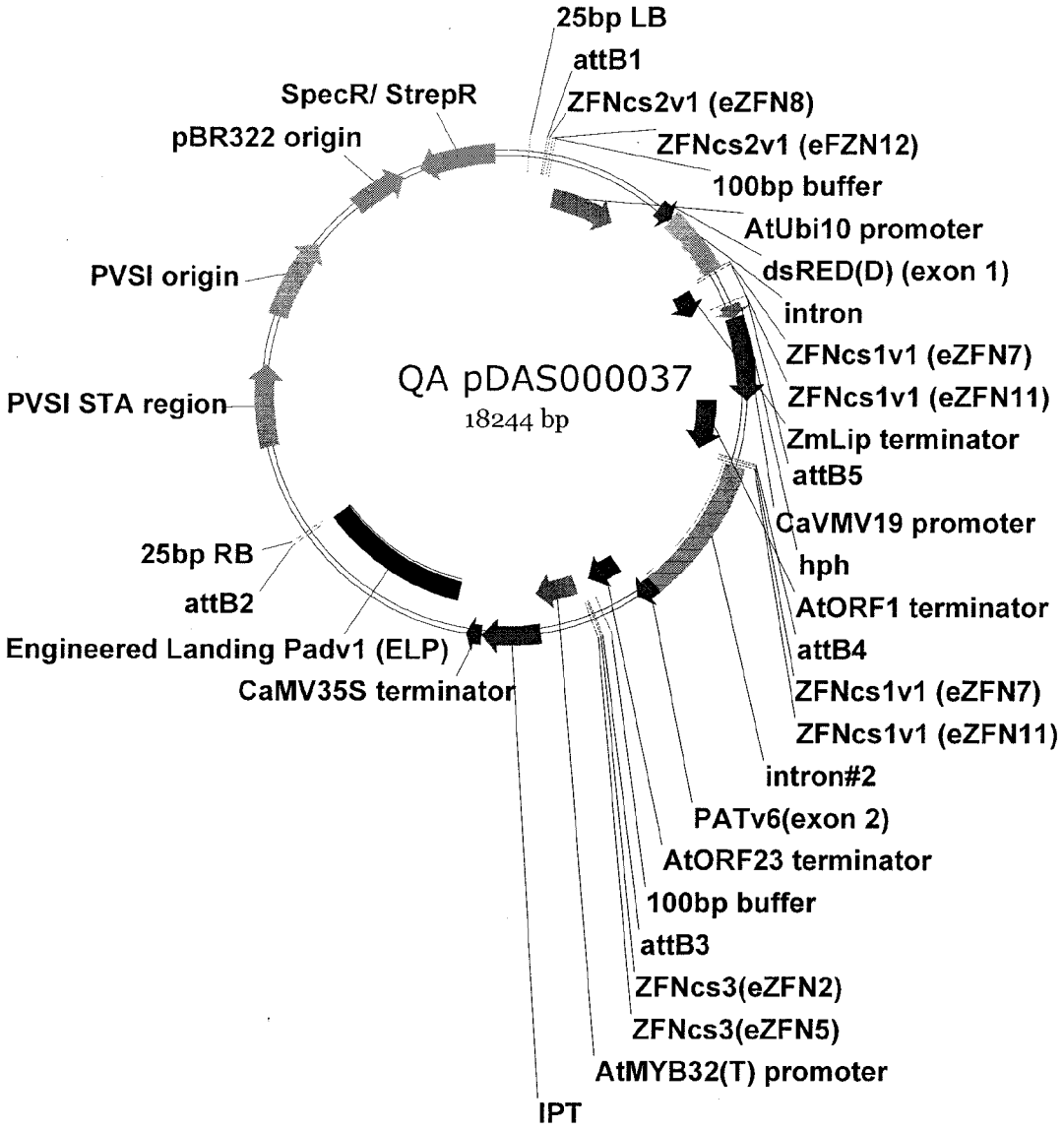
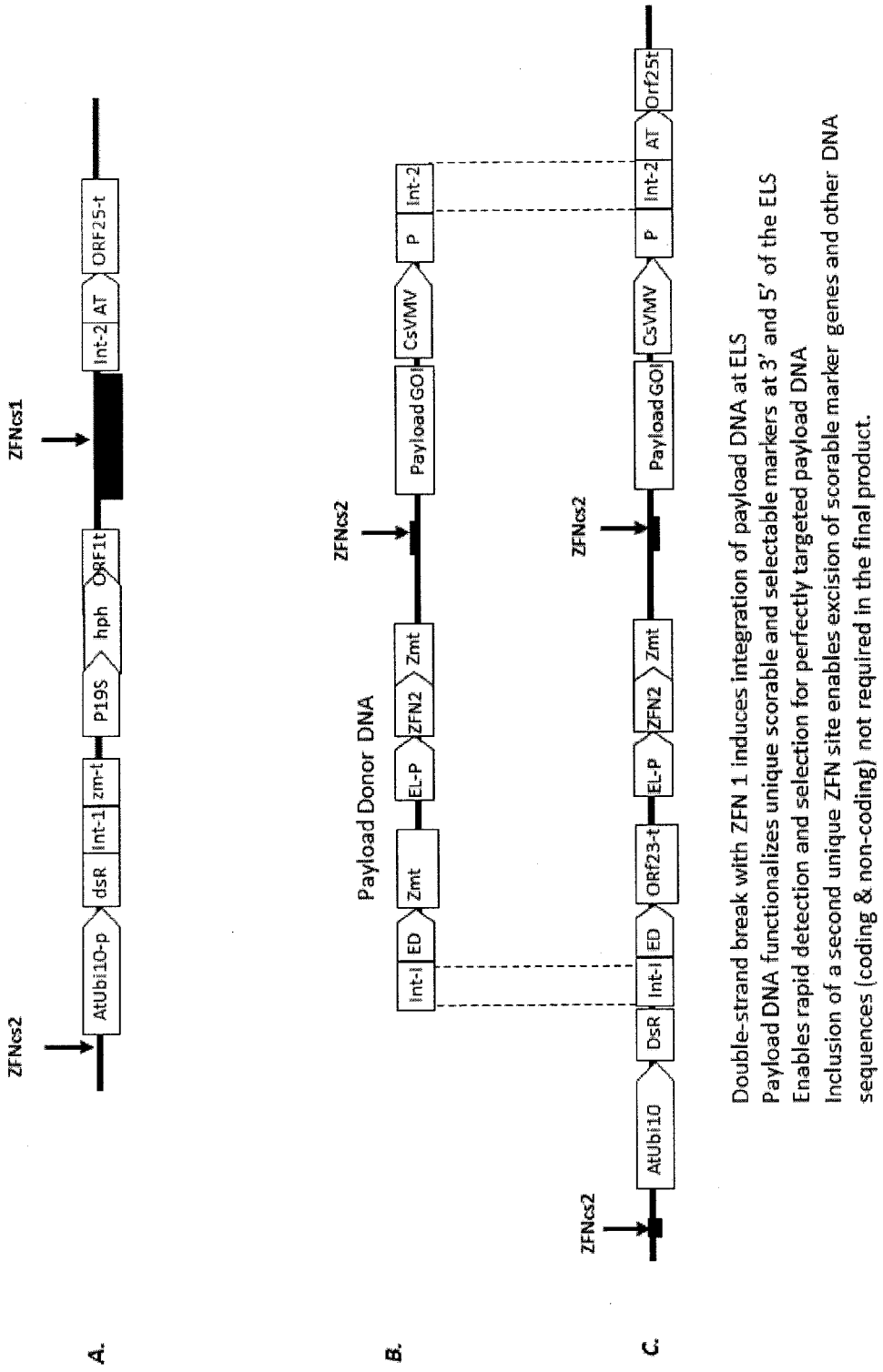

25bp LB
attB1
ZFNcs2v1 (eZFN8)
ZFNcs2v1 (eFZN12)
100bp buffer
AtUbi10 promoter
dsRED(D) (exon 1)
intron
ZFNcs1v1 (eZFN7)
ZFNcs1v1 (eZFN11)
ZmLip terminator
attB5
CaVMV19 promoter
hph
AtORF1 terminator
attB4
ZFNcs1v1 (eZFN7)
ZFNcs1v1 (eZFN11)
intron#2
PATv6(exon 2)
AtORF23 terminator
100bp buffer
attB3
ZFNcs3(eZFN2)
ZFNcs3(eZFN5)
AtMYB32(T) promoter
IPT
CaMV35S terminator
Engineered Landing Padv1 (ELP)
attB2
25bp RB
PVSI STA region
PVSI origin
pBR322 origin
SpecR/ StrepR
QA pDAS000037
18244 bp

FIG. 16



Double-strand break with ZFN 1 induces integration of payload DNA at ELS
Payload DNA functionalizes unique scorable and selectable markers at 3' and 5' of the ELS
Enables rapid detection and selection for perfectly targeted payload DNA
Inclusion of a second unique ZFN site enables excision of scorable marker genes and other DNA sequences (coding & non-coding) not required in the final product.

FIG. 17

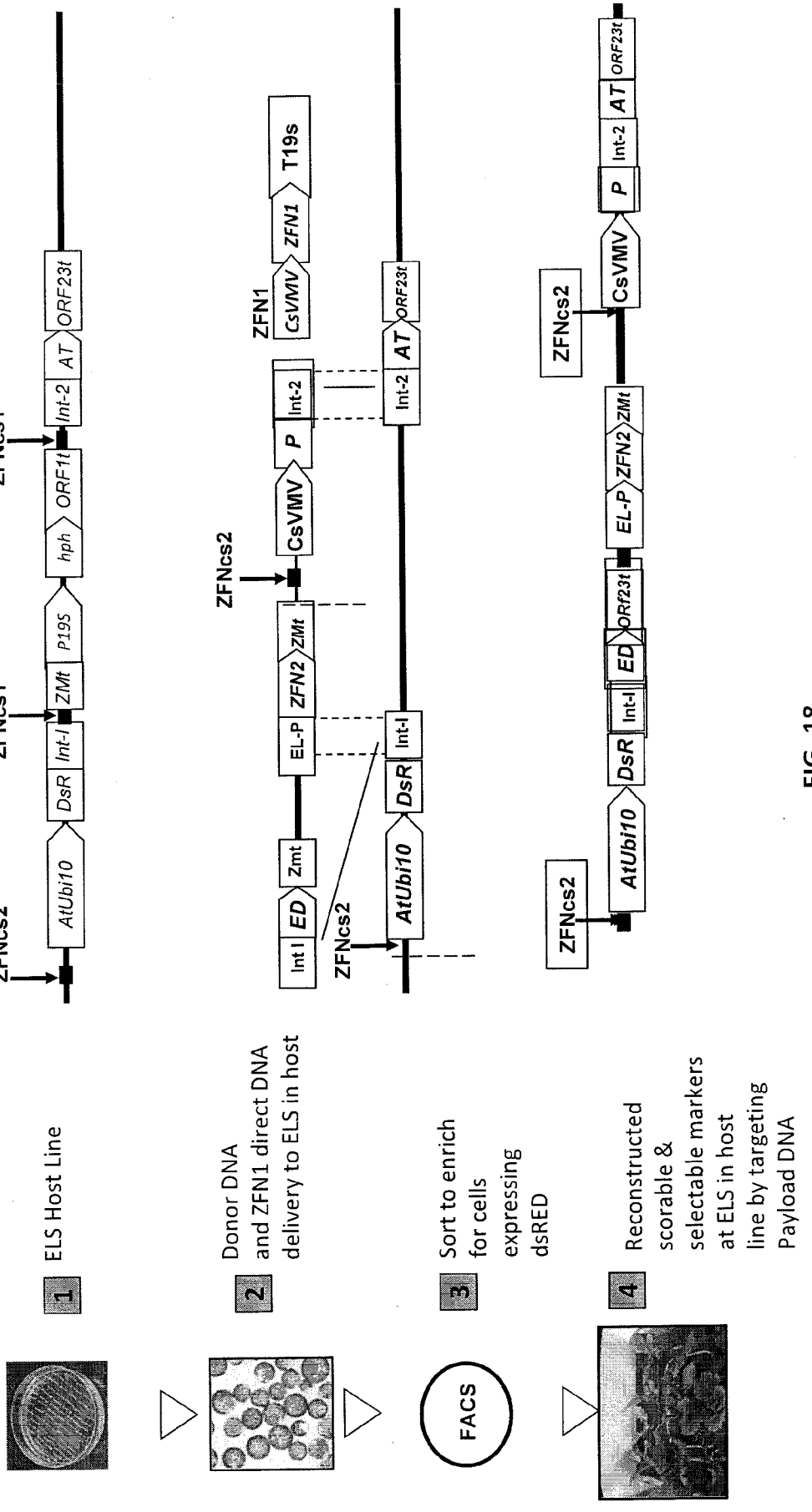

ZFNcs2
AtUbi10
DsR
Int-I
ZFNcs1
ZMt
P19S
hph
ORF11t
ZFNcs1
Int-2
AT
ORF23t
ZFNcs2
Int I
ED
Zmt
EL-P
ZFN2
ZMt
CsVMV
P
Int-2
ZFN1
CsVMV
ZFN1
T19s
ZFNcs2
AtUbi10
DsR
Int-I
Int-2
AT
ORF23t
ZFNcs2
AtUbi10
DsR
Int-I
ED
ORf23t
EL-P
ZFN2
ZMt
ZFNcs2
CsVMV
P
Int-2
AT
ORF23t
1
ELS Host Line
2
Donor DNA
and ZFN1 direct DNA
delivery to ELS in host
3
Sort to enrich
for cells
expressing
dsRED
FACS
4
Reconstructed
scorable &
selectable markers
at ELS in host
line by targeting
Payload DNA

FIG. 18

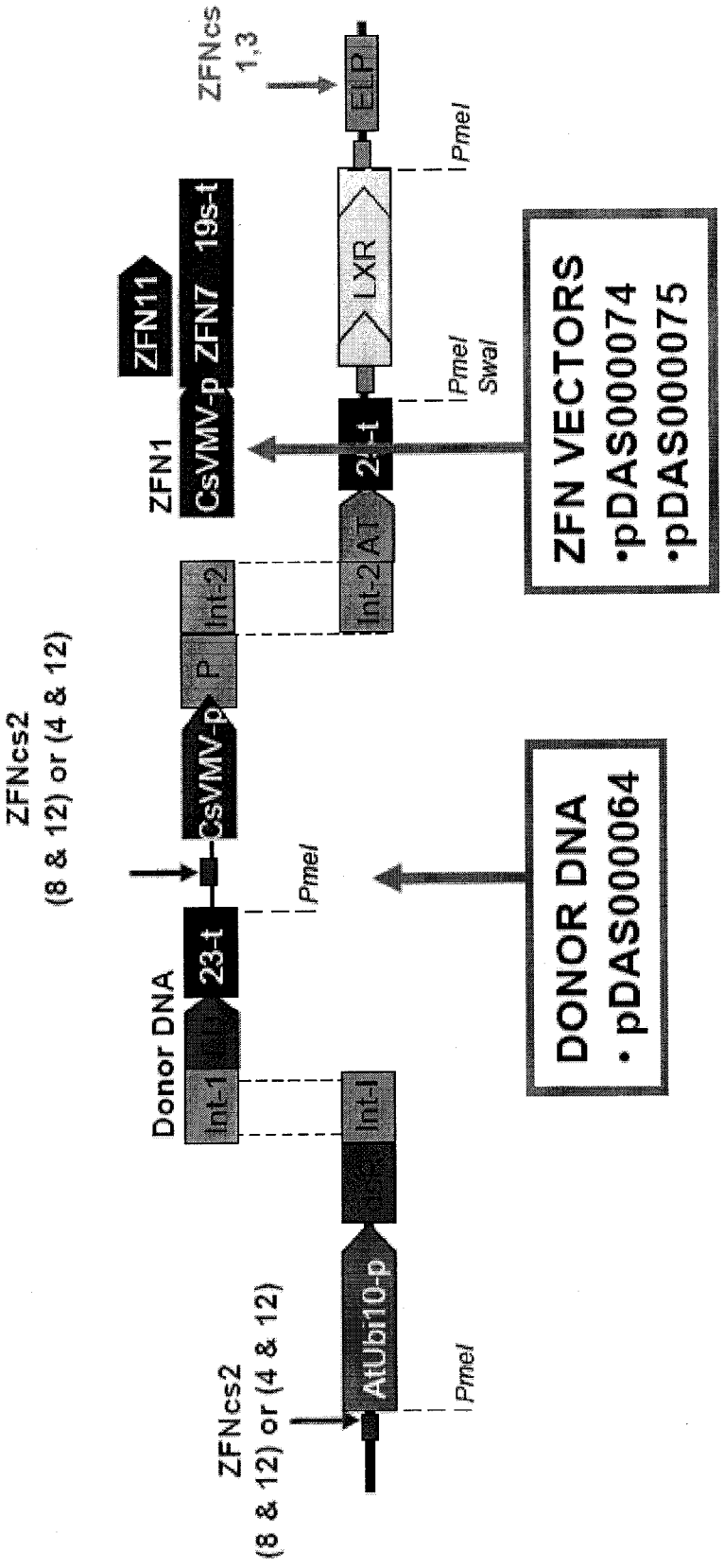

ZFNcs
1,3
ELP
PmeI
LXR
PmeI
SwaI
2-t
ZFN1
CsVMV-p
ZFN7
19s-t
ZFN11
Int-2 AT
Int-2
P
CsVMV-p
ZFNcs2
(8 & 12) or (4 & 12)
PmeI
23-t
Donor DNA
Int-1
Int-I
AtUbi10-p
PmeI
ZFNcs2
(8 & 12) or (4 & 12)
ZFN VECTORS
•pDAS000074
•pDAS000075
DONOR DNA
• pDAS000064

FIG. 19A

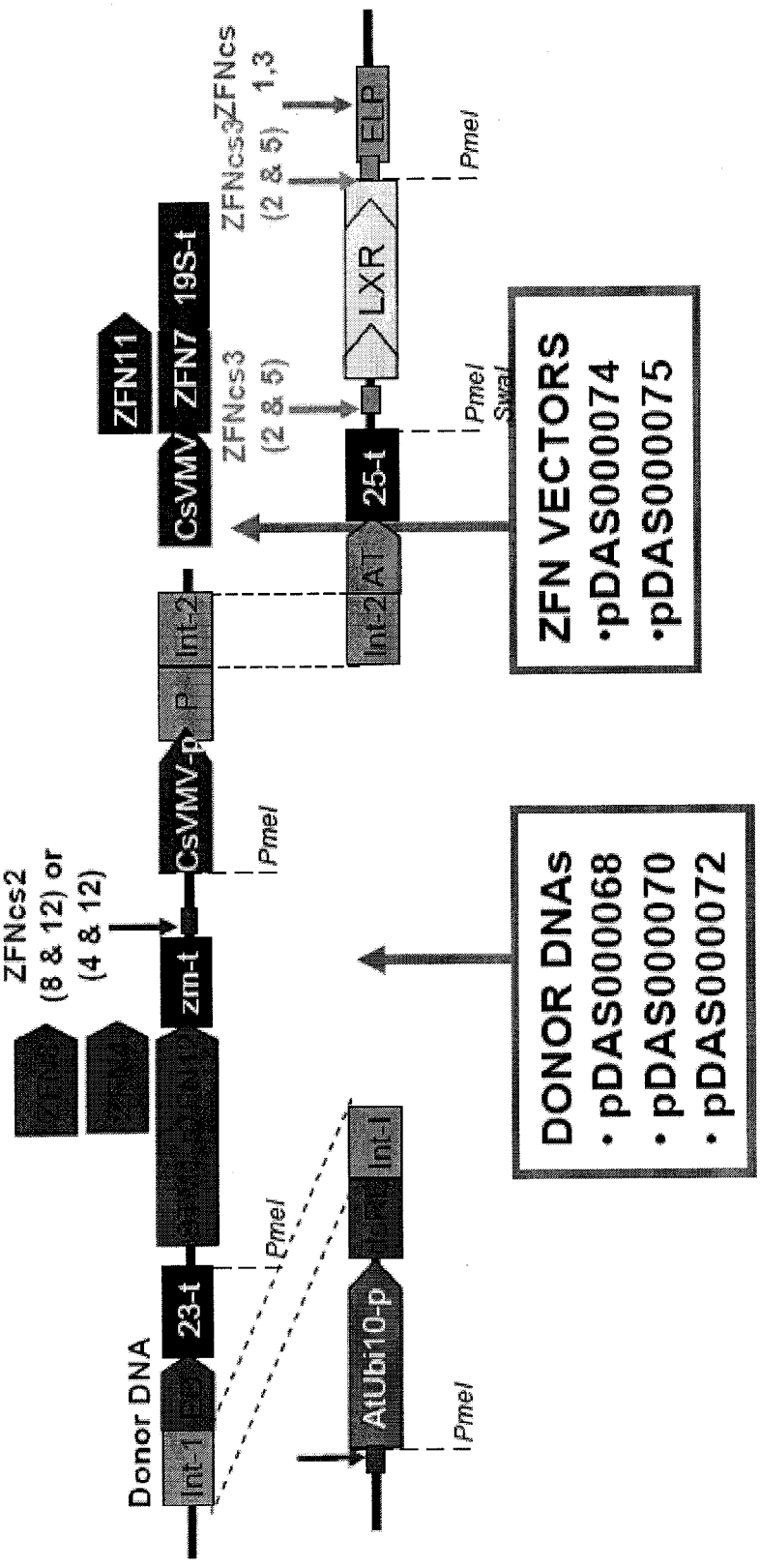
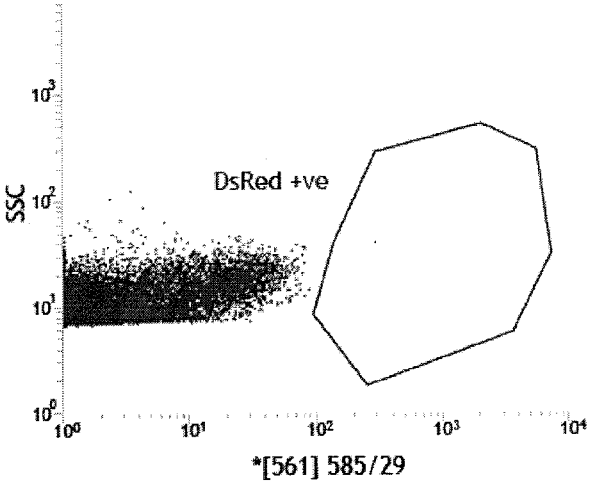
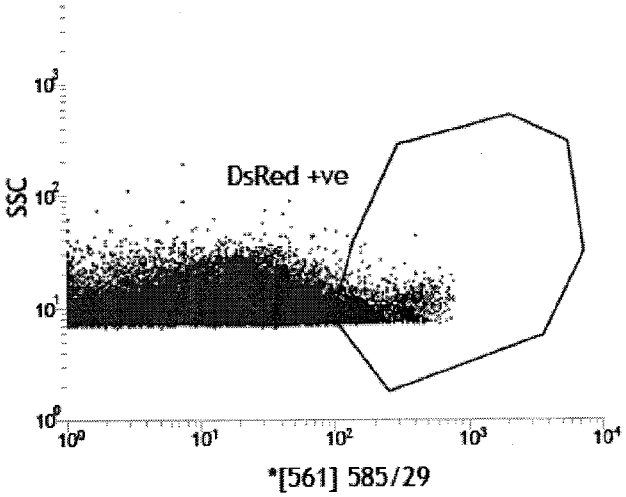

ZFN VECTORS
•pDAS000074
•pDAS000075
DONOR DNAs
• pDAS000068
• pDAS000070
• pDAS000072
Donor DNA
Int-1
ELP
23-t
AtUbi10-p
Int-1
PR
PmeI
ZFNcs2
(8 & 12) or
(4 & 12)
zm-t
CsVMV-p
P
Int-2
PmeI
ZFNcs3
(2 & 5)
25-t
AT
Int-2
PmeI
SwaI
ZFN11
ZFN7
19S-t
CsVMV
ZFNcs3
(2 & 5)
LXR
ELP
ZFNcs
1,3
PmeI

FIG. 19B



Statistics: neg control_001

Populations	Events	% Parent
All Events	50,000	####
Cells (DsRed)	48,433	96.87%
DsRed +ve	1	0.00%



Statistics: DS-RED PDAS31 POS CONT

Populations	Events	% Parent
All Events	50,000	####
Cells (DsRed)	48,801	97.60%
DsRed +ve	2,432	4.98%

FIG. 20

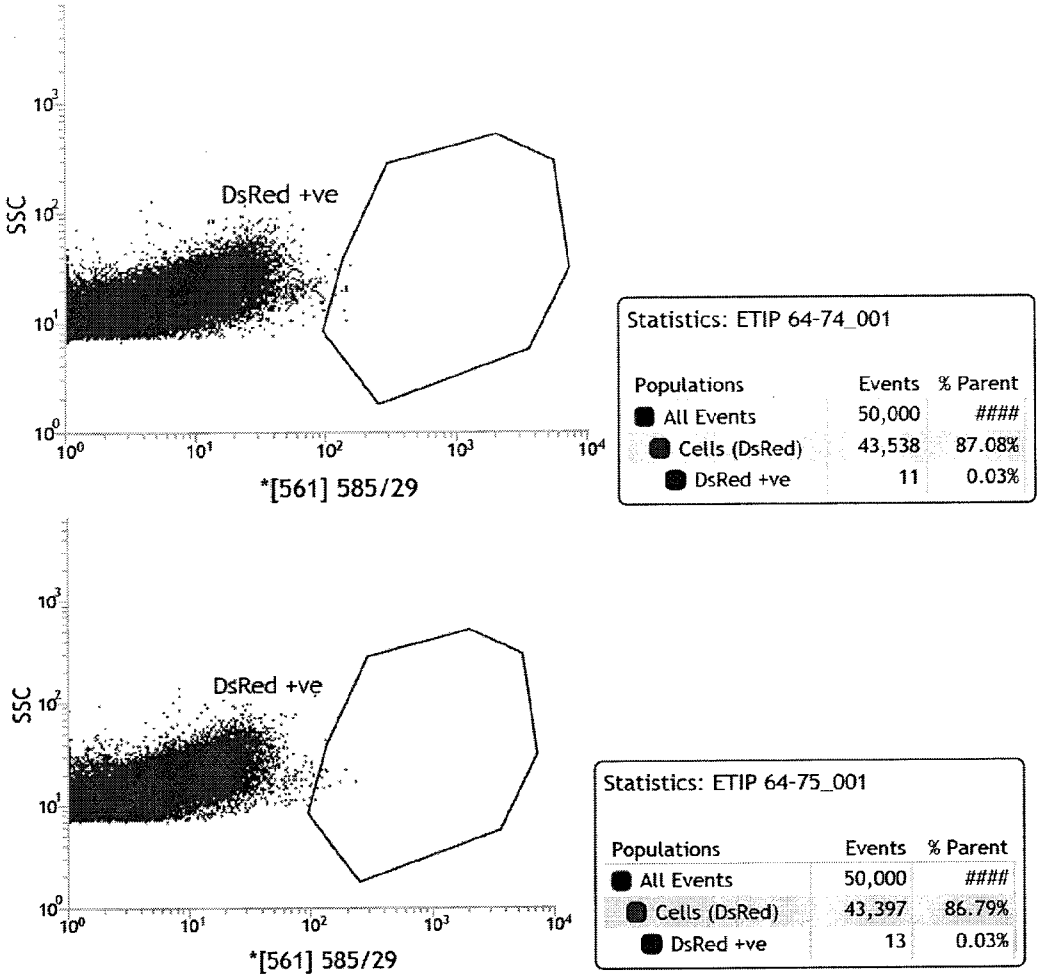
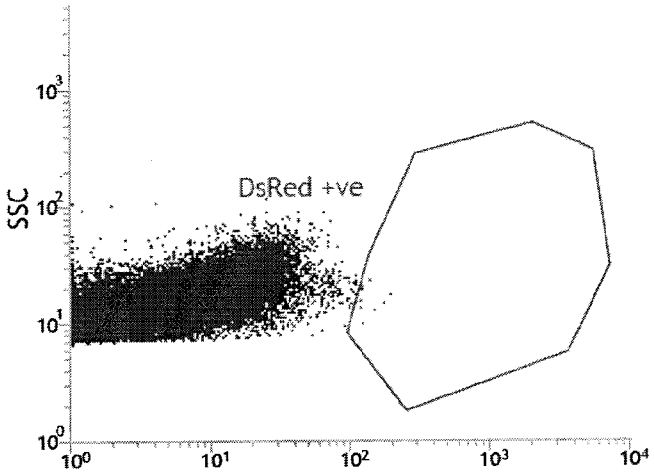
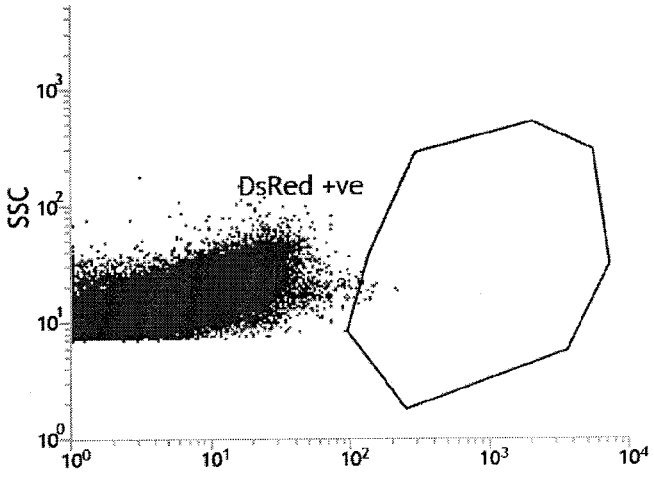

DsRed +ve
SSC
*[561] 585/29
Statistics: ETIP 64-74_001
Populations Events % Parent
All Events 50,000 ####
Cells (DsRed) 43,538 87.08%
DsRed +ve 11 0.03%
DsRed +ve
SSC
*[561] 585/29
Statistics: ETIP 64-75_001
Populations Events % Parent
All Events 50,000 ####
Cells (DsRed) 43,397 86.79%
DsRed +ve 13 0.03%

FIG. 21



Statistics: ETIP 68-74_001

Populations	Events	% Parent
All Events	50,000	####
Cells (DsRed)	43,397	86.79%
DsRed +ve	11	0.03%



Statistics: ETIP 68-75_001

Populations	Events	% Parent
All Events	50,000	####
Cells (DsRed)	43,905	87.81%
DsRed +ve	19	0.04%

FIG. 22

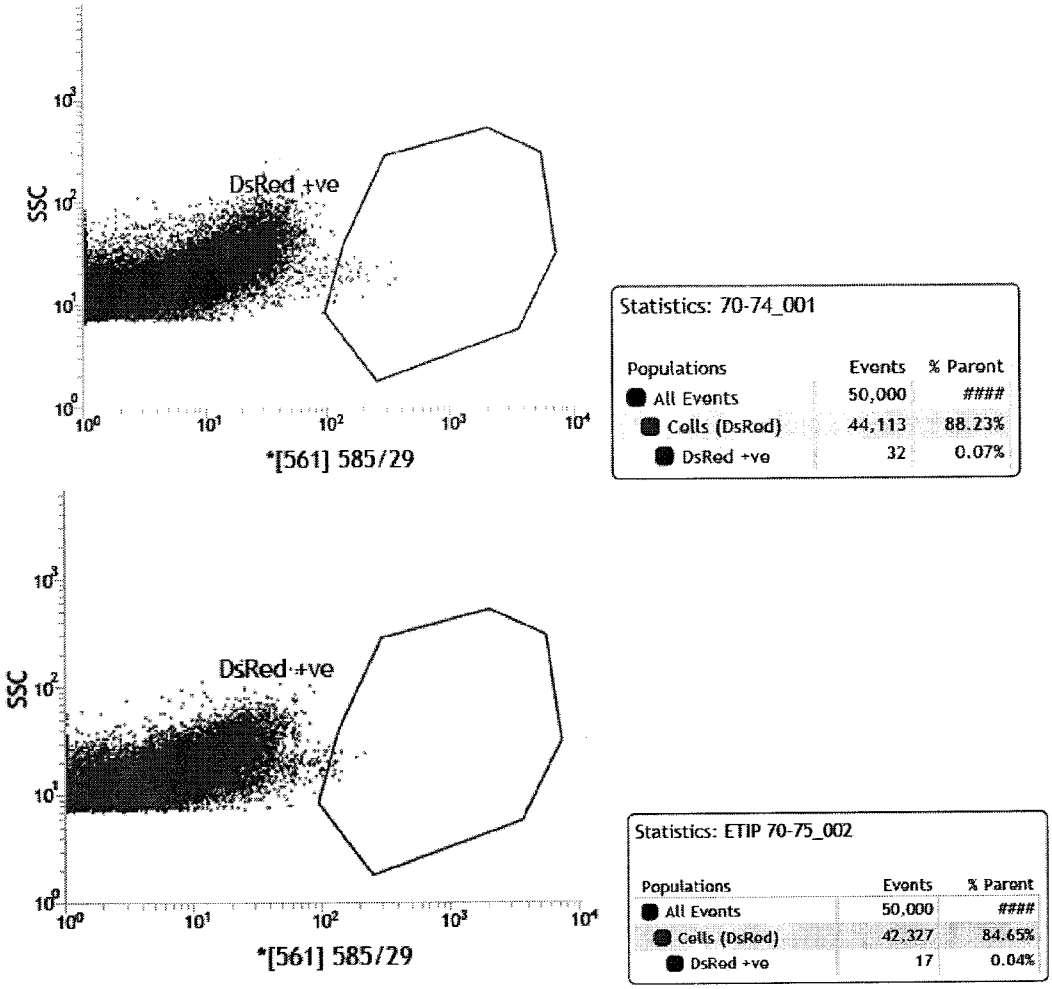

DsRed +ve
SSC
*[561] 585/29
Statistics: 70-74_001
Populations Events % Parent
All Events 50,000 ####
Cells (DsRed) 44,113 88.23%
DsRed +ve 32 0.07%
DsRed +ve
SSC
*[561] 585/29
Statistics: ETIP 70-75_002
Populations Events % Parent
All Events 50,000 ####
Cells (DsRed) 42,327 84.65%
DsRed +ve 17 0.04%

FIG. 23

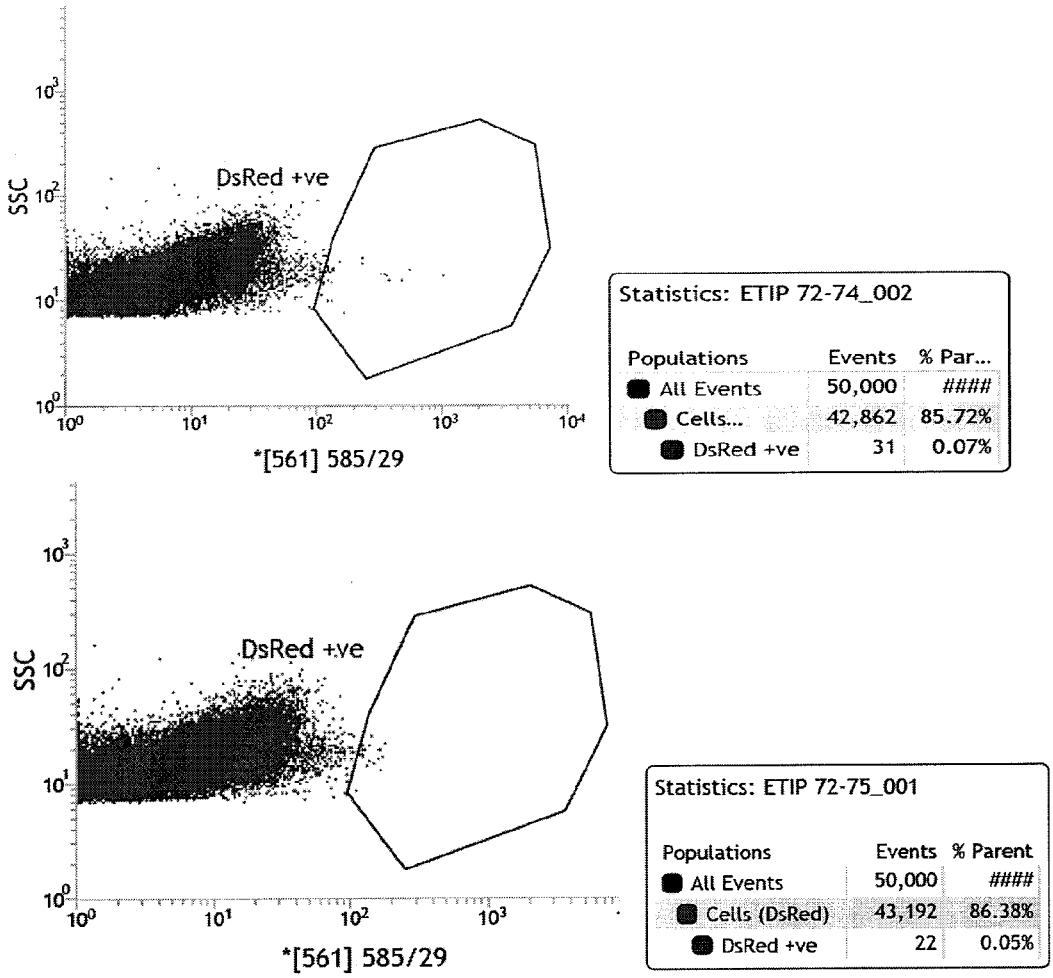

SSC
DsRed +ve
*[561] 585/29
Statistics: ETIP 72-74_002
Populations Events % Par...
All Events 50,000 ####
Cells... 42,862 85.72%
DsRed +ve 31 0.07%
SSC
DsRed +ve
*[561] 585/29
Statistics: ETIP 72-75_001
Populations Events % Parent
All Events 50,000 ####
Cells (DsRed) 43,192 86.38%
DsRed +ve 22 0.05%

FIG. 24

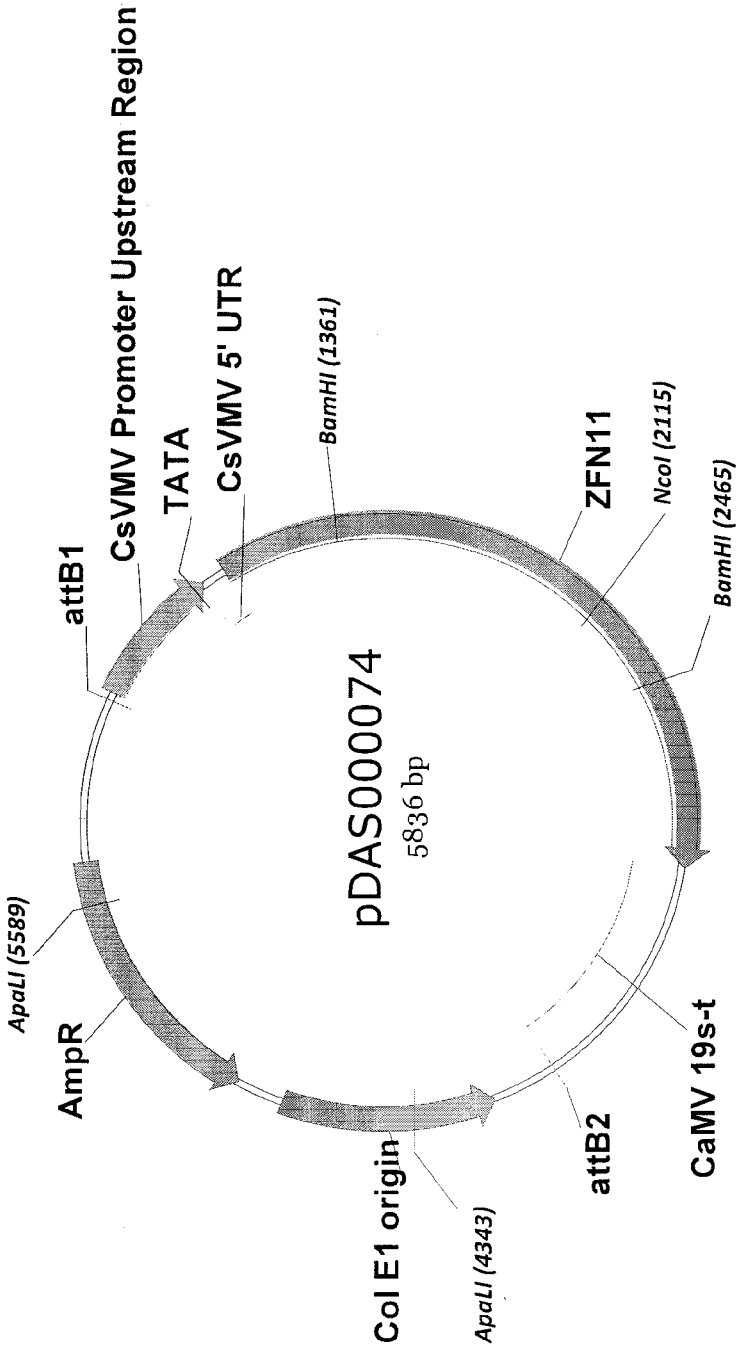

pDAS000074
5836 bp
attB1
CsVMV Promoter Upstream Region
TATA
CsVMV 5' UTR
BamHI (1361)
ZFN11
NcoI (2115)
BamHI (2465)
CaMV 19s-t
attB2
ApaLI (4343)
Col E1 origin
AmpR
ApaLI (5589)

FIG. 25

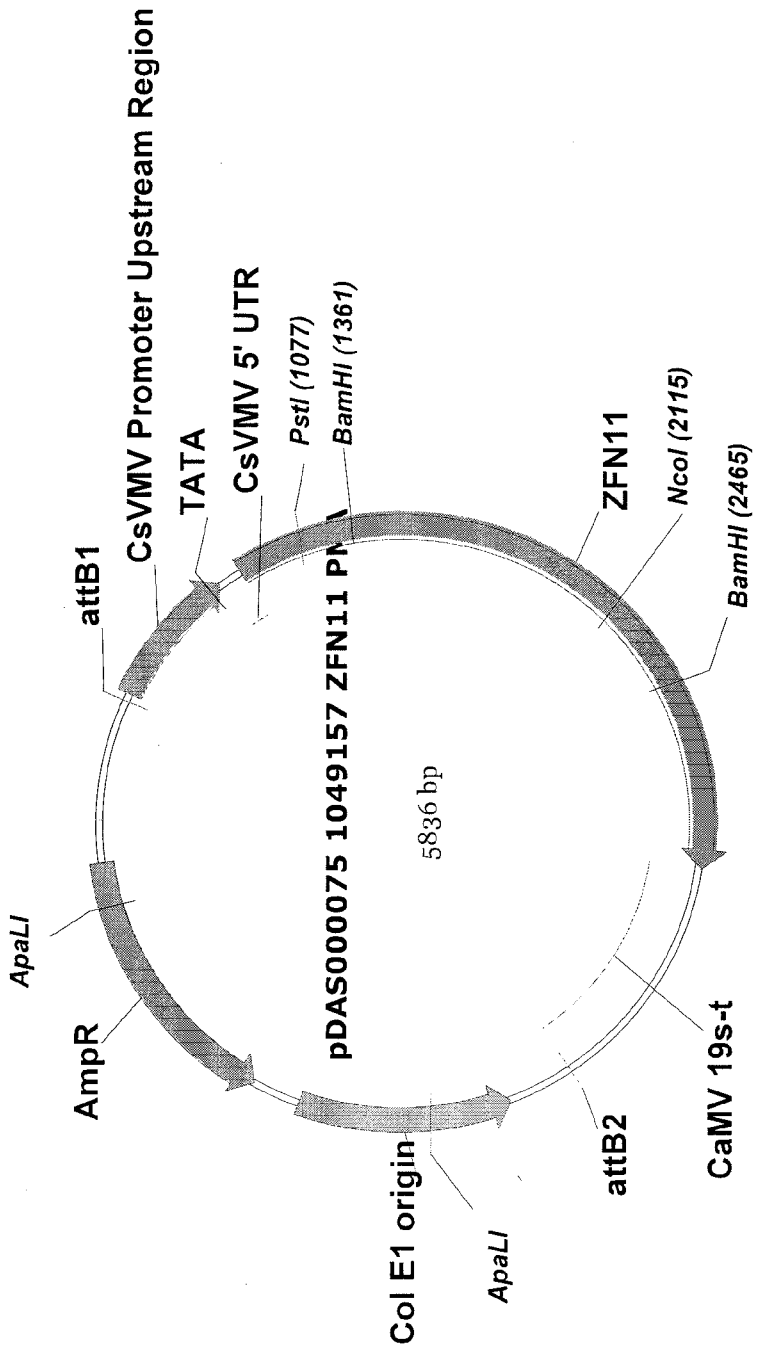

attB1
CsVMV Promoter Upstream Region
TATA
CsVMV 5' UTR
PstI (1077)
BamHI (1361)
ZFN11
NcoI (2115)
BamHI (2465)
pDAS000075 1049157 ZFN11 PM
5836 bp
ApaLI
AmpR
Col E1 origin
ApaLI
attB2
CaMV 19s-t

FIG. 26

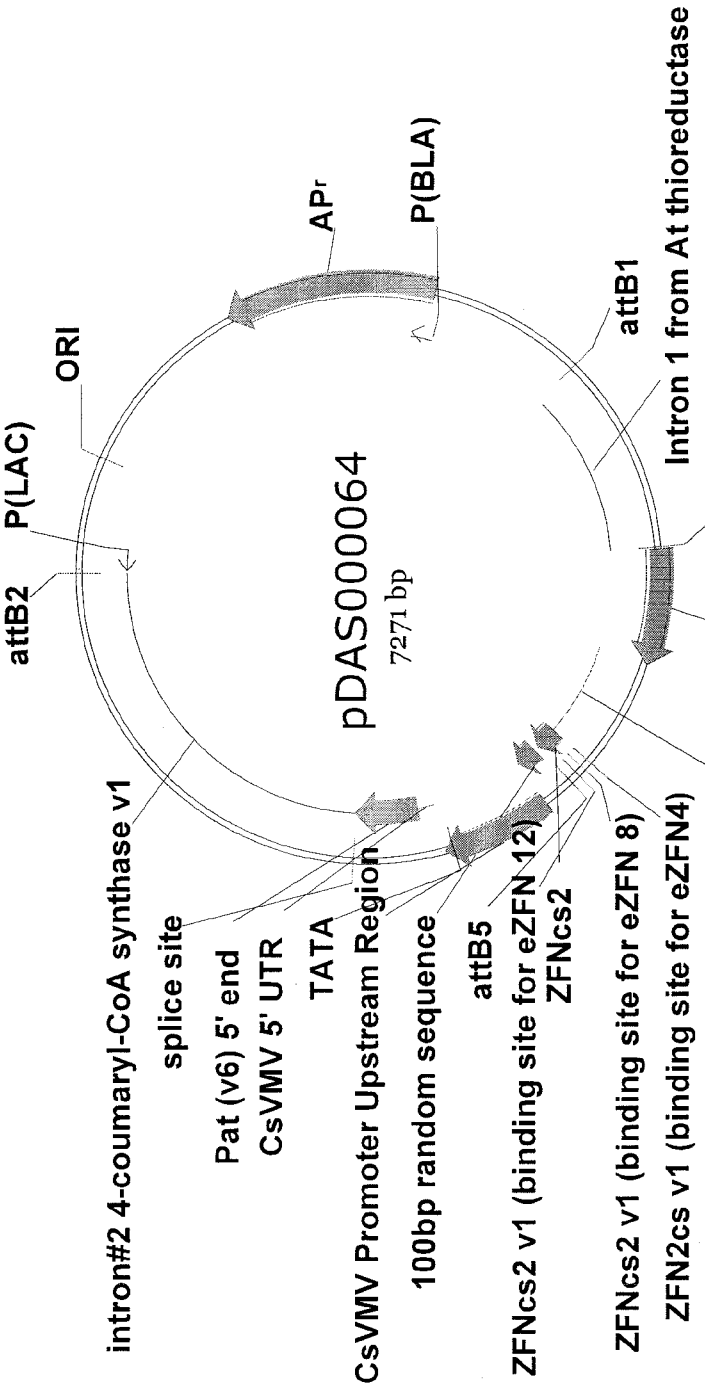

pDAS000064
7271 bp
attB2
P(LAC)
ORI
APr
P(BLA)
attB1
Intron 1 from At thioreductase
PmeI
3' half of dsRED
At ORF23 terminator
ZFN2cs v1 (binding site for eZFN4)
ZFNcs2 v1 (binding site for eZFN 8)
ZFNcs2
ZFNcs2 v1 (binding site for eZFN 12)
attB5
100bp random sequence
CsVMV Promoter Upstream Region
TATA
CsVMV 5' UTR
Pat (v6) 5' end
splice site
intron#2 4-coumaryl-CoA synthase v1

FIG. 27

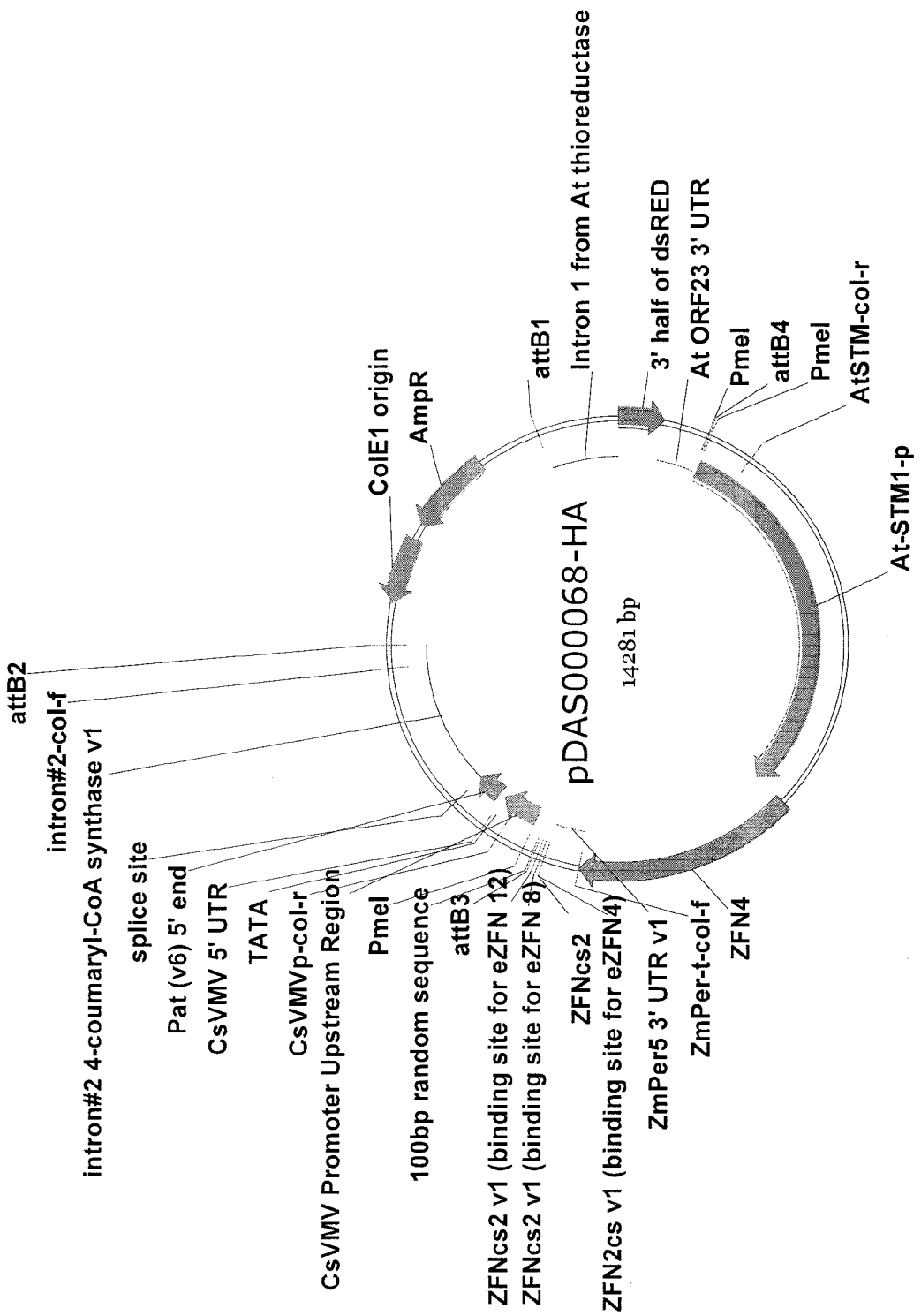

pDAS000068-HA
14281 bp
attB2
intron#2-col-f
intron#2 4-coumaryl-CoA synthase v1
splice site
Pat (v6) 5' end
CsVMV 5' UTR
TATA
CsVMVp-col-r
CsVMV Promoter Upstream Region
PmeI
100bp random sequence
attB3
ZFNcs2 v1 (binding site for eZFN 12)
ZFNcs2 v1 (binding site for eZFN 8)
ZFNcs2
ZFN2cs v1 (binding site for eZFN4)
ZmPer5 3' UTR v1
ZmPer-t-col-f
ZFN4
At-STM1-p
AtSTM-col-r
PmeI
attB4
PmeI
At ORF23 3' UTR
3' half of dsRED
Intron 1 from At thioreductase
attB1
ColE1 origin
AmpR

FIG. 28

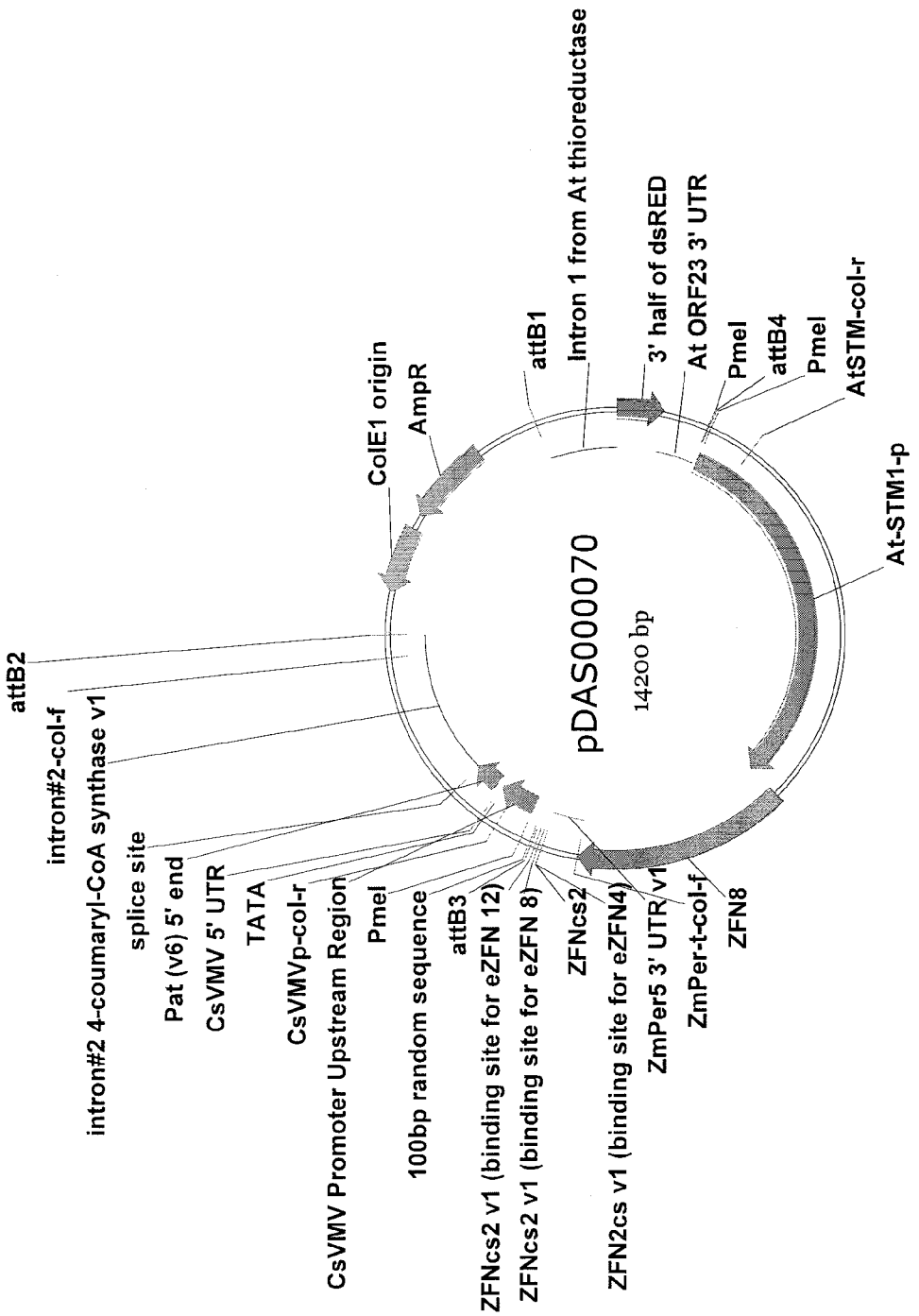

pDAS000070
14200 bp
attB2
intron#2-col-f
intron#2 4-coumaryl-CoA synthase v1
splice site
Pat (v6) 5' end
CsVMV 5' UTR
TATA
CsVMVp-col-r
CsVMV Promoter Upstream Region
PmeI
100bp random sequence
attB3
ZFNcs2 v1 (binding site for eZFN 12)
ZFNcs2 v1 (binding site for eZFN 8)
ZFNcs2
ZFN2cs v1 (binding site for eZFN4)
ZmPer5 3' UTR v1
ZmPer-t-col-f
ZFN8
At-STM1-p
AtSTM-col-r
PmeI
attB4
PmeI
At ORF23 3' UTR
3' half of dsRED
Intron 1 from At thioreductase
attB1
ColE1 origin
AmpR

FIG. 29

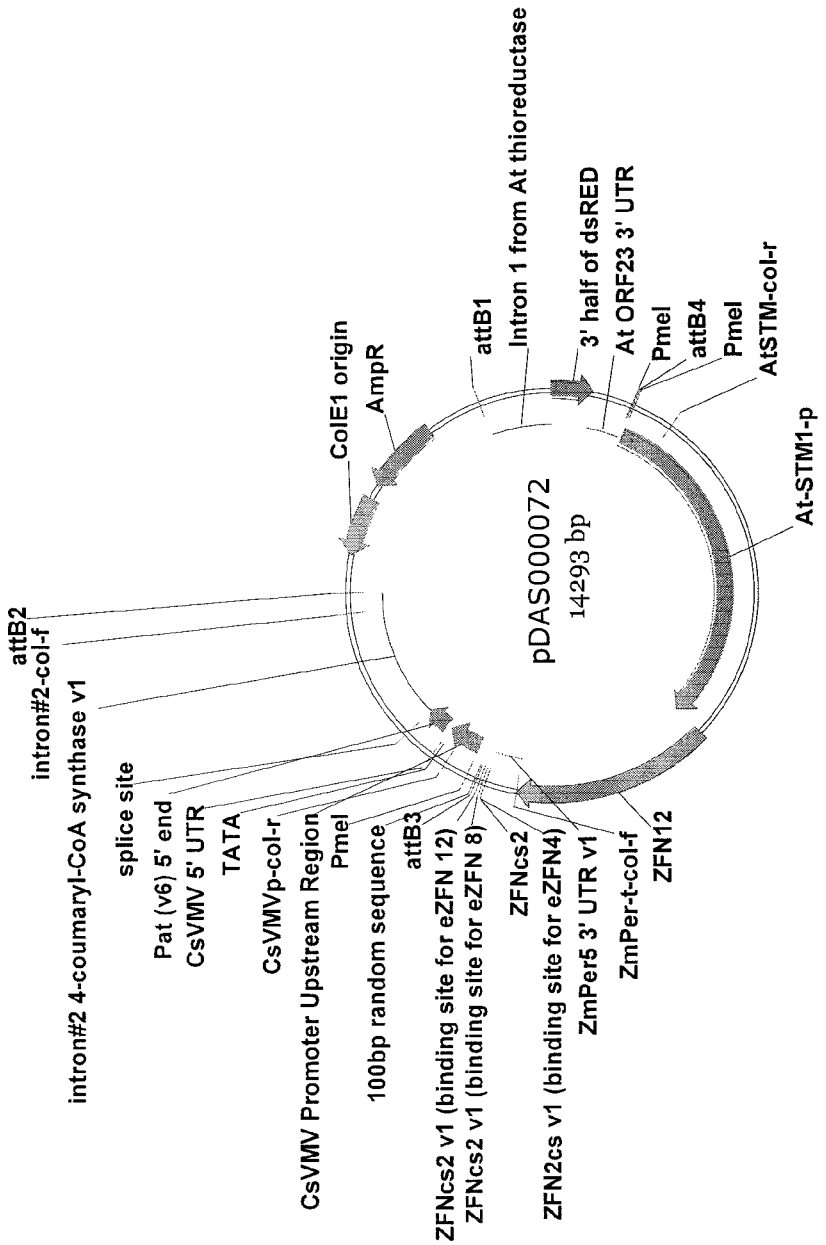

pDAS000072
14293 bp
attB2
intron#2-col-f
intron#2 4-coumaryl-CoA synthase v1
splice site
Pat (v6) 5' end
CsVMV 5' UTR
TATA
CsVMVp-col-r
CsVMV Promoter Upstream Region
PmeI
100bp random sequence
attB3
ZFNcs2 v1 (binding site for eZFN 12)
ZFNcs2 v1 (binding site for eZFN 8)
ZFNcs2
ZFN2cs v1 (binding site for eZFN4)
ZmPer5 3' UTR v1
ZmPer-t-col-f
ZFN12
At-STM1-p
AtSTM-col-r
PmeI
attB4
PmeI
At ORF23 3' UTR
3' half of dsRED
Intron 1 from At thioreductase
attB1
ColE1 origin
AmpR

FIG. 30

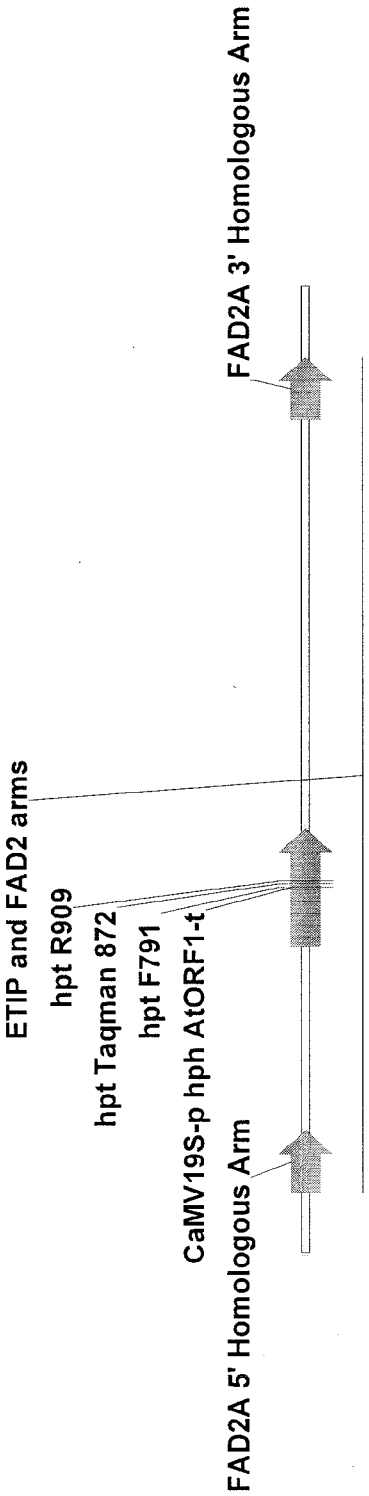

FAD2A 3' Homologous Arm
ETIP and FAD2 arms
hpt R909
hpt Taqman 872
hpt F791
CaMV19S-p hph AtORF1-t
FAD2A 5' Homologous Arm
FAD2A locus with perfect ETIP integration -hph qPCR primer binding sites
15612 bp

FIG. 31

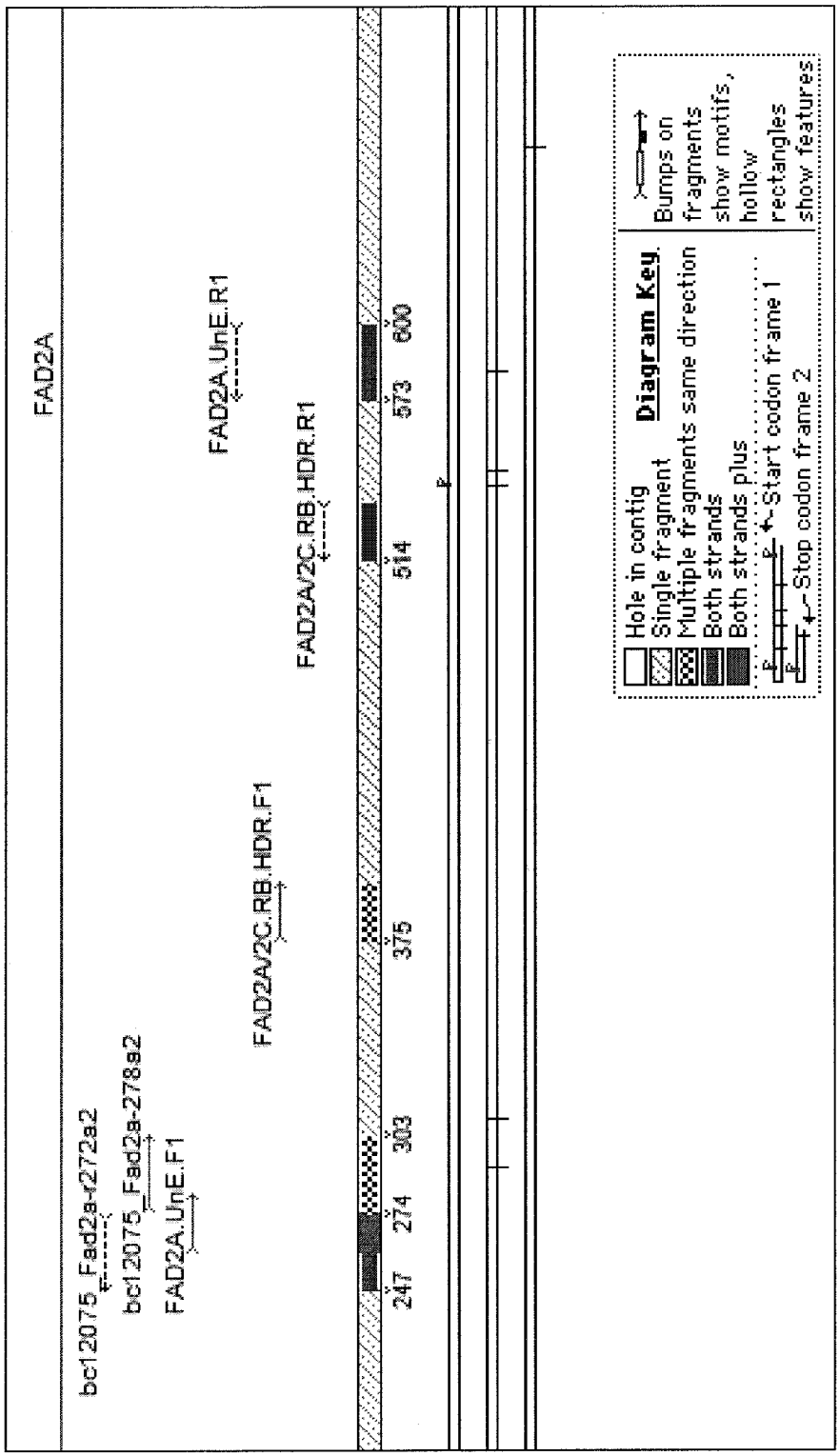

FAD2A
bc12075_Fad2a-r272a2
bc12075_Fad2a-278a2
FAD2A.UnE.F1
FAD2A/2C.RB.HDR.F1
FAD2A.UnE.R1
FAD2A/2C.RB.HDR.R1
247
274
303
375
514
573
600
Diagram Key
Hole in contig
Single fragment
Multiple fragments same direction
Both strands
Both strands plus
Start codon frame 1
Stop codon frame 2
Bumps on fragments show motifs, hollow rectangles show features

FIG. 32

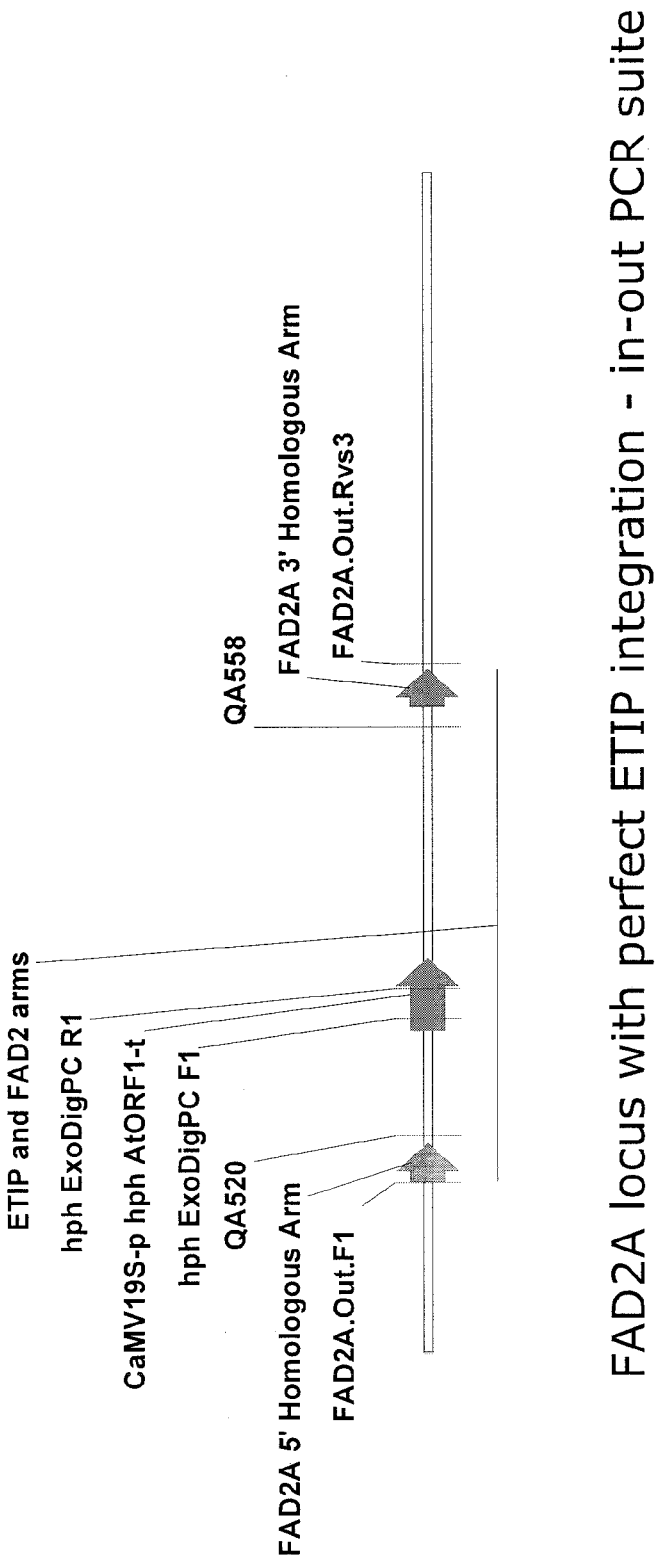

FAD2A locus with perfect ETIP integration - in-out PCR suite
31000 bp
ETIP and FAD2 arms
hph ExoDigPC R1
CaMV19S-p hph AtORF1-t
hph ExoDigPC F1
QA520
FAD2A 5' Homologous Arm
FAD2A.Out.F1
QA558
FAD2A 3' Homologous Arm
FAD2A.Out.Rvs3

FIG. 33

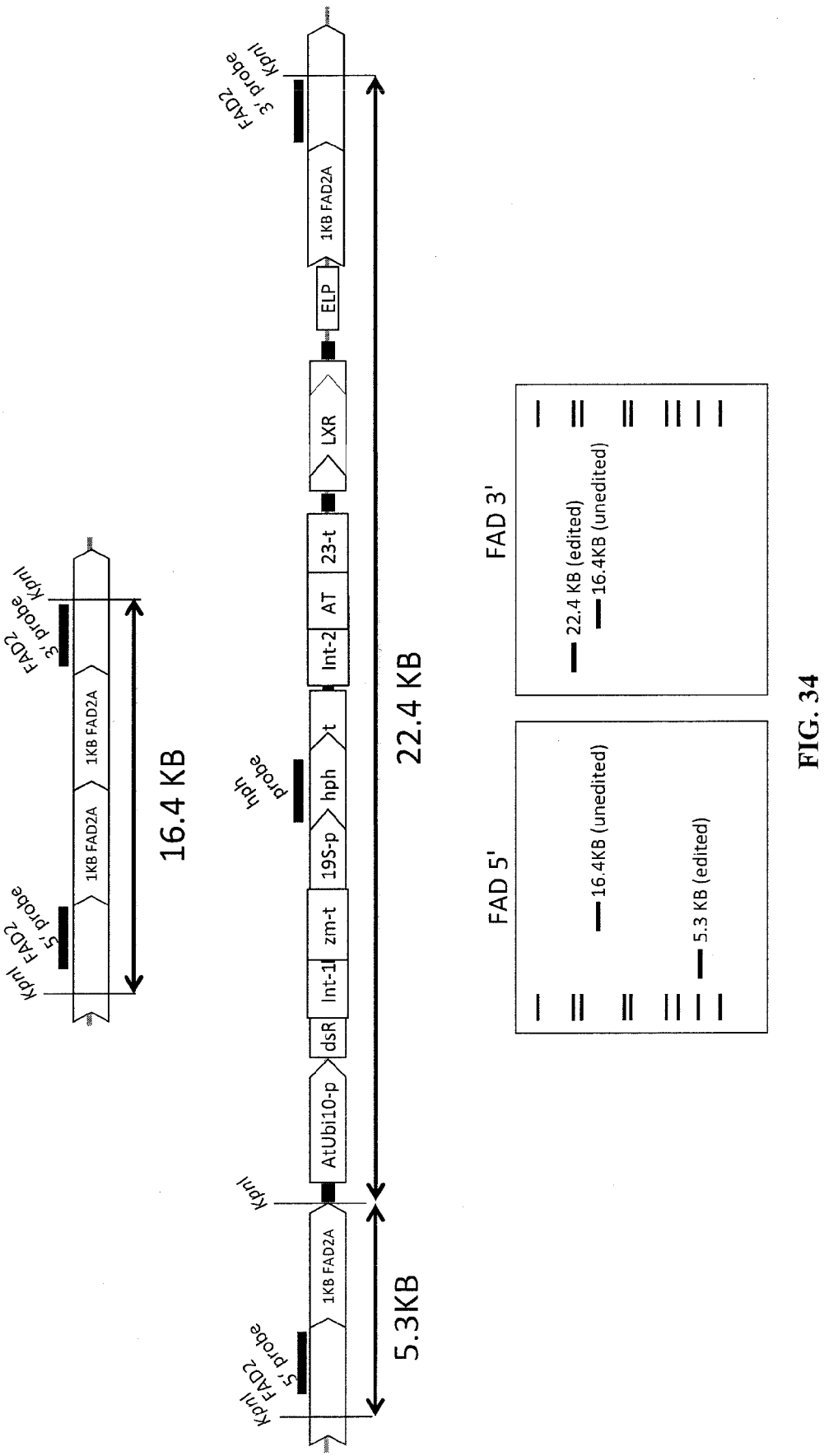

KpnI
FAD2 3' probe
1KB FAD2A
ELP
LXR
23-t
AT
Int-2
t
hph
19S-p
zm-t
Int-1
dsR
AtUbi10-p
KpnI
1KB FAD2A
KpnI
FAD2 5' probe
22.4 KB
hph probe
5.3KB
16.4 KB
FAD 3'
22.4 KB (edited)
16.4KB (unedited)
FAD 5'
16.4KB (unedited)
5.3 KB (edited)

FIG. 34

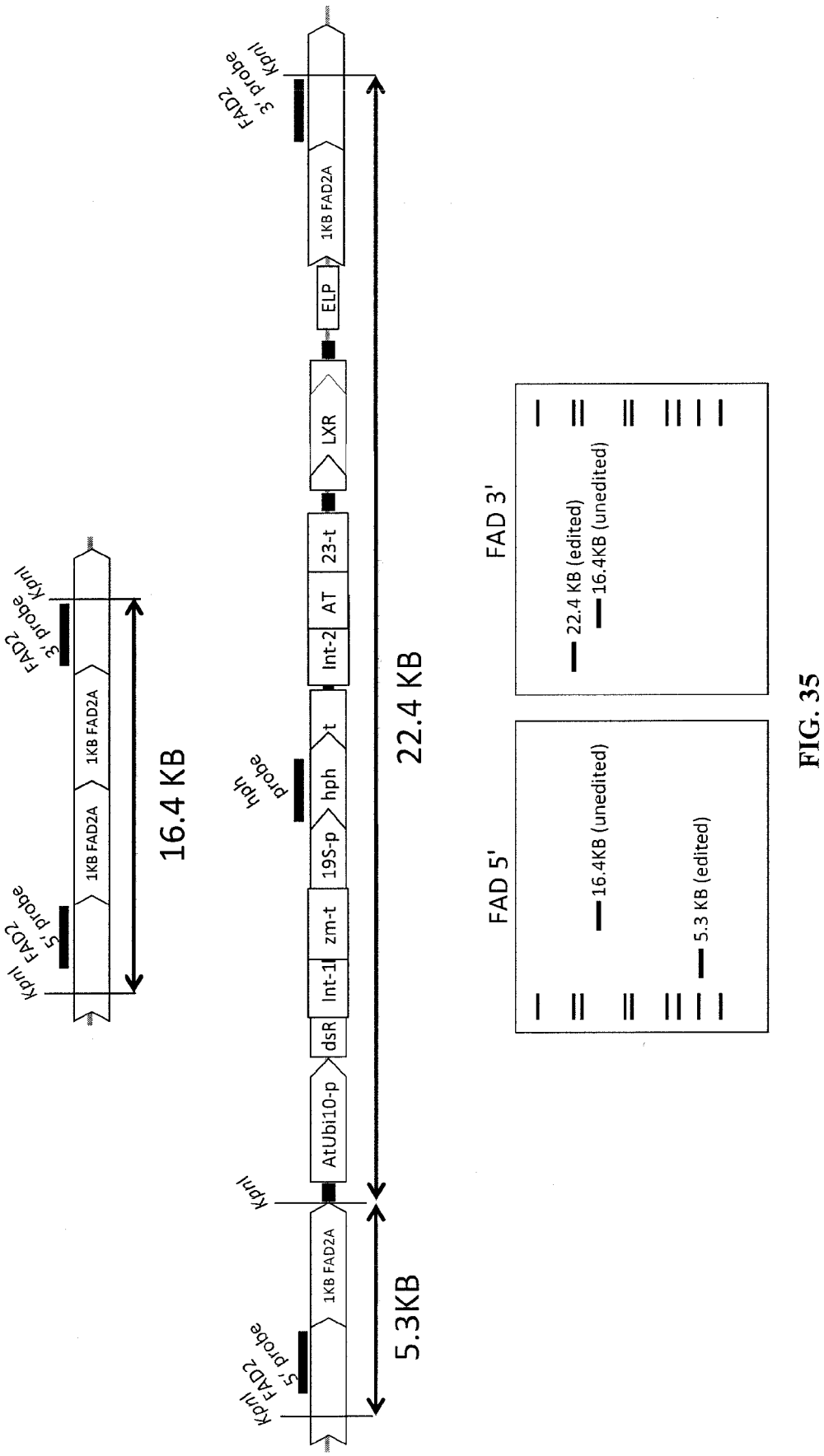

KpnI
FAD2 3' probe
1KB FAD2A
ELP
LXR
23-t
AT
Int-2
t
hph
19S-p
zm-t
Int-1
dsR
AtUbi10-p
KpnI
1KB FAD2A
KpnI
FAD2 5' probe
22.4 KB
5.3KB
hph probe
16.4 KB
FAD 3'
22.4 KB (edited)
16.4KB (unedited)
FAD 5'
16.4KB (unedited)
5.3 KB (edited)

FIG. 35

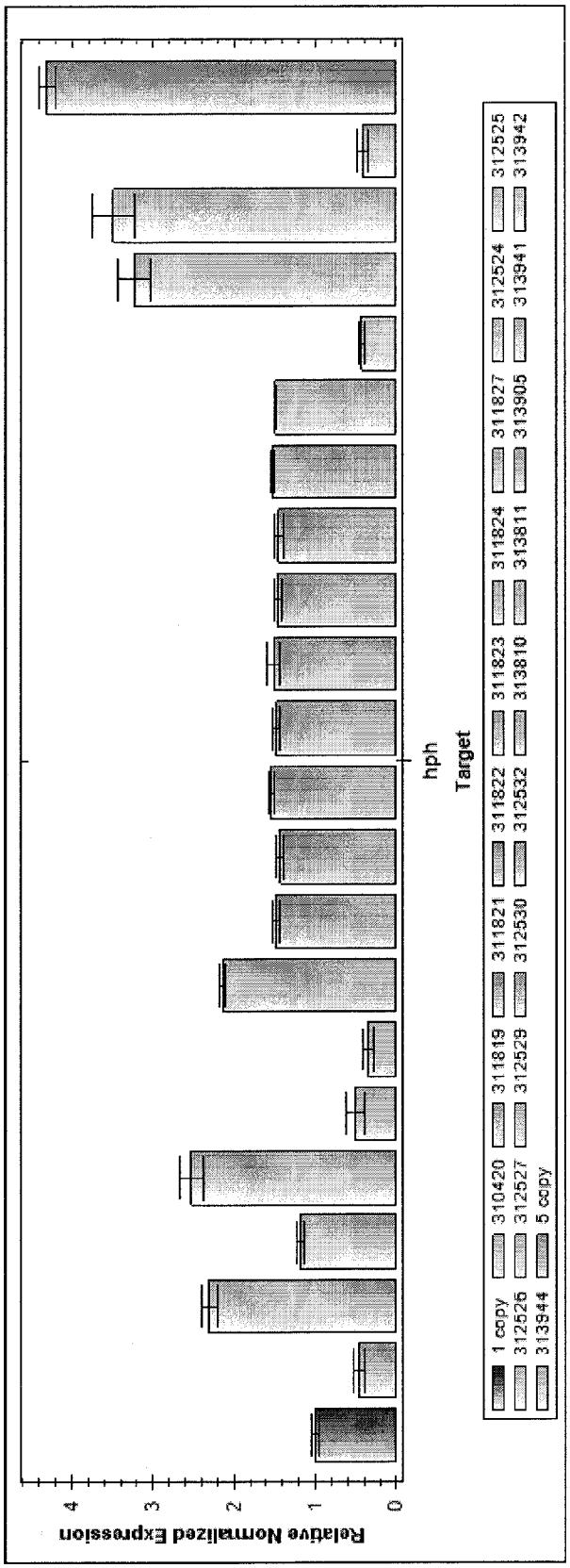

Relative Normalized Expression
0
1
2
3
4
hph
Target
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FIG. 36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/058766

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/82(2006.01)i, C12Q 1/24(2006.01)i, A01H 5/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/82; C12N 5/14; A01H 5/00; C12Q 1/68; C12Q 1/24

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: plant protoplast, fluorescent marker, sodium alginate, encapsulation, microcalli, nuclease, homologous recombination

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BARGMANN, B. O. R. et al., `Fluorescence activated cell sorting of plant protoplasts` Journal of Visualized Experiments, 18 February 2010, Vol. 36, pp. 1-4. See abstract; page 1, line 18-35 and page 2, line 11-36; figures 1 and 3-4.	1-18
Y	US 2010-0257638 A1 (CAI, Q. et al.) 7 October 2010 See abstract; paragraphs [0289], [0294] and [0377]; claims 17-36 and 61-72.	1-17
A		18
Y	VIJAYALAXMI, G. et al., `Plant Regeneration from Protoplasts of Indica Rice cv tellahamsa` Proc. Indian Natn. Sci. Acad., 1997, Vol. 63, No. 6, pp. 631-638. See abstract; page 632, right column, line 11- page 633, right column, line 20; figures 1 and 3-4.	1-18
A	US 2003-0219763 A1 (SHEEN, J.) 27 November 2003 See abstract; claims 1, 6-7, 12, 16 and 21-24.	1-18
A	EP 2455454 A1 (UNIVERSITY OF ALBERT LUDWIGS FREIBURG) 23 May 2012 See abstract; claims 1 and 9-13	1-18

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 December 2013 (23.12.2013)	**23 December 2013 (23.12.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
HEO, Joo Hyung
Telephone No. +82-42-481-8150

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/058766

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2010-0257638 A1	07/10/2010	AR 062336 A1	29/10/2008
		AU 2007-284748 A1	21/02/2008
		AU 2007-284748 B2	16/05/2013
		CA 2660485 A1	21/02/2008
		CN 101528924 A	09/09/2009
		EP 2049663 A1	22/04/2009
		EP 2395081 A1	14/12/2011
		IL 196942 D0	01/08/2011
		JP 2010-500029 A	07/01/2010
		KR 10-2009-0054986 A	01/06/2009
		TW 200815593 A	01/04/2008
		WO 2008-021207 A2	21/02/2008
		ZA 200900787 A	26/05/2010
US 2003-0219763 A1	27/11/2003	US 2004-0181829 A1	16/09/2004
		US 6613959 B1	02/09/2003
		US 7084323 B1	01/08/2006
EP 2455454 A1	23/05/2012	WO 2012-066147 A1	24/05/2012

SEQUENCE LISTING

<110> Spangenberg, German
Sahab, Sareena
Mason, John

<120> ENGINEERED TRANSGENE INTEGRATION PLATFORM (ETIP)

<130> 2971-P10992.1US (72292-US-NP)

<160> 480

<170> PatentIn version 3.5

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tcagaaataa aaaatcgaat tccttttaac ttaataaata ttagattgtt ctttgtaaat 300

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gatgttttct taaaacctcg taatgataca ggttgaagta attaaaaaaa aattaagtaa 540

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aaaacctaac tattaaatgc tgaattttat gaagttataa cataaagtga ttaatgaaca 780

aacgtcttat caattaatta tgcttccgca aagcatcatg ttcattgccc tataaaatca 840

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ccacatcgga ttttgatatt tacgtcgaat ctcccgatga atctctattt gaagaagtcg 1080

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taaatgggaa gagatttcta cacagattag attaatgaac atgatatcag attcattgac 3420

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tacaaaattg cggattggtt ttgatgttca cctctagttg ttttgtctct tctacagagc 3900

aaagaggatg tcaatagaga ttggaccaag tatgatcatt acggcagcgc cactatgatc 3960

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gagtgatttc agaactcgag ttttagggga agctcgcgga gagaatctat gttgtggctt 4140

ctaagctcac cgataacagt ctttaccatg tctatgtgtt ttctaataac attgttgcta 4200

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1 5

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Trp Ser Ser Ser Leu Tyr Tyr
1 5

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Asn Ser Arg Asn Leu Arg Asn
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Asp Gln Ser Thr Leu Arg Asn
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Asp Arg Ser Asn Leu Ser Arg
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Asp Arg Ser Asn Leu Trp Arg
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Asp Arg Ser Ala Leu Ser Arg
1 5

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Arg Ser Asp Ala Leu Ala Arg
1 5

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Arg Ser Asp Ala Leu Ser Arg
1 5

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Asp Arg Ser Asp Leu Ser Arg
1 5

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Arg Ser Asp His Leu Thr Gln
1 5

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Gln Ser Gly Ala Leu Ala Arg
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Gln Ser Gly Asn Leu Ala Arg
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Asp Arg Ser Asn Leu Ser Arg
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Asp Ser Ser Ala Arg Asn Thr
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Asp Arg Ser Ser Arg Lys Arg
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Gln Ser Gly Asp Leu Thr Arg
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Leu Ala His His Leu Val Gln
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Arg Ser Asp Asn Leu Ser Thr
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His Ser His Ala Arg Ile Lys
1 5

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Thr Ser Gly His Leu Ser Arg
1 5

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Arg Ser Asp Asn Leu Ser Val
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Ile Arg Ser Thr Leu Arg Asp
1 5

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Thr Ser Gly His Leu Ser Arg
1 5

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Asp Arg Ser Asn Leu Ser Arg
1 5

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His Arg Ser Ser Leu Arg Arg
1 5

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Thr Ser Gly Asn Leu Thr Arg
1 5

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Met Ser His His Leu Arg Asp
1 5

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Arg Ser Asp Ala Leu Ser Arg
1 5

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Asp Arg Ser Asp Leu Ser Arg
1 5

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Arg Ser Asp His Leu Thr Gln
1 5

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Gln Ser Gly Ala Leu Ala Arg
1 5

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Gln Ser Gly Asn Leu Ala Arg
1 5

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Arg Ser Ala Val Leu Ser Glu
1 5

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<400> 373

Thr Asn Ser Asn Arg Ile Thr
1 5

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Leu Lys Gln His Leu Asn Glu
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Gln Ser Gly Ala Leu Ala Arg
1 5

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Gln Ser Gly Asn Leu Ala Arg
1 5

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Asp Arg Ser Asn Leu Ser Arg
1 5

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<211> 7
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Gln Ser Gly Asp Leu Thr Arg
1 5

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Gln Ser Gly Ala Leu Ala Arg
1 5

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Asp Arg Ser Asn Leu Ser Arg
1 5

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Gln Arg Thr His Leu Thr Gln
1 5

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Arg Ser Asp Asn Leu Ser Asn
1 5

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Thr Asn Ser Asn Arg Ile Lys
1 5

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Gln Ser Ser Asp Leu Ser Arg
1 5

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Gln Ser Ser Asp Leu Arg Arg
1 5

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Asp Arg Ser Asn Arg Ile Lys
1 5

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Arg Ser Ala Asn Leu Ala Arg
1 5

<210> 388
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Arg Ser Asp Asn Leu Thr Thr
1 5

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Gln Ser Gly Glu Leu Ile Asn
1 5

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Arg Ser Ala Asp Leu Ser Arg
1 5

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<400> 391

Arg Ser Asp Asn Leu Ser Glu
1 5

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Asp Arg Ser His Leu Ala Arg
1 5

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Asp Arg Ser His Leu Ala Arg
1 5

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Arg Ser Asp Asn Leu Ser Glu
1 5

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Ser Lys Gln Tyr Leu Ile Lys
1 5

<210> 396
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Glu Arg Gly Thr Leu Ala Arg
1 5

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Arg Ser Asp His Leu Thr Thr
1 5

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Gln Ser Gly Ala Leu Ala Arg
1 5

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Gln Ser Gly Asn Leu Ala Arg
1 5

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Asp Arg Ser His Leu Ala Arg
1 5

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Asp Arg Ser Asp Leu Ser Arg
1 5

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Arg Ser Asp Asn Leu Thr Arg
1 5

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Asp Arg Ser His Leu Ala Arg
1 5

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Arg Ser Asp Asn Leu Ser Glu
1 5

<210> 405
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Ser Lys Gln Tyr Leu Ile Lys
1 5

<210> 406
<211> 7

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Glu Arg Gly Thr Leu Ala Arg
1 5

<210> 407
<211> 7
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<400> 407

Arg Ser Asp His Leu Thr Thr
1 5

<210> 408
<211> 7
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Asp Arg Ser Asp Leu Ser Arg
1 5

<210> 409
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Arg Ser Asp Asn Leu Thr Arg
1 5

<210> 410
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Gln Arg Thr His Leu Thr Gln
1 5

<210> 411
<211> 7
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Arg Ser Asp Asn Leu Ser Glu
1 5

<210> 412
<211> 7
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Ala Ser Lys Thr Arg Lys Asn
1 5

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Arg Ser Asp Thr Leu Ser Glu
1 5

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<211> 7
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Gln Ser His Asn Arg Thr Lys
1 5

<210> 415
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Gln Ser Asp His Leu Thr Gln
1 5

<210> 416
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Arg Ser Ser Asp Leu Ser Arg
1 5

<210> 417
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Gln Ser Ser Asp Leu Ser Arg
1 5

<210> 418
<211> 7
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<220>
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<400> 418

Arg Ser Asp His Leu Thr Gln
1 5

<210> 419
<211> 7
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<400> 419

Arg Ser Asp Ser Leu Ser Arg
1 5

<210> 420
<211> 7
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<220>
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<400> 420

Arg Lys Asp Ala Arg Ile Thr
1 5

<210> 421
<211> 7
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Asp Arg Ser His Leu Ser Arg
1 5

<210> 422
<211> 7
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Gln Ser Gly Asn Leu Ala Arg
1 5

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<211> 7
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<220>
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Gln Ser Ser Asp Leu Ser Arg
1 5

<210> 424
<211> 7

<212> PRT
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<220>
<223> 24832 Finger 6

<400> 424

Asp Arg Ser Ala Leu Ala Arg
1 5

<210> 425
<211> 7
<212> PRT
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<220>
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<400> 425

Arg Ser Asp Asp Leu Ser Lys
1 5

<210> 426
<211> 7
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<400> 426

Arg Ser Asp Thr Arg Lys Thr
1 5

<210> 427
<211> 7
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Asp Arg Ser Asn Leu Ser Arg
1 5

<210> 428
<211> 7
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<220>
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Asp Arg Ser Asn Leu Trp Arg
1 5

<210> 429
<211> 7
<212> PRT
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<220>
<223> 24833 Finger 5

<400> 429

Arg Ser Asp Ser Leu Ser Arg
1 5

<210> 430
<211> 7
<212> PRT
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<223> 24833 Finger 6

<400> 430

Asn Asn Asp His Arg Lys Thr
1 5

<210> 431
<211> 694
<212> DNA
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<220>
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ttaaggagag aaactttgct gattaattca aattagtgat cattaagatt ccaaagattc 120

cgatttagaa aagtcaaaga ttcaaagaac aagtctaggt cctcatggct catgttgcat 180

ccgattcacc atccactcat ctttcatatc ttcctccact gtctctctag aaacaactca 240

tttaatttag aaaactcctt tttcaattta gaaatattaa agtttatcac aatgtatcaa 300

ttaaatatta tccgatgact cattcatagt caggaccttg ctgtctgtgt cgtccgtaat 360

tattatttca atacaaaaca aatatatgtt cactcagaaa attacggcgc aatcatctaa 420

ttttgtggac caaaataaat agcgtagctt cgagatttca aagttgtgtt caaatttaat 480

tttgatttcc gttcctcgta tactctttta tgtatagaaa ataataatat ccactactag 540

tagttgataa ctacattaca tatattaaaa ttatgatgtc acattgcgga cgtttttaat 600

gtactgaatt aacgccgaat tgaattcgag ctcggtaccc ggggatcctc tagagtcgac 660

ctgcaggcat gcaagcttag cttgagcttg gatc 694

<210> 432
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<212> DNA
<213> artificial sequence

<220>
<223> em02-5788-1-1 Left border flanking

<400> 432
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tccctcatta accagatctg gccggcctac ccagctttct tgtacacata gcgaccgagc 180

tcgagccgaa gttcggtcgc tgtttcactg tttgggaaag catcagtaac gcagaagaca 240

taattaaaaa ttaattatat atggtagttt ttctagattc tccactatac ctcattgtga 300

ttgaaaaaca actatatata tatatatata tgtatttaaa attaagaaat cattaaatcg 360

taccataatg cagaaaactt tataagttcc tattcttttg tcaagatgag tagatgacac 420

atcatgtacc acataacata accataatgg tgcgatacca taccaaaact taatttagaa 480

actaattaaa attttggtag tttaagattc ctcctaatgg ttgtcaaaaa aaaaaaaaga 540

ttcctcctaa tacatggtag aatatatttt tgagttaaaa tgaaagtaaa atcttcaaat 600

tagtcataag gaaattctaa aaaacatcga ctttctttat aaagatccca ttgtaatttt 660

agatgattaa ttttatccca atccaattaa gaattgtaca catcggcctc tatatatatc 720

aaacacctaa aa 732

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<211> 643
<212> DNA
<213> artificial sequence

<220>
<223> Event details: ad58-5784-2-1 Left border flanking

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aaactttatc agcctgtgta gtgattattt caatttgata aagaaaaaaa aaggcttagc 120

tttatttggt tttttgttac aatcttgatt aattttagat tagcactctg attctagcgg 180

aacatgagag tggttccatc aaacctcaga cagtgagcac agtggttgca gcaaaccatt 240

tgggtgagag ctcttcagtt tctttgctat tagctggttc tggctcttct cttcaagaag 300

ctgcttctca agctgcctct tgtcatcctt ctgtttccga ggtaactaac tacattcttc 360

atttgtcctt ttttcttgtg ggtcttaaat gttygtgctt ttctttatag gtacttgttg 420

ctgattcaga tagatttgag tacccttag ctgaaccttg ggctaaactg gttgactttg 480

ttcgccaaca aagagattac tctcacatcc ttgccttcct ctagctcatt tggcaagaac 540

atacttcctc tagacaactt aataacacat tgcggacgtt tttaatgtac tgaattaacg 600

ccgaattgaa ttcgagctcg gtacccgggg atcctctaga gtc 643

<210> 434
<211> 653
<212> DNA
<213> artificial sequence

<220>
<223> ad58-5784-2-1 Right border flanking

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tacttgaagg catggagtat aagccatgtt cctttcagag gggactgtac ttctgtagat 120

tactttccct cattaaccag atctggccgg cctacccagc tttcttgtac aaagtggtga 180

taaactatcg ccggcctacc tcgcgttgct gctcttttag atgtctctcc tgttactgat 240

gttgtcaaaa tcttaggatc caatgagttt atcaggtata cttctatcat gtattgcttg 300

agattttgga gtgttagtaa agatttcaat aaaagaattt tttcaaacaa aattttgggg 360

gcttgaagct aatgtttgga aatatgtaac ggagtttaaa tcttttggca ggcctatata 420

tgcgggaaac gccttgtgta gagttcgcta tactggtgct ggtccttgtg tgttgactat 480

tagaactaca tcttttcctg ttaccccaat aactgagtca aagaaagcta ctatctctca 540

gattgatctc tcgaaattca aagaaggttt gtttagtatt attctcttgt gcatagcctt 600

tttgcttttt ttttttttat aaaaaaagtt gagtatgctt attgcccatt gca 653

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<211> 450
<212> DNA
<213> artificial sequence

<220>
<223> Event details: ad58-5898-10-1 Left border flanking

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ggttaggaat aaaataaaga acgtggacat cgtggaataa aaatattcag acaaggaaac 120

taacaataaa acagtaatga acatggttct gaatctcatc tttgtgtatc tccaatggaa 180

tccaccgcca cgaatcagac tccttctcca agctccaccg tcgacgatga caatggcgac 240

ggtgtctata ccgacgaatt taccaaacta cccccggacc cggggatcct ctagagtcaa 300

caaattgacg cttagacaac ttaataacac attgcggacg tttttaatgt actgaattaa 360

cgccgaattg aattcgagct cggtacccgg ggatcctcta gagtcgacct gcaggcatgc 420

aagcttagct tgagcttgga tcagattgtc 450

<210> 436
<211> 644
<212> DNA
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<220>
<223> ad58-5898-10-1 Right border flanking

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aaatttgatt tttcattaca taataatcca ttaattttca cgcacgtgga acccatttgg 120

tgtacttcca cgtcctctaa gagagcactg acccactcat caaaaagata catctttata 180

agccccttca tcgtcacaca gacacacact tccctctcaa ttattccata ttctcctaat 240

ttctaattgt tacacctcaa cacattagat tcgtaccaaa caaaaacgat tagctcccaa 300

gcctaagctt ttatttcctt ataatttttc ttgggtttct ctctataaag aatgcaaatg 360

actgagagag gccgagccat gtggcacacg tccctagcct cggcattccg cacagctcta 420

gcttgcacaa tcgttggtgc ggctacgctc tacggacccg agtggatcct ccgttttgtg 480

gcattcccgg cgttttctta cgtcacggtc attctcatca ttacggacgc cacgctaggc 540

gacacactac gtggctgctg gctagccctt tacgccacat gtcagagcgt tgcaccggct 600

atcattacac taaggcttat aggaccagct cggctcacgg ccgg 644

<210> 437
<211> 674
<212> DNA
<213> artificial sequence

<220>
<223> 1f31-6139-2-3 Left border flanking

<400> 437
tttctttcca tcagttcttc ggcaccttcc tggctctgcg tctatctttc tttccatccc 60

ggcccatctc gtacacattc cacccaacta cacaaacacg ttatagtctt ttacattatg 120

accaaatcaa ccctaaagat acagccttta taaaaaatat aggggtcaaa gcaaagaaga 180

gaaagtttgc ttacagtgtg gagaaaagaa gttggaagat gaggagtaag aagaagaaga 240

gaagagaaag ggtcttctga tgaggaatag gagataaggt ggaactggaa tgtttgccgc 300

taaatacttg aagacaagag cttgattttc aagctcttcc cattgtgact cagtgaaagg 360

gatcctagtc gtcatgaaga gatagagaaa attgatgatg aaggaggtct gatgaagaga 420

agagagagag agagatatta cacaaagagg gttgtattgc aaaattgaaa gtgtagagag 480

agtagtaggt aagtttttat taataatgtt gttcacacct gcagtctgca gaatattgtg 540

gtgtgaacaa attgacgctt agacaactta ataacacatt gcggacgttt ttaatgtact 600

gaattaacgc cgaattgaat tcgagctcgg tacccgggga tcctctagag tcgacctgca 660

ggcatgcaag ctta 674

<210> 438
<211> 544
<212> DNA
<213> artificial sequence

<220>
<223> [00213] lf31-6139-2-3 Right border flanking

<400> 438
ttcaatctac ttgaaggcat ggagtataag ccatgttcct ttcagagggg actgtacttc 60

tgtagattac tttccctcat taaccagatc tggccggcct acccagcttt cttgtacaaa 120

gtggtgataa actatcagtg tttgaacata tatatacgca taatattctc agaacccgac 180

ccattggttg actcggatca agatcgaccc gatccgaccc ggttaaaagc acgtcgtctc 240

ctttggttcg ccccttatat tcgacgagtg agttcgattg gatcgctgtt tgtcatttct 300

cactacctta agaaaaaaaa aaaggtgcgt ctctctctca cctttaccgc tcacttacct 360

ctcagatctg acatcgattt tccaaatctt ctccaggtac tctctctcct ggccgttgac 420

ggtcccgtcc cggccgtgga tctgatttcg ccgcgatctg aggtcaagca tggcggcagc 480

taacgccccc atcacgatga aggaggtcct aacggtgagt cccgcctcca tttttagtaa 540

cata 544

<210> 439
<211> 569
<212> DNA
<213> artificial sequence

<220>
<223> bm56-6315-1-1 Left border flanking

<400> 439
tacatcgcga ttcatcctgg tttgattaga atgacgagga agttgtcata ttcccaaaca 60

ggaaaattgg gatcgcctta tttgaaagtg ggataacttc ttcatcttaa ttcttatgag 120

aattattcca cttcctggtg attctccact actttttgta tataaataca gcttcttaca 180

tcgcgattca tcctgatttg attagaatga cgagaaagtt gtcatattcc caaacaggaa 240

aactgggatc acctgatttg aaagtgggat aacttcttca tcctaattct tatgagattt 300

attccacttc ctggtgattc tccacttctt tatgtatcca aatacatctt cttacatcgc 360

gattcatcct ggtttgatta gaatgacgag gaagttgtca tattcccaaa catgaaaact 420

gggaaagtgg gattgacgct tagacaactt aataacacat tgcggacgtt tttaatgtac 480

tgaattaacg ccgaattgaa ttcgagctcg gtacccgggg atcctctaga gtcgacctgc 540

aggcatgcaa gcttagcttg agcttggat 569

<210> 440
<211> 685
<212> DNA
<213> artificial sequence

<220>
<223> bm56-6315-1-1 Right border flanking

<400> 440
ccaccatctc aaacaataga acttcctaag tgaagcaatg acttcaaatc tacttgaagg 60

catggagtat aagccatgtt cctttcagag gggactgtac ttctgtagat tactttccct 120

cattaaccag atctggccgg cctacccagc tttcttgtac aaagtgacga taaactatca 180

gtctttcaaa gcgcatctat ggctagtcat cacggttttt aactgtttta cgaagtcgcg 240

gagaggctcg tcttctccct aggataaact gcagaggtcg acatcggagg tttctcgatc 300

tatgaacata gagtactgtt tgagaaactc cgaggcgagt tggcggaaac tccctataga 360

gtttttcttt agacaagaga accactcaag cgctgcttcg cggaggttct cgacgaagag 420

gcggcattcg ccggcatctc tttctctatc tttaaacttg gctcttccca tcgcgatctg 480

gaaagcctgc aagtgtgcct taggatcggt tgtaccatcg taaggagcca ctttgacttt 540

atcaggatcc gaaaccgtag tttcggtgat gcgggtggtg aagggcgttt ttcgagactc 600

ctcgaggagt agggtgatat cgagtgcagt actagtagcg tgatgatttg ggatttcacc 660

gctctaacct ccgcagccgt tttca 685

<210> 441
<211> 734
<212> DNA
<213> artificial sequence

<220>
<223> ad58-6372-1-1 Left border flanking

<400> 441
ttttacagtg ttagaagaag tggatgaagc tgaaattgaa ttttcaaact cgttcagctt 60

gactagaggt gggagagagt caaagcctcc catcaagtac caaaacatgg aatagaagac 120

agtccgaggg agaggaaacc gtggccgacg aggccgtggc tcctatcatt aactagtgtc 180

tttcttacta ttaatggttt attaagtctc agcctttgtt gtttcattgg tttgagattc 240

actcatacat gaaacttgtt tcattccagc ttttccaaac tataagaata tttccaatct 300

tatcttgtaa tagtttaagt tttaaattga aagcccttag ttcaaaaaac aaaaaaaaaa 360

aattagccct tgaatttata tataatcacg acggccatat ttggcagcta cactgatatg 420

ttttcaattg gctgacaggc cttgagcagg gtttgctggg tatattggta ggaagatgtg 480

ttgcgaggtt gaagcctcat ttaggcaata taaacatgat cattagcgtt atgtcattag 540

ttatacttat acgtagacta agtaacccac taaaggttgc tgattccttt tgtatcgact 600

aacacattgc ggacgttttt aatgtactga attaacgccg aattgaattc gagctcggta 660

cccggggatc ctctagagtc gacctgcagg catgcaagct tagcttgagc ttggatcaga 720

ttgtcgtttc ccgc 734

<210> 442
<211> 521
<212> DNA
<213> artificial sequence

<220>
<223> ad58-6372-1-1 Right border flanking

<400> 442
catgttcctt tcagagggga ctgtacttct gtagattact ttccctcatt aaccagatct 60

ggccggccta cccagctttc ttgtacaaag tggtgataaa ctatcagtgt ttgactgaat 120

tttaatttct aatttttgta aaaaatttgt ataacctcaa attattaaaa ggcggatttt 180

attagaatta taactaaatt atctataact ccaaaatttt gacaatcaat catgtctata 240

tctttatttt tttgctaaat tatcatgtct atatcttttc tttcttccaa acttacttga 300

gactaaaagt ctttataaat tttgatagga gttccacaca caaacaaaaa caaaacaaat 360

atttttcatc aagggatact tatttaacat cacggattca cagtttatta acaaaaatcc 420

aaacaaagac tgaaagacag aagattcaat ctaacaatag tcggcaaaca ccagtgatta 480

actaacgaaa taaattaaca agtggtcaga tcttcgggaa a 521

<210> 443
<211> 450
<212> DNA
<213> artificial sequence

<220>
<223> ad58-6620-4-1 Left border flanking

<400> 443
taatttcatt ttcgtcattt tggtaaagta acaaaagaca gaatattggt tagtcgtgtt 60

ggttaggaat aaaataaaga acgtggacat cgtggaataa aaatattcag acaaggaaac 120

taacaataaa acagtaatga acatggttct gaatctcatc tttgtgtatc tccaatggaa 180

tccaccgcca cgaatcagac tccttctcca agctccaccg tcgacgatga caatggcgac 240

ggtgtctata ccgacgaatt taccaaacta cccccggacc cggggatcct ctagagtcaa 300

caaattgacg cttagacaac ttaataacac attgcggacg tttttaatgt actgaattaa 360

cgccgaattg aattcgagct cggtacccgg ggatcctcta gagtcgacct gcaggcatgc 420

aagcttagct tgagcttgga tcagattgtc 450

<210> 444
<211> 775
<212> DNA
<213> artificial sequence

<220>
<223> ad58-6620-4-1 Right border flanking

<400> 444
cataaccacc atctcaaaca atagaacttc ctaagtgaag caatgacttc aaatctactt 60

gaaggcatgg agtataagcc atgttccttt cagaggggac tgtacttctg tagattactt 120

tccctcatta accagatctg gccggcctac ccagctttct tgtacaaagt ggtgataaac 180

tatcagtgtt tgaaataatc ggatatttaa ttttcttaga cagttcatta gtagttgatc 240

ttaaacattc acgttttatt ttcttttctt ttcgaatgct agactctagt ttggtaccca 300

taggattcga gttacatgaa gctattgaca ctgggagtgc ttcaagatct gtaagaggca 360

aagattcaca aacagaacgt gatttcttgg atagtgatgt ggagattgtg ataagaacca 420

gcatgagtat tacttttact gcccctgctg tggtgaagac atcaccaaaa cagtcaagct 480

cgtgaagaaa tcagatattc aacccgcaaa aaaatctgac aatgcaaata aacctattga 540

cactaagaat ggttcaagat ccgaagacaa gaagacaaaa aatttgtcct ggctccctgc 600

ttatctccag aagctgtttc tttctgttta tggccacatc aaagacaaag gtacccttc 660

ttttggtgcc tttggtgtgg acaggtgtga ttgactttgg tttcttggca gattcaggca 720

agatagaggt tgattcaaag tcaactaaca atgatcttgg taccaatagt gagga 775

<210> 445
<211> 808
<212> DNA
<213> artificial sequence

<220>
<223> ad58-6620-17-1 Left border flanking

<400> 445
ccgccttgaa caaccgctcc gccgttgctc agtacattat cgaggttacc aaaaagttca 60

atcctttatg cttgttttgg cgtgtgattc gttacgaatg agtaaaattg atttggtttt 120

tttctttgaa cagcatggtg gagatgtgaa tgcgacggat catacggggc agactgcgtt 180

gcattggagt gcggttcgtg gtgcggtgca agttgcggag cttttgcttc aagagggtgc 240

aagggttgat gctacggata tgtatggata tcaggttcta accacttcct ctttctttgt 300

ggagattgtc tttttgtttc aatgctagtc aacttctttc tttctttcac aaaactaagt 360

agtattgctt gttttgttgt cgttgcattt tttcttatgg ctgtgttctg aggttcatgt 420

aggtatataa gcacttcgta ctttgccact tgtttcattt aggcaacatt gtgcacatat 480

ctaagtagtt ggtctttgta aaattagttt gtttgtcttc aaagtatatt gagcagtttc 540

atgactcatt attcaaaggt ttgtctaaat tagagagaac tttcattttg cctggattta 600

atcagcattt agaatgttta tagcgatatc atttttagtt gaaaaaatct caacaaattg 660

acgcttagac aacttaataa cacattgcgg acgtttttaa tgtactgaat taacgccgaa 720

ttgaattcga gctcggtacc cggggatcct ctagagtcga cctgcaggca tgcaagctta 780

gcttgagctt ggatcagatt gtcgtttc 808

<210> 446
<211> 578
<212> DNA
<213> artificial sequence

<220>
<223> ad58-6620-17-1 Right border flanking

<400> 446
gtataagcca tgttcctttc agaggggact gtacttctgt agattacttt ccctcattaa 60

ccagatctgg ccggcctacc cagctttctt gtacaaagtg gtgataaact atcagtgtta 120

gatccccgac cgaccgccca tcctggacgg cctcgtgcat gctgatgttg tcaaaatctt 180

aggatccaat gagtttatca ggtatacttc tatcatgtat tgcttgagat tttggagtgt 240

tagtaaagat ttcaataaaa gaatttttc aaacaaaatt ttgggggctt gaagctaatg 300

tttggaaata tgtaacggag tttaaatctt ttggcaggcc tatatatgcg ggaaacgcct 360

tgtgtagagt tcgctatact ggtgctggtc cttgtgtgtt gactattaga actacatctt 420

ttcctgttac cccaataact gagtcaaaga aagctactat ctctcagatt gatctctcga 480

aattcaaaga aggtttgttt agtattattc tcttgtgcat agccttttg stttttttt 540

ttttataaaa aaagttgagt atgcttattg cccattgc 578

<210> 447
<211> 24
<212> DNA
<213> artificial sequence

<220>
<223> primer sequence

<400> 447
cttacatgct taggatcgga cttg 24

<210> 448
<211> 22
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 448
agttccagca ccagatctaa cg 22

<210> 449
<211> 24
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 449
ccctgagccc aagcagcatc atcg 24

<210> 450
<211> 18
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 450
cggagagggc gtggaagg 18

<210> 451
<211> 22
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 451
ttcgatttgc tacagcgtca ac 22

<210> 452
<211> 22
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 452
aggcaccatc gcaggcttcg ct 22

<210> 453
<211> 22
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 453
cttccactcc ttcctcctcg tc 22

<210> 454
<211> 21
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 454
gcgtcccaaa gggttgttga g 21

<210> 455
<211> 22
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 455
tctctactgg gcctgccagg gc 22

<210> 456
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> primer

<400> 456
ccccgagacg ttgaaggcta agtacaaa 28

<210> 457
<211> 24
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 457
ttgcgctgac ggattctaca agga 24

<210> 458
<211> 24
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 458
tccatcagtc caaacagcag caga 24

<210> 459
<211> 23

<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 459
catagcagtc tcacgtcctg gtc 23

<210> 460
<211> 26
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 460
ggaagctaag ccattacact gttcag 26

<210> 461
<211> 23
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 461
catagcagtc tcacgtcctg gtc 23

<210> 462
<211> 20
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 462
cctgatccgt tgacctgcag 20

<210> 463
<211> 20
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 463
gtgtgaggtg gctaggcatc 20

<210> 464
<211> 26
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 464
ggaagctaag ccattacact gttcag 26

<210> 465
<211> 22
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 465
agagaggaga cagagagaga gt 22

<210> 466
<211> 23
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 466
agacagcatc aagatttcac aca 23

<210> 467
<211> 21
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 467
caacggcgag cgtaatctta g 21

<210> 468
<211> 22
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 468
gttccctgga attgctgata gg 22

<210> 469
<211> 20
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 469
tgttggtgga agaggatacg 20

<210> 470
<211> 18
<212> DNA
<213> artificial sequence

<220>
<223> primer

<400> 470
atcagcagca gcgatagc 18

<210> 471
<211> 118
<212> DNA
<213> brassica napus

<400> 471
gacatcaagt ggtacggcaa gtacctcaac aacccgctag gacgcacggt gatgctaacc 60

gtccagttca agctcggctg gccgttgtac ttagccttca acgtctcggg aagacctt 118

<210> 472
<211> 118
<212> DNA
<213> brassica napus

<400> 472
gacatcaagt ggtacggcaa gtacctcaac aacccgctag gacgcacggt gatgctaacc 60

gtccagttca cgctcggctg gccgttgtac ttagccttca acgtctctgg aagacctt 118

<210> 473
<211> 118
<212> DNA
<213> brassica napus

<400> 473
gacatcaagt ggtacggcaa gtacctcaac aacccctttgg gacgcacggt gatgttaacg 60

gttcagttca ctctcggctg gcctttgtac ttagccttca acgtctcggg aagacctt 118

<210> 474
<211> 118
<212> DNA
<213> brassica napus

<400> 474
gacatcaagt ggtacggcaa gtacctcaac aacccctttgg gacgcacggt gatgttaacg 60

gttcagttca ctctcggctg gccgttgtac ttagccttca acgtctcggg aagacctt 118

<210> 475
<211> 20
<212> DNA

<213> brassica napus

<400> 475
cagacatcaa gtggtacggc 20

<210> 476
<211> 28
<212> DNA
<213> brassica napus

<400> 476
acggcaagta cctcaacaac cctttggg 28

<210> 477
<211> 28
<212> DNA
<213> brassica napus

<400> 477
cgcaccgtga tgttaacggt tcagttca 28

<210> 478
<211> 28
<212> DNA
<213> brassica napus

<400> 478
cggcaagtac ctcaacaacc ctttggga 28

<210> 479
<211> 28
<212> DNA
<213> brassica napus

<400> 479
accgtgatgt taacggttca gttcactc 28

<210> 480
<211> 20
<212> DNA
<213> brassica napus

<400> 480
cttagcttca acgtctcggg 20

摘要

描述了一种工程化转基因整合平台（ETIP），它能在植物基因组中随机地或在靶定位置处插入，从而推动快速选择和检测完美靶定（3’和5’端二者）在ETIP基因组位置处的GOI。本发明中的一个要件是在ETIP内引入特定双链断裂。在一些实施方案中，描述了一种ETIP，它使用锌指核酸酶结合位点，但是可利用其它靶向技术，诸如大范围核酸酶、TAL、CRISPR、或亮氨酸拉链。还描述了转基因植物的组合物和用于生成转基因植物的方法，其中供体或有效载荷DNA表达稳定整合入植物细胞中ETIP的外源核酸序列的一种或多种产物（例如蛋白质或RNA）。在多个实施方案中，从构思贯穿开发阶段，ETIP推动测试基因候选和植物表达载体。