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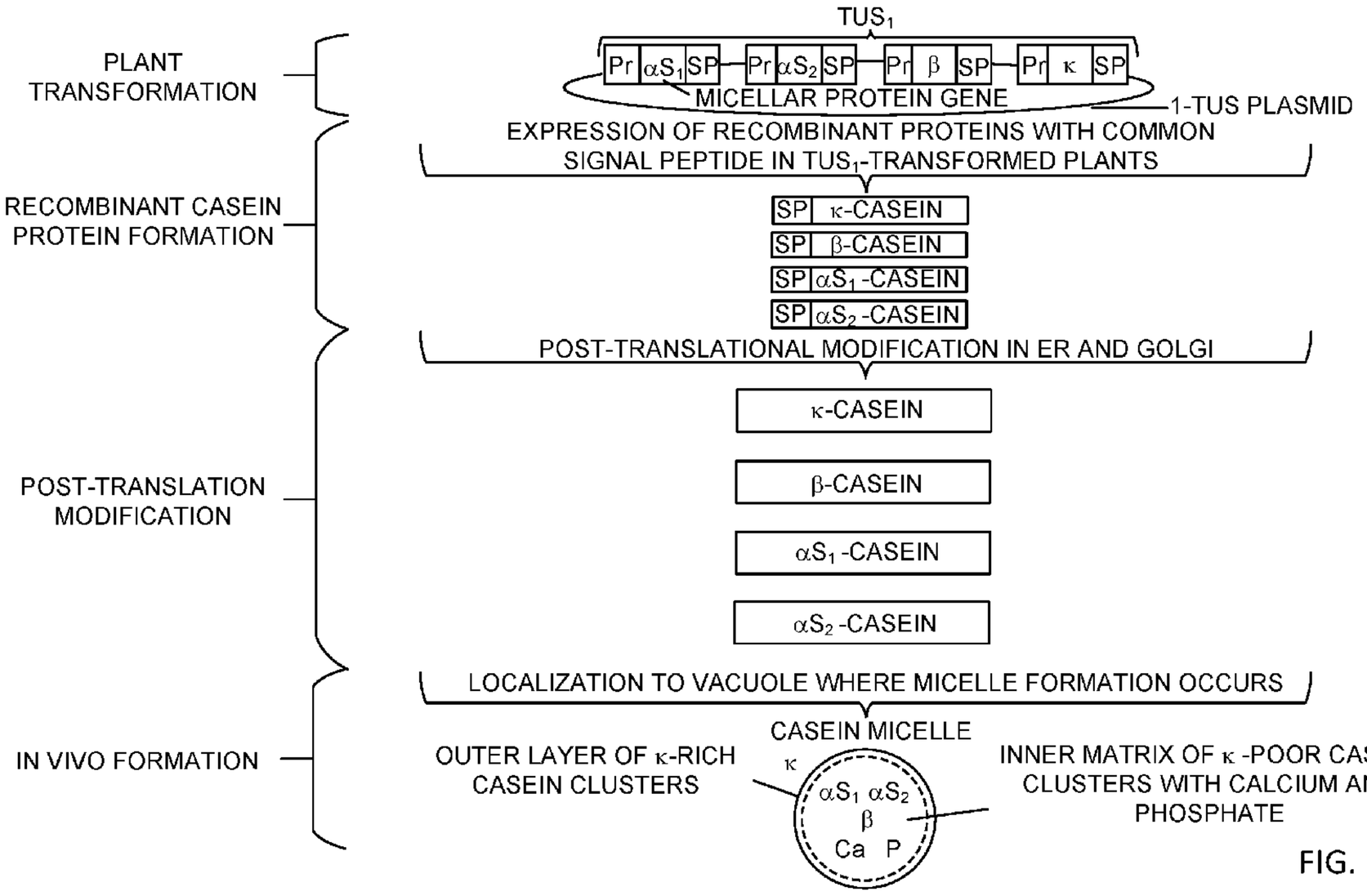


FIG. 1

(57) Abstract: A method of in vivo assembly of a recombinant micelle including: introducing a plasmid into a plant cell, wherein: the plasmid includes a segment of deoxyribonucleic acid (DNA) for encoding a ribonucleic acid (RNA) for a protein in a casein micelle, the segment of DNA is transcribed and translated; forming recombinant casein proteins in the plant cell, wherein: the recombinant casein proteins include a κ-casein and at least one of an αS_1-casein, an αS_2-casein, a β-casein; and assembling in vivo a recombinant micelle within the plant cell, wherein: an outer layer of the recombinant micelle is enriched with the κ-casein, an inner matrix of the recombinant micelle include at least one of the αS_1-casein, the αS_2-casein, the β-casein.

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RECOMBINANT MICELLE AND METHOD OF IN VIVO ASSEMBLY

REFERENCE TO A SEQUENCE LISTING SUBMITTED VIA EFS-WEB

[0001] The content of the ASCII text file of the sequence listing named "0107-001P-PCT_Sequence_Listing.txt", which is 31 kb in size was created on 11 January 2020 and electronically submitted via EFS-Web for corresponding US application number 16/741,680 on 13 January 2020 and also electronically submitted via EFS-Web for this International PCT Application on 20 October 2020, is incorporated herein by reference in its entirety.

CROSS REFERENCE TO RELATED APPLICATION(S)

[0002] This application claims the benefit of U.S. Provisional Patent Application serial number 62/939,247 filed November 22, 2019 and U.S. Non Provisional Patent Application serial number 16/741,680 filed January 13, 2020, and the subject matter thereof are incorporated herein by reference thereto.

TECHNICAL FIELD

[0003] An embodiment of the present disclosure relates generally to a micelle and more particularly to recombinant micelle and method of in vivo assembly in a plant cell.

BACKGROUND

[0004] Casein micelles account for more than 80% of the protein in bovine milk and are a key component of all dairy cheeses. Casein micelles include individual casein proteins are produced in the mammary glands of bovines and other ruminants. The industrial scale production of the milk that is processed to yield these casein micelles, primarily in the form of curds for cheese production, typically occurs on large-scale dairy farms and is often inefficient, damaging to the environment, and harmful to the animals. Dairy cows contribute substantially to greenhouse gasses, consume significantly more water than the milk they produce, and commonly suffer from dehorning, disbudding, mastitis, routine forced insemination, and bobby calf slaughter.

[0005] Accordingly, there is a need for an in vivo plant-based casein expression system which allows for purification of biologically active casein proteins that is cost effective at industrial scale.

[0006] Solutions to these problems have been long sought but prior developments have not taught or suggested any solutions and, thus, solutions to these problems have long eluded those skilled in the art.

DISCLOSURE OF INVENTION

[0007] An embodiment of the present invention provides a method of in vivo assembly of a recombinant micelle including: introducing a plasmid into a plant cell, wherein: the plasmid includes a segment of deoxyribonucleic acid (DNA) for encoding a ribonucleic acid (RNA) for a protein in a casein micelle, the segment of DNA is transcribed and translated; forming recombinant casein proteins in the plant cell, wherein: the recombinant casein proteins include a κ-casein and at least one of an αS_1-casein, an αS_2-casein, a β-casein; and assembling in vivo a recombinant micelle within the plant cell, wherein: an outer layer of the recombinant micelle is enriched with the κ-casein, an inner matrix of the recombinant micelle include at least one of the αS_1-casein, the αS_2-casein, the β-casein.

[0008] An embodiment of the present invention provides a recombinant micelle including: an outer layer enriched with a κ-casein; and an inner matrix including at least one of a αS_1-casein, a αS_2-casein, a β-casein.

[0009] An embodiment of the present invention provides a plasmid including a segment of deoxyribonucleic acid (DNA) for encoding a protein in a casein micelle wherein the segment of DNA includes a promoter and a N-terminal signal peptide.

[0010] Certain embodiments of the disclosure have other steps or elements in addition to or in place of those mentioned above. The steps or elements will become apparent to those skilled in the art from a reading of the following detailed description when taken with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] FIG. 1 is an example of a flow for forming in vivo casein micelles in an embodiment.

[0012] FIG. 2 is an example of a schematic illustration of a plasmid used in FIG. 1.

[0013] FIG. 3A is an example with additional details from the plant transformation to the post-translation modification.

[0014] FIG. 3B is an example with additional details for the in vivo formation.

[0015] FIG. 3C is an example of a schematic illustration of a transcription of proteins which impart herbicide resistance to the transformed plant.

[0016] FIG. 3D is an example of a schematic illustration of suppression of native seed storage proteins by RNAi transcribed by a portion of the plasmid of FIG. 1.

[0017] FIG. 3E is an example of a schematic illustration of a transcription of a portion of the plasmid of FIG. 1 and resulting proteins used to increase calcium concentrations in the plant cell.

[0018] FIG. 3F is an example of a schematic illustration of a transcription of a portion of the plasmid of FIG. 1 and resulting proteins used to increase phosphate concentrations in the plant cell.

[0019] FIG. 3G is an example with further additional details of the in vivo formation.

[0020] FIG. 4 is an example of a schematic illustration of a portion of a plasmid in Arabidopsis.

[0021] FIG. 5 is an example of a schematic illustration of a portion of a plasmid in Arabidopsis for a screenable marker in plants.

[0022] FIG. 6 is an example of a schematic illustration of a portion of a plasmid in soybean.

[0023] FIG. 7 is an example of a schematic illustration of a portion of a plasmid in soybean for herbicide resistance in plants.

[0024] FIG. 8 is an example of a schematic illustration of a portion of the plasmid of FIG. 1 for soybean for suppression of native seed storage proteins in plants.

[0025] FIG. 9 is an example of a schematic illustration of a portion of a plasmid for soybean to regulate intracellular concentrations of minerals which can enhance micelle formation.

[0026] FIG. 10 is an example of a flow for the purification of micelles formed in vivo in soybean.

DETAILED DESCRIPTION

[0027] The following embodiments are described in sufficient detail to enable those skilled in the art to make and use the invention. It is to be understood that other embodiments would be evident based on the present disclosure, and that system, process, or mechanical changes may be made without departing from the scope of an embodiment of the present disclosure.

[0028] In the following description, numerous specific details are given to provide a thorough understanding of the invention. However, it will be apparent that the invention may be practiced without these specific details. In order to avoid obscuring an embodiment of the present disclosure, some well-known techniques, system configurations, and process steps are not disclosed in detail.

[0029] The drawings showing embodiments of the system are semi-diagrammatic, and not to scale and, particularly, some of the dimensions are for the clarity of presentation and are shown

exaggerated in the drawing figures. Similarly, although the views in the drawings for ease of description generally show similar orientations, this depiction in the figures is arbitrary for the most part. Generally, the invention can be operated in any orientation.

[0030] The term "invention" or "present invention" as used herein is not meant to be limiting to any one specific embodiment of the invention but applies generally to any and all embodiments of the invention as described in the claims and specification.

[0031] Referring now to FIG. 1, therein is shown an example of a flow for forming in vivo casein micelles in an embodiment. In this example, FIG. 1 depicts the flow for forming the casein micelles by a plant transformation, a recombinant casein protein formation, a post-translation modification, and an in-vivo formation. As a specific example, FIG. 1 is a schematic illustration of the elements of a plasmid of this embodiment and its use in creation of micelles in vivo in a plant cell.

[0032] In this example for the plant transformation, a plant is transformed using a plasmid including a single transcription unit set. As used herein "plasmid " is a deoxyribonucleic acid (DNA) molecule capable of replication in a host cell and to which another DNA segment can be operatively linked so as to bring about replication of the attached DNA segment. As it relates to this example, methods for plant transformation include microprojectile bombardment as illustrated in U.S. Pat. Nos. 5,015,580; 5,550,318; 5,538,880; 6,153,812; 6,160,208; 6,288,312 and 6,399,861, all of which are incorporated herein by reference. Methods for plant transformation also include Agrobacterium-mediated transformation as illustrated in U.S. Pat. Nos. 5,159,135; 5,824,877; 5,591,616 and 6,384,301, all of which are incorporated herein by reference. Recipient cells for the plant transformation include, but are not limited to, meristem cells, callus, immature embryos, hypocotyls explants, cotyledon explants, leaf explants, and gametic cells such as microspores, pollen, sperm and egg cells, and any cell from which a fertile plant may be regenerated, as described in U.S. Pat. Nos. 6,194,636; 6,232,526; 6,541,682 and 6,603,061 and U.S. Patent Application publication US 2004/0216189 A1, all of which are incorporated herein by reference.

[0033] Continuing this example for the plant transformation, the plasmid including the single transcription unit set is shown and abbreviated in FIG. 1 as 1-TUS PLASMID. The transcription unit set included on this plasmid is transcription unit set 1 shown and abbreviated in FIG. 1 as TUS_1. As used herein "transcription unit set" is a segment of DNA including one or more transcription units. The purpose of a transcription unit set includes but is not limited to protein expression, gene suppression, regulatory ribonucleic acid (RNA) production, and herbicide resistance. As used herein "transcription unit" is a segment of DNA including at least a promoter

DNA and transcribable DNA. As used herein "promoter" means regulatory DNA for initiating RNA transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells whether or not its origin is a plant cell. As used herein "terminator" means any DNA sequence that causes RNA transcription to terminate.

[0034] Further continuing this example for the plant transformation shown in FIG.1 as an embodiment, the transcription unit set 1 includes four segments of DNA; each encoding RNA for one of the four proteins found in a casein micelle: an αS_1 casein, an αS_2 casein, a β casein, and a κ casein. For clarity and as an example, the genes encoding the αS_1 casein, the αS_2 casein, the β casein, and the κ casein are shown and abbreviated in FIG. 1 as αS_1, as αS_2, as β, and as κ, respectively, and shown and annotated in FIG. 1 as MICELLAR PROTEIN GENE. Each DNA segment encoding RNA for one of the four proteins found in a casein micelle is operably linked to a promoter, shown and abbreviated in FIG. 1 as P, and includes a plant-derived, tissue specific, N-terminal signal peptide, shown and abbreviated in FIG. 1 as SP. As used herein "operably linked" is the association of two or more DNA fragments in a DNA construct such that the function of one is controlled by the other, for example DNA encoding a protein associated with DNA encoding a promoter. In some embodiments, the N-terminal signal peptide targets the recombinant casein proteins to the plant vacuoles. In other embodiments, the recombinant casein proteins are targeted to and retained in the endoplasmic reticulum.

[0035] As an example for the recombinant casein protein formation, when the four segments of DNA included in transcription unit set 1 are transcribed and translated in a transgenic plant (not shown), four recombinant casein proteins, each including a plant-derived tissue specific signal peptide, are formed in the cytoplasm of the plant cell. The recombinant casein proteins are shown and abbreviated in FIG. 1 as αS_1-CASEIN, as αS_2-CASEIN, as β-CASEIN, and as κ-CASEIN, respectively, and are also referred to herein as "recombinant casein proteins" for brevity. As used herein, "transgenic" plant is a plant whose genome has been altered by the stable integration of recombinant DNA. As an example of stable integration, the transgenic plant includes a plant regenerated from an originally-transformed plant cell and progeny transgenic plants from later generations or crosses of a transformed plant. As used herein "recombinant DNA" refers to DNA which has been synthesized, assembled or constructed outside of a cell. Examples of recombinant DNA can include DNA containing naturally occurring DNA or complementary DNA (cDNA) or synthetic DNA.

[0036] As it relates to this example for the post-translation modification shown in FIG. 1 as an embodiment, the four recombinant casein proteins in the cytoplasm of the plant cell include the αS_1-casein, the αS_2-casein, the β-casein, and the κ-casein, each including a signal peptide (SP)

that localizes the recombinant casein protein to specific organelles, for example the secretory pathway and protein storage vacuoles, in the plant cell. The signal peptide is removed from the recombinant casein proteins during post-translational modification that occurs in the endoplasmic reticulum and the Golgi apparatus of the plant cell. For clarity in this example, the endoplasmic reticulum and the Golgi apparatus are shown and abbreviated in FIG. 1 as ER, and as GOLGI, respectively. In this embodiment and example, phosphorylation occurs on the recombinant casein proteins prior to, during, or after migration to a specific tissue, shown in FIG. 1 as circles enclosing the letter "P" attached to each of the recombinant casein proteins. In other embodiments and examples, one or more post-translational modifications of the recombinant casein proteins can occur, including phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis. In other embodiments no post-translational modifications occur on the recombinant casein proteins, or in other words, the post-translation modification is optional.

[0037] Continuing this example for the in vivo formation as an embodiment, an outer layer of the micelle is enriched in recombinant κ-casein shown and abbreviated in FIG. 1 as κ, and an inner matrix of the micelle includes the recombinant αS_1-casein, the recombinant αS_2-casein, the recombinant β-casein, the calcium and the phosphate, shown in and annotated in FIG. 1 as αS_1, αS_2, as αS, and β, respectively. Micelle formation is enhanced by the presence of intracellular calcium and phosphate, shown and abbreviated in FIG. 1 as Ca and P, respectively.

[0038] Referring now to FIG. 2, therein is shown an example of a schematic illustration of a plasmid used in FIG. 1. As a specific example, FIG. 2 is a schematic illustration of the elements of a plasmid of this embodiment.

[0039] In this example for the plant transformation of FIG. 1, an embodiment provides a plant that is transformed with one or more transfer DNAs including one or more transcription unit sets. As used herein "transfer DNA" (T-DNA) is DNA which integrates or is integrated into a genome.

[0040] For example, an Agrobacterium-mediated transformation T-DNA is part of a binary plasmid, which is flanked by T-DNA borders, and the binary plasmid is transferred into an Agrobacterium tumefaciens strain carrying a disarmed tumor inducing plasmid. Also for example, for a biolistic mediated transformation a gene gun is used for delivery of T-DNA, which is typically a biolistic construct containing promoter and terminator sequences, reporter genes, and border sequences or signaling peptides, to cells.

[0041] Continuing the example of a T-DNA used to transform a plant in an embodiment, the T-DNA includes four transcription unit sets: a transcription unit set 1, a transcription unit set 2, a

transcription unit set 3, and a transcription unit set 4. For clarity, the transcription unit set 1, the transcription unit set 2, the transcription unit set 3, and the transcription unit set 4 are shown and abbreviated in FIG. 2 as TUS_1, as TUS_2, as TUS_3, and as TUS_4, respectively.

[0042] In this example as an embodiment, TUS_1 includes one transcription unit for each of the four casein proteins found in a casein micelle of FIG. 2: a transcription unit 1-1 includes DNA encoding αS_1-casein, a transcription unit 1-2 includes DNA encoding β-casein, a transcription unit 1-3 includes DNA encoding κ-casein, and a transcription unit 1-4 includes DNA encoding αS_1-casein. For clarity and brevity, the transcription unit 1-1, the transcription unit 1-2, the transcription unit 1-3, and the transcription unit 1-4 are shown and abbreviated in FIG. 2 as TU_{1-1}, as TU_{1-2}, as TU_{1-3}, and as TU_{1-4}, respectively. Each transcription unit in TUS_1 can also include DNA encoding the same plant-derived signal peptide. Additionally, each transcription unit in TUS_1 includes a promoter and a transcriptional terminator.

[0043] Continuing this example as an embodiment, TUS_2 includes one transcription unit, shown and abbreviated in FIG. 2 as TU_{2-1}, that includes a promoter, DNA encoding phosphinothricin acetyltransferase, and a transcriptional terminator. In other embodiments, TUS_2 can include one or more genes encoding a selectable marker that can impart herbicide or antibiotic resistance which enables the selection of transformed plants that produce micelles in vivo. Genes enabling selection of transformed plants include those conferring resistance to antibiotics, including as examples kanamycin, hygromycin B, gentamicin, and bleomycin. Genes enabling selection of transformed plants also include those conferring resistance to herbicides, including as examples a glyphosate herbicide, a phosphinothricin herbicide, an oxynil herbicide, an imidazolinone herbicide, a dinitroaniline herbicide, a pyridine herbicide, a sulfonylurea herbicide, a bialaphos herbicide, a sulfonamide herbicide, and a glufosinate herbicide. Examples of such selectable markers are illustrated in U.S. Pat. Nos. 5,550,318; 5,633,435; 5,780,708 and 6,118,047, all of which are incorporated herein by reference. In other embodiments, TUS_2 includes one or more genes expressing a screenable marker which enables the visual identification of transformed plants that produce micelles in vivo. Genes expressing a screenable marker include genes encoding a colored or fluorescent protein, including as examples luciferase or green fluorescent protein (U.S. Pat. No. 5,491,084, herein incorporated by reference), and genes expressing β-glucuronidase or uidA gene (U.S. Pat. No. 5,599,670, herein incorporated by reference) for which various chromogenic substrates are known. In some embodiments, each of the genes encoding a selectable or screenable marker are operably linked to an inducible promoter, such as for example a NOS promoter, or a tissue-specific promoter, such as for example a promoter from

the soybean a' subunit of β-conglycinin, such that the translation of the selectable or screenable markers can be regulated.

[0044] Continuing this example as an embodiment, TUS_3 includes two transcription units that yield untranslated RNA molecules that suppress native seed protein gene translation. The first transcription unit in TUS_3, a transcription unit 3-1, includes the sense strand, or coding strand, of DNA encoding soybean Glycinin1, and the antisense strand, or non-coding strand, of DNA encoding soybean Glycinin1 separated by the potato IV2 intron. For clarity and brevity, the transcription unit 3-1 the sense strand or coding strand of DNA encoding soybean Glycinin1, and the antisense strand or non-coding strand of DNA encoding soybean Glycinin1, the potato IV2 intron are shown and annotated in FIG. 2 as $TU_{3\text{-}1}$ as GY1 SENSE, as GY1 ANTISENSE, and as IV2 INTRON, respectively. The second transcription unit in TUS_3, a transcription unit 3-2 includes the sense strand, or coding strand, of DNA encoding β-conglycinin 1 and the antisense strand, or non-coding strand, of DNA encoding β-conglycinin 1 separated by the potato IV2 intron. For clarity and brevity, the transcription unit 3-2, the sense strand or coding strand of DNA encoding β-conglycinin 1, the antisense strand or non-coding strand of DNA encoding β-conglycinin 1, and the potato IV2 intron are shown and annotated in FIG. 2 as $TU_{3\text{-}2}$ as CG1 SENSE, as CG1 ANTISENSE, and as IV2 INTRON, respectively.

[0045] In other embodiments, TUS_3 includes other transcription units that yield untranslated RNA molecules that suppress native seed protein gene translation. As an example, in other embodiments, TUS_3 includes one transcription unit, a transcription unit 3-1, that includes a promoter from the soybean GY4 gene (SEQ ID NO:15), a miR319a microRNA from Arabidopsis thaliana that has been modified such that the homologous arms of the microRNA hairpin contain 21 nucleotide sequences matching a portion of the soybean GY1 gene sequence (SEQ ID NO:10), and a NOS transcriptional terminator (SEQ ID NO:35) (not shown).

[0046] Continuing this example as an embodiment, TUS_4 includes two transcription units that encode proteins which alter the intracellular environment in a manner that optimizes the production of micelles having requisite attributes including size, mineral content, protein content, protein distribution, and mass. The first transcription unit in TUS_4, a transcription unit 4-1 includes a promoter, DNA encoding oxalate decarboxylase, and a transcriptional terminator. For clarity and brevity, the transcription unit 4-1 is shown and abbreviated in FIG. 2 as $TU_{4\text{-}1}$. The second transcription unit in TUS_4, a transcription unit 4-2, includes a promoter, DNA encoding phytase, and a transcriptional terminator. For clarity and brevity, the transcription unit 4-2 is shown and abbreviated as $TU_{4\text{-}2}$. In this embodiment, transcription and translation of $TU_{4\text{-}1}$ yields an oxalate-degrading enzyme which increases the amount of free intracellular calcium available

for capture and inclusion during micelle formation. Also in this embodiment, transcription and translation of $TU_{4\text{-}2}$ yields a phytase enzyme which increases the amount of free intracellular phosphate available for capture and inclusion during micelle formation. In some embodiments, each of the genes encoding oxalate-degrading enzymes or phytase enzymes are operably linked to a constitutive promoter, tissue specific promoter or an inducible promoter, such as for example, a nopaline synthase promoter or a promoter from the soybean β-conglycinin gene, such that the translation of proteins which alter the intracellular environment can be regulated. In some embodiments, TUS_4 includes both a transcription unit 4-1 that increases the intracellular calcium concentration and a transcription unit 4-2 that increases the intracellular phosphate concentration. In other embodiments, TUS_4 includes only a transcription unit 4-1 that increases the intracellular calcium concentration. In other embodiments, TUS_4 includes only a transcription unit 4-2 that increases the intracellular phosphate concentration.

[0047] In other embodiments, TUS_4 includes transcription units that increase the intracellular calcium concentration by expressing an oxalate oxidase enzyme (not shown). As an example, in other embodiments, TUS_4 includes one transcription unit, a transcription unit 4-1, that includes a promoter from the soybean GY4 gene (SEQ ID NO:15), the coding sequence for the oxalate oxidase 1 coding sequence from wheat that has been codon optimized for expression in soybean (SEQ ID NO:9), and the NOS transcriptional terminator (SEQ ID NO:35) (not shown). In other embodiments, TUS_4 includes transcription units that increase the intracellular phosphate concentration by suppressing the expression of the soybean myo-inositol-3-phosphate synthase (MIPS1) gene. As an example, in other embodiments, TUS_4 includes one transcription unit, a transcription unit 4-2, that includes a promoter from the soybean GY4 gene (SEQ ID NO:15), a portion of the MIPS1 coding sequence lacking a start codon (SEQ ID NO:21), the IV2 intron from potato (SEQ ID NO:25), the antisense of the MIPS1 sequence (SEQ ID NO:22), and the NOS transcriptional terminator (SEQ ID NO:35) (not shown).

[0048] In some embodiments of the disclosure, transcription unit sets are assembled in numeric order. In other embodiments, transcription unit sets can be assembled in any order.

[0049] In some embodiments of the disclosure, the plant is transformed with a plasmid that contains transcription unit sets TUS_1, TUS_2, TUS_3, and TUS_4. In other embodiments of the disclosure, the plant is transformed with a plasmid that contains only transcription unit set TUS_1.

[0050] In some embodiments of the disclosure, the plant is transformed with a plasmid that contains transcription unit sets TUS_1, and TUS_2. In other embodiments of the disclosure, the plant is transformed with a plasmid that contains transcription unit sets TUS_1, TUS_2, and TUS_3.

In other embodiments of the disclosure, the plant is transformed with a plasmid that contains transcription unit sets TUS_1, TUS_2, and TUS_4.

[0051] In other embodiments of the disclosure, the plant is transformed with a plasmid that contains transcription unit sets TUS_1, and TUS_3. In other embodiments of the disclosure, the plant is transformed with a plasmid that contains transcription unit sets TUS_1, TUS_3, and TUS_4.

[0052] In other embodiments of the disclosure, the plant is transformed with a plasmid that contains transcription unit sets TUS_1, and TUS_4. In some embodiments of the disclosure, transgenic plants are prepared by crossing a first plant that has been transformed with a plasmid containing one or more transcription unit sets with a second untransformed plant. In other embodiments of the disclosure, transgenic plants are prepared by crossing a first plant that has been transformed with a plasmid containing one or more but not all transcription unit sets required for micelle formation in vivo with a second plant having one or more transcription unit sets, wherein at least one of the transcription unit sets is present in the second plant and not present in the first plant.

[0053] In some embodiments of the disclosure, transgenic plants are prepared by crossing a first plant that has been transformed with a plasmid containing one or more transcription unit sets enabling micelle formation in vivo with a second plant having another trait, such as herbicide resistance or pest resistance.

[0054] In some embodiments of the disclosure, transgenic plants are prepared by growing progeny generations of a plant that has been transformed with a plasmid containing one or more transcription unit sets enabling micelle formation in vivo. In other embodiments, transgenic plants are prepared by growing progeny generations of a transgenic plant produced by crossing one or more plants that have been transformed with a plasmid containing one or more transcription unit sets enabling micelle formation in vivo.

[0055] Further to this example shown in FIG. 2 as an embodiment, the promoters in the four transcription unit sets include the promoters of genes coding for soybean Glycinin1, soybean β-conglycinin1, soybean Glycinin4, soybean Bowman-Birk protease inhibitor, Agrobacterium nopaline synthase, soybean Glycinin5, soybean lectin, and soybean Glycinin3. For clarity and brevity, the promoters of genes coding for soybean Glycinin1 is shown and annotated in FIG. 2 as GY1 PROMOTER. Also for clarity and brevity, the soybean β-conglycinin1 is shown and annotated in FIG. 2 as CG1 promoter. Further for clarity and brevity, the soybean Glycinin4 is shown and annotated in FIG. 2 as GY4 promoter. Yet further for clarity and brevity, the Bowman-Birk protease inhibitor promoter is shown and annotated in FIG. 2 as D-II promoter. Yet further for clarity and brevity, the Agrobacterium nopaline synthase is shown and annotated

in FIG. 2 as NOS promoter. Yet further for clarity and brevity, the soybean Glycinin5 is shown and annotated in FIG. 2 as GY5 promoter. Yet further for clarity and brevity, the soybean lectin is shown and annotated in FIG. 2 as LE1 promoter. Yet further for clarity and brevity, the soybean Glycinin3 is shown and annotated in FIG. 2 as GY3 promoter.

[0056] In other embodiments and examples, promoters in one or more of the four transcription unit sets include a promoter capable of initiating transcription in plant cells whether or not an origin of the promoter is a plant cell. For example, Agrobacterium promoters are functional in plant cells. The promoters capable of initiating transcription in plant cells include promoters obtained from plants, plant viruses and bacteria such as Agrobacterium.

[0057] As specific examples of promoters under developmental control include promoters that preferentially initiate transcription in certain tissues, such as leaves, roots, or seeds. Such promoters are referred to as "tissue preferred". Also as specific examples of promoters that initiate transcription only in certain tissues are referred to as "tissue specific". Further as a specific example, a "cell type specific" promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. Yet further a specific example, an "inducible" or "repressible" promoter is a promoter which is under environmental control. Examples of environmental conditions that may affect transcription by inducible or repressible promoters include anaerobic conditions, or certain chemicals, or the presence of light. Tissue preferred, tissue specific, cell type specific, and inducible or repressible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter which is active under most conditions.

[0058] Returning to this example in FIG. 2 as an embodiment, the transcriptional terminators in the four transcription unit sets include the termination sequence of the nopaline synthase gene, shown and annotated in FIG. 2 as NOS terminator. In other embodiments, the transcriptional terminators in one or more of the four transcription unit sets includes transcriptional terminators from the native soybean Glycinin genes, or any other plant transcriptional terminators.

[0059] In this example as an embodiment, the T-DNA used to transform a plant also includes DNA encoding an origin of replication, a gene conferring antibiotic resistance, a right boundary for the T-DNA, and a left boundary for the T-DNA, shown and annotated in FIG. 2 as pUC origin, ampicillin resistance, RB, and LB, respectively. In this embodiment, the gene conferring antibiotic resistance is a gene conferring resistance to the antibiotic ampicillin. In other embodiments, the gene conferring antibiotic resistance is a gene conferring resistance to any other antibiotic, including kanamycin and chloramphenicol.

[0060] Referring now to FIG. 3A, therein is shown an example with additional details from the plant transformation to the post-translation modification. The plant transformation and the post-translation modification are also described in FIG. 1. The example depicted in FIG. 3A also depicts the recombinant casein protein formation, also described in FIG. 1. As a specific example, FIG. 3A schematically illustrates the transcription of casein proteins from genes in TUS_1 as well as post transcriptional alterations that occur as the proteins move towards their subcellular specific destination encoded by the common signal peptide.

[0061] In the example shown in FIG. 3A, the purpose of transcription unit set 1 is forming casein micelles in vivo in an embodiment. In this example, the plant transformation depicts a plant transformed using a T-DNA including four transcription unit sets shown and annotated in FIG. 3A as 4-TUS plasmid. The T-DNA includes transcription unit set 1, shown and abbreviated in FIG. 3A as TUS_1, which includes one transcription unit for each of the four casein proteins found in a casein micelle, with each transcription unit including DNA encoding the same plant-derived signal peptide, a promoter and a transcriptional terminator as described in FIG. 2. Upon transcription and translation of TUS_1 in the transgenic plant during the recombinant casein protein formation, the four recombinant casein proteins (αS_1-casein, αS_2-casein, β-casein, and κ-casein) are formed in the cytoplasm, each including a signal peptide that localizes the recombinant protein to a specific tissue, for example the secretory pathway and protein storage vacuoles, in the plant cell. In this example, the signal peptide is removed from the recombinant casein proteins during post-translational modification that occurs in the endoplasmic reticulum, abbreviated as ER, of the plant cell.

[0062] Continuing this example and embodiment for the post-translation modification, phosphorylation occurs on the recombinant casein proteins prior to, during, or after migration to a specific tissue. The phosphorylation is shown in FIG. 3A as circles enclosing the letter "P" that are added to and then attached to each of the recombinant casein proteins to form phosphorylated casein proteins. The phosphorylated casein proteins are then localized to the vacuole where micelle assembly occurs in vivo. In some embodiments, proteins encoded by TUS_2 transcription units (not shown) are also phosphorylated, glycosylated, or a combination thereof. In other embodiments, the casein proteins encoded by TUS_4 transcription units (not shown) are also phosphorylated or glycosylated or both. In other embodiments, no post-translational modifications occur to proteins encoded by TUS_1, TUS_2, TUS_3, or TUS_4 (not shown). As another example and embodiment, a kinase gene may optionally be included to generate a kinase protein that ensures phosphorylation of the casein proteins encoded by TUS_4 transcription units (not shown).

[0063] Referring now to FIG. 3B, therein is shown an example with additional details for the in vivo formation. The in vivo formation is also described in FIG. 1. As a specific example, FIG. 3B schematically illustrates the in vivo formation of recombinant micelles inside a plant cell.

[0064] Upon localization to the vacuole, each of the four recombinant casein proteins assemble with the other recombinant casein proteins to form micelles in vivo. In this example, the outer layer of the micelle is enriched in recombinant κ-casein shown and abbreviated in FIG. 3B as κ, and the inner matrix of the micelle includes recombinant αS_1-casein and αS_2-casein, shown and abbreviated as αS_1 and αS_2, respectively, in FIG. 3B, and β-casein, shown and abbreviated in FIG. 3B as β.

[0065] Referring now to FIG. 3C, therein is shown an example of a schematic illustration of a transcription of proteins which impart herbicide resistance to the transformed plant. FIG. 3C depicts an example of the purpose of transcription unit set 2 in an embodiment.

[0066] In this example, a plant is transformed using a T-DNA including four transcription unit sets shown and annotated in FIG. 3C as 4-TUS plasmid. The T-DNA includes transcription unit set 2, shown in FIG. 3C and abbreviated as TUS_2 which includes one transcription unit that includes DNA encoding phosphinothricin acetyltrasnferase that imparts herbicide resistance and allow for selection of transformed cells producing micelles, shown and abbreviated as AC-PT in FIG. 3C, and a promoter and a transcriptional terminator (not shown).

[0067] Referring now to FIG. 3D, therein is shown an example of a schematic illustration of suppression of native seed storage proteins by interference RNA (RNAi) transcribed by a portion of the plasmid of FIG. 1. As a specific example, FIG. 3D schematically illustrates suppression of native seed storage proteins by RNAi transcribed by one or more genes in TUS_3.

[0068] FIG. 3D depicts an example of the purpose of transcription unit set 3 in an embodiment. In this example, a plant is transformed using a T-DNA including four transcription unit sets, shown and annotated in FIG. 3D as 4-TUS plasmid. The T-DNA includes transcription unit set 3, shown and abbreviated in FIG. 3D as TUS_3, which includes one or more transcription units that yield untranslated RNA molecules that suppress native seed protein gene translation thereby freeing cellular resources to produce micelles in vivo. Transcription of the DNA in TUS_3 yields RNAi, shown and abbreviated in FIG. 3D as RNAi, that targets messenger RNA of native plant proteins or native plant peptides, shown and annotated in FIG. 3D as mRNA NATIVE PROTEIN, and suppresses the expression of those messenger RNAs through messenger RNA degradation such that the recombinant casein proteins encoded by TUS_1, described in FIG. 1, FIG. 3A, and FIG. 3B, can be translated at higher quantities, thereby yielding higher concentrations of micelles in vivo (not shown). In some embodiments, DNA encoding RNAi is

operably linked to a constitutive promoter or an inducible promoter (not shown), such as for example a nopaline synthase promoter or soybean α' subunit of β-conglycinin, such that the suppression of native seed protein gene translation by RNAi can be regulated.

[0069] Referring now to FIG. 3E and FIG. 3F, therein are shown examples of schematic illustrations of a transcription of a portion of the plasmid of FIG. 1 and resulting proteins used to alter the intracellular conditions of the plant cell. As specific examples, FIG. 3E and FIG. 3F schematically illustrate the transcription of TUS_4 genes and the resulting proteins used to alter the conditions in the cytoplasm of the cell.

[0070] FIG. 3E depicts an example of the purpose of transcription unit set 4 in an embodiment. In this example, a plant is transformed using a T-DNA including four transcription unit sets, shown and annotated in FIG. 3E as 4-TUS plasmid. The T-DNA includes transcription unit set 4, shown and abbreviated in FIG. 3E as TUS_4, which includes one or more transcription units that encode proteins which increase the concentration of intracellular minerals, including calcium and phosphate. In this example, TUS_4 includes one transcription unit, a $TU_{4\text{-}1}$, that includes a promoter, DNA encoding oxalate decarboxylase, and a transcriptional terminator (not shown). Transcription and translation of $TU_{4\text{-}1}$ yields the enzyme oxalate decarboxylase, shown and abbreviated in FIG. 3E as OD, that breaks down the calcium oxalate and increases calcium levels in the plant cell. The increased intracellular calcium enhances the formation of recombinant casein micelles in the plant cell (not shown).

[0071] FIG. 3F depicts an example of the purpose of transcription unit set 4 in an embodiment. In this example, a plant is transformed using a T-DNA including four transcription unit sets, shown and annotated in FIG. 3F as 4-TUS plasmid. The T-DNA includes transcription unit set 4, shown and abbreviated in FIG. 3F as TUS_4, which includes one or more transcription units that encode proteins which increase the concentration of intracellular minerals, including calcium and phosphate. In this example, TUS_4 includes one transcription unit, a $TU_{4\text{-}2}$, that includes a promoter, DNA encoding a phytase enzyme, and a transcriptional terminator (not shown). Transcription and translation of $TU_{4\text{-}2}$ yields the phytase enzyme, shown and abbreviated in FIG. 3F as PE, that breaks down the phytic acid and increases phosphate levels in the plant cell. The increased intracellular phosphate enhances the formation of recombinant casein micelles in the plant cell (not shown).

[0072] Referring now to FIG. 3G, therein is shown an example of further additional details of the in vivo formation. The in vivo formation is also described in FIG. 1, FIG. 3A, and FIG. 3B. As a specific example, FIG. 3G schematically illustrates the in vivo formation of recombinant micelles inside a plant cell.

[0073] In the example shown in FIG. 3G, the in vivo formation of recombinant micelles inside a plant cell in which the four micellar proteins are produced by transcription and translation of transcription unit set 1 as depicted and described in FIG. 3A. The levels of calcium in plant cell vacuoles is increased by the presence of oxalate decarboxylase produced by transcription and translation of transcription unit set 4 as depicted and described in FIG. 3E. In this example, the four casein proteins encoded by transcription unit 1 are phosphorylated and localized to the plant cell vacuole where the intracellular calcium and the intracellular phosphate enhances the formation of recombinant casein micelles in the plant cell vacuole.

[0074] Aspects of the disclosure can be further illustrated by a specific embodiment in which a casein micelle is assembled in vivo from its constituent proteins in Arabidopsis thaliana as further described in FIG. 4 and FIG. 5.

[0075] Referring now to FIG. 4, therein is shown an example of a schematic illustration of a portion of a plasmid in Arabidopsis. The example shown in FIG. 4 is also described in FIG. 2. As a specific example, FIG. 4 schematically illustrates elements of plasmids that encode micellar component proteins. Transcription units depicted are components of TUS_1 in Arabidopsis.

[0076] The example in FIG. 4 depicts a transcription unit set which can be used for creation of casein micelles in vivo in Arabidopsis thaliana. The transcription unit set includes one transcription unit for each of the four casein proteins found in a casein micelle, abbreviated and shown in FIG. 4 as $TU_{1\text{-}1}$, $TU_{1\text{-}2}$, $TU_{1\text{-}3}$, and $TU_{1\text{-}4}$. The transcription unit set abbreviated and shown in FIG. 4 as TUS_1. Each of the four transcription units includes a promoter, a plant-derived N-terminal signal peptide, DNA encoding one of the four proteins found in a casein micelle, and a transcriptional terminator.

[0077] Continuing this example, $TU_{1\text{-}1}$ includes a double 35S promoter containing the tobacco mosaic virus omega leader sequence (SEQ ID NO:29), a signal peptide from the Arabidopsis CLV3 gene (SEQ ID NO:27), the αS_1-casein coding sequence codon optimized for expression in Arabidopsis with a C-terminal HDEL peptide for retention in the endoplasmic reticulum (SEQ ID NO:5), and the nopaline synthase terminator (SEQ ID NO:35), annotated and shown in FIG. 4 as 2X35S promoter + TMVΩ, signal peptide, αS_1 casein, and NOS terminator, respectively.

[0078] Further continuing this example, $TU_{1\text{-}2}$ includes a 35S short promoter containing a truncated version of the cauliflower mosaic virus promoter and the tobacco mosaic virus omega leader sequence (SEQ ID NO:31), a signal peptide (SEQ ID NO:27), the β-casein coding sequence codon optimized for expression in Arabidopsis with a C-terminal HDEL peptide for retention in the endoplasmic reticulum (SEQ ID NO:7), and the nopaline synthase terminator

(SEQ ID NO:35), abbreviated and shown in FIG. 4 as 35S SHORT PROMOTER + TMVΩ, SIGNAL PEPTIDE, β-CASEIN, and NOS TERMINATOR, respectively.

[0079] Further continuing this example, $TU_{1\text{-}3}$ includes the mannopine synthase promoter from Agrobacterium tumefaciens (SEQ ID NO:32), a signal peptide (SEQ ID NO:27), the κ-casein coding sequence codon optimized for expression in Arabidopsis with a C-terminal HDEL peptide for retention in the endoplasmic reticulum (SEQ ID NO:6), and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 4 as MANNOPINE SYNTHASE PROMOTER (A. TUMEFACIENS), SIGNAL PEPTIDE, κ-CASEIN, and NOS TERMINATOR, respectively.

[0080] Further continuing this example, $TU_{1\text{-}4}$ includes the mannopine synthase promoter from Agrobacterium tumefaciens, a signal peptide (SEQ ID NO:32), the αS_2-casein coding sequence codon optimized for expression in Arabidopsis with a C-terminal HDEL peptide for retention in the endoplasmic reticulum (SEQ ID NO:8), and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 4 as MANNOPINE SYNTHASE PROMOTER (A. TUMEFACIENS), SIGNAL PEPTIDE, αS_2 CASEIN, and NOS TERMINATOR, respectively.

[0081] Referring now to FIG. 5, therein is shown an example of a schematic illustration of a portion of a plasmid in Arabidopsis for a screenable marker in plants. As a specific example, FIG. 5 schematically illustrates elements of plasmids that provide for a screenable marker in plants. Transcription units depicted are components of TUS_2 in Arabidopsis.

[0082] The example shown in FIG. 5 depicts a transcription unit set which can be used to identify plant cells that have been transformed. The transcription unit set, abbreviated and shown in FIG. 5 as TUS_2, includes a single transcription unit, abbreviated and shown in FIG. 5 as $TU_{2\text{-}1}$.

[0083] Continuing this example for a portion of the plant transformation shown in FIG. 5 as an embodiment, $TU_{2\text{-}1}$ includes the nopaline synthase constitutive promoter (SEQ ID NO:28), the enhanced green fluorescence protein coding sequence modified to enhance fluorescence brightness and codon optimized for expression in Arabidopsis (SEQ ID NO:33), and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 5 as NOS PROMOTER, EGFP, and NOS TERMINATOR, respectively.

[0084] As a specific example, subsequent steps in the plant transformation for creation of casein micelles in vivo in Arabidopsis thaliana, a plasmid including TUS_1 and TUS_2 can be introduced into Arabidopsis thaliana cotyledons using Agrobacterium tumefaciens and the FAST transient expression method. Seedlings are soaked in a solution containing Agrobacterium two days after germination which results in some cotyledon cells being transformed. Transformed Arabidopsis

cells can be identified as containing the T-DNA by observing fluorescence exhibited by the enhanced green fluorescence protein. Successfully transformed Arabidopsis cells display green fluorescence while unsuccessfully transformed cells show little or no green fluorescence.

[0085] Also as a specific example of the in vivo formation of micelles in Arabidopsis thaliana as an embodiment, immunogold labeling techniques can be used to identify the location and morphology of the casein micelles formed in vivo. For this example for the in vivo formation of micelles as an embodiment, embryonic tissue can be obtained from Arabidopsis thaliana that has been transformed with a plasmid including TUS_1, and optionally TUS_2, shown in FIG. 4 and FIG 5, respectively. The embryonic tissue can be treated with casein-specific antibodies using immunogold labeling techniques, and imaged with transmission electron microscopy to identify the location and morphology of the micelles formed in vivo. In tissue obtained from the transformed Arabidopsis thaliana, the casein micelles are visualized as gold-antibody labeled subcellular structures that range in size from 50nm to 600nm, which is similar to the size of bovine casein micelles. As a control, no casein micelles are visualized using immunogold labeling techniques in tissue obtained from untransformed Arabidopsis thaliana.

[0086] Continuing this specific example of the in vivo formation of micelles in Arabidopsis thaliana as an embodiment, protein extraction and high performance liquid chromatography (HPLC) analysis can be used to evaluate the protein composition of the casein micelles formed in vivo. In this example for the in vivo formation of micelles as an embodiment, embryonic tissue can be obtained from Arabidopsis thaliana that has been transformed with a plasmid including TUS_1, and optionally TUS_2, shown in FIG. 4 and FIG 5, respectively. Proteins extracted from the embryonic tissue can be separated using HPLC and detected by ultraviolet absorbance. Proteins extracted from the transformed Arabidopsis thaliana tissue and subjected to HPLC analysis show peaks associated with each four proteins found in a casein micelle, including αS_1 casein, αS_2 casein, β casein, and κ casein, that display retention times similar to those reported by Bordin et al. for each of the four casein proteins found in bovine casein micelles. As a control, proteins extracted from the untransformed Arabidopsis thaliana tissue and subjected to HPLC analysis do not show peaks associated with any of the four casein proteins.

[0087] Further continuing this specific example of the in vivo formation of micelles in Arabidopsis thaliana as an embodiment, the amount of each casein protein found in micelles formed in vivo can be quantified by measuring the area under the peaks produced upon HPLC analysis. Quantification of the peaks produced upon HPLC analysis of proteins extracted from transformed Arabidopsis thaliana produces measurements showing that αS_1 casein is the most abundant, followed by β casein as the next most abundant, then αS_2 casein and κ casein as the

least abundant casein proteins, which correlates to the relative abundances of each of the four casein proteins in bovine casein micelles as previously reported in the Handbook of Dairy Foods and Nutrition, Table 1.1.

[0088] Aspects of the disclosure can be further illustrated by a specific embodiment in which a casein micelle is assembled in vivo from its constituent proteins in soybean and further described in FIG. 6 through FIG. 9.

[0089] Referring now to FIG. 6, therein is shown an example of a schematic illustration of a portion of a plasmid in soybean. As a specific example, FIG. 6 schematically illustrates elements of plasmids that encode micellar component proteins. Transcription units depicted are components of TUS_1 in soybean.

[0090] In this example, FIG. 6 depicts a transcription unit set which can be used for creation of casein micelles in vivo in soybean. The transcription unit set includes one transcription unit for each of the four casein proteins found in a casein micelle, abbreviated and shown in FIG. 4 as $TU_{1\text{-}1}$, $TU_{1\text{-}2}$, $TU_{1\text{-}3}$, and $TU_{1\text{-}4}$. The first transcription unit set is abbreviated and shown in FIG. 6 as TUS_1. Each of the four transcription units includes a promoter, a plant-derived N-terminal signal peptide, DNA encoding one of the four proteins found in a casein micelle, and a transcriptional terminator.

[0091] Continuing this example for a portion of the plant transformation shown in FIG. 6 as an embodiment, $TU_{1\text{-}1}$ includes a promoter from the soybean glycinin GY1 gene (SEQ ID NO:13), a signal peptide (SEQ ID NO:26), the αS_1 casein coding sequence codon optimized for expression in soybean (SEQ ID NO:1), and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 6 as GY1 PROMOTER, SIGNAL PEPTIDE, αS_1 CASEIN, and NOS TERMINATOR, respectively.

[0092] Further continuing this example for a portion of the plant transformation shown in FIG. 6 as an embodiment, $TU_{1\text{-}2}$ includes the promoter from the soybean CG1 gene (SEQ ID NO:14), a signal peptide (SEQ ID NO:26), the β casein coding sequence codon optimized for expression in soybean (SEQ ID NO:3), and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 6 as CG1 PROMOTER, SIGNAL PEPTIDE, β CASEIN, and NOS TERMINATOR, respectively.

[0093] Further continuing this example for a portion of the plant transformation shown in FIG. 6 as an embodiment, $TU_{1\text{-}3}$ includes the promoter from the soybean glycinin GY4 gene (SEQ ID NO:15), a signal peptide (SEQ ID NO:26), the κ casein coding sequence codon optimized for expression in soybean (SEQ ID NO:2), and the nopaline synthase terminator (SEQ ID NO:35),

abbreviated and shown in FIG. 6 as GY4 PROMOTER, SIGNAL PEPTIDE, κ CASEIN, and NOS TERMINATOR, respectively.

[0094] Further continuing this example for a portion of the plant transformation shown in FIG. 6 as an embodiment, TU_{1-4} includes the promoter from the soybean D-II Bowman-Birk proteinase isoinhibitor gene (SEQ ID NO:16), a signal peptide (SEQ ID NO:26), the αS_2 casein coding sequence codon optimized for expression in soybean (SEQ ID NO:4), and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 6 as D-II PROMOTER, SIGNAL PEPTIDE, αS_2 CASEIN, and NOS TERMINATOR, respectively.

[0095] Referring now to FIG. 7, therein is shown an example of a schematic illustration of a portion of a plasmid in soybean for herbicide resistance in plants. As a specific example, FIG. 7 schematically illustrates elements of plasmids that provide for herbicide resistance in plants. Transcription units depicted are components of TUS_2 in soybean.

[0096] FIG. 7 is an example of a portion of the plant transformation that depicts a transcription unit set which can be used to select for plant cells that have been transformed. The transcription unit set abbreviated and shown in FIG. 7 as TUS_2 includes a single transcription unit abbreviated and shown in FIG. 7 as TU_{2-1}.

[0097] Continuing this example for a portion of the plant transformation shown in FIG. 7 as an embodiment, TU_{2-1} includes nopaline synthase promoter (SEQ ID NO:28), the phosphinothricin acetyltransferase coding sequence codon optimized for expression in soybean (SEQ ID NO:34) which confers resistance to the herbicide glufosinate, and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 7 as NOS PROMOTER, PHOSPHINOTHRICIN ACETYLTRANSFERASE, and NOS TERMINATOR, respectively.

[0098] Referring now to FIG. 8, therein is shown an example of a schematic illustration of a portion of a plasmid in soybean for suppression of native seed storage proteins in plants. As a specific example, FIG. 8 schematically illustrates elements of plasmids that provide for suppression of native seed storage proteins in plants. Transcription units depicted are components of TUS_3 in soybean.

[0099] FIG. 8 is an example of a portion of the plant transformation that depicts a transcription unit set which can be used for enhancing the creation of casein micelles in vivo in soybean. The third transcription unit set abbreviated and shown in FIG. 8 as TUS_3 includes two transcription units abbreviated and shown in FIG. 8 as TU_{3-1} and TU_{3-2}. The transcription of TU_{3-1} and TU_{3-2} produces RNA with a hairpin structure where the arms are homologous to a portion of a native soybean gene or gene family and are sufficient to cause down regulation of those native genes or gene families (not shown).

[0100] Continuing this example for a portion of the plant transformation shown in FIG. 8 as an embodiment, $TU_{3\text{-}1}$ includes a promoter from the soybean glycinin GY4 gene (SEQ ID NO:15), a portion of the soybean glycinin GY1 coding sequence that is lacking a start codon and is highly homologous among the glycinin gene family (SEQ ID NO:24), the potato IV2 intron (SEQ ID NO:25), the antisense of the soybean glycinin GY1 sequence (SEQ ID NO:17), and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 8 as GY4 PROMOTER, GY1 SENSE, IV2 INTRON, GY1 ANTISENSE, and NOS TERMINATOR, respectively.

[0101] Further continuing this example for a portion of the plant transformation shown in FIG. 8 as an embodiment, $TU_{3\text{-}2}$ includes a promoter from the soybean glycinin GY5 gene (SEQ ID NO:18), a portion of the soybean β-conglycinin 1 coding sequence that is lacking a start codon and is highly homologous among the β-conglycinin gene family (SEQ ID NO:19), the potato IV2 intron (SEQ ID NO:25), the antisense of the soybean β-conglycinin 1 sequence (SEQ ID NO:20), and the nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 8 as GY5 PROMOTER, CG1 SENSE, IV2 INTRON, CG1 ANTISENSE, and NOS TERMINATOR, respectively.

[0102] Referring now to FIG. 9, therein is shown an example of a schematic illustration of a portion of a plasmid in soybean for regulating cytoplasmic concentrations of minerals which can enhance micelle formation. As a specific example, FIG. 9 schematically illustrates elements of plasmids that regulate cytoplasmic concentrations of minerals which can enhance micelle formation. Transcription units depicted are components of TUS_4 in soybean.

[0103] FIG. 9 is an example of a portion of the plant transformation that depicts a transcription unit set which can be used for enhancing the creation of casein micelles in vivo in soybean. The fourth transcription unit set abbreviated and shown in FIG. 9 as TUS_4 includes two transcription units abbreviated and shown in FIG. 9 as $TU_{4\text{-}1}$ and $TU_{4\text{-}2}$. Proteins encoded by $TU_{4\text{-}1}$ and $TU_{4\text{-}2}$ alter the intracellular environment in a manner that optimizes the formation of micelles in vivo.

[0104] Continuing this example for a portion of the plant transformation shown in FIG. 9 as an embodiment, $TU_{4\text{-}1}$ includes a promoter from the soybean LE1 gene (SEQ ID NO:23), a coding sequence for oxalate decarboxylase from Flammulina velutipes codon optimized for expression in soybean (SEQ ID NO:12), and nopaline synthase terminator (SEQ ID NO:35), abbreviated and shown in FIG. 9 as LE1 PROMOTER, OXALATE DECARBOXYLASE CDS, and NOS TERMINATOR, respectively.

[0105] Further continuing this example for a portion of the plant transformation shown in FIG. 9 as an embodiment, $TU_{4\text{-}2}$ includes the glycinin GY3 promoter (SEQ ID NO:30), the coding sequence for a soybean phytase enzyme (SEQ ID NO:11), and the nopaline synthase terminator

(SEQ ID NO:35), abbreviated and shown in FIG. 9 as GY3 PROMOTER, PHYTASE, and NOS TERMINATOR, respectively.

[0106] In this example, subsequent steps in the plant transformation for creation of casein micelles in vivo in soybean, a plasmid including TUS_1, TUS_2, and optionally TUS_3, and optionally TUS_4, shown in FIG. 6, FIG. 7, FIG. 8, and FIG. 9, respectively, can be introduced into soybean callus using standard biolistic transformation methods. Transformed soybean plants can be selected on a medium containing glufosinate herbicide, and the genomes of transformed soybean plants can be screened for insertion of the plasmid using standard PCR mapping methods. Transformed soybean plants including TUS_1, TUS_2, and optionally TUS_3, and optionally TUS_4 in their genome can be transferred to a greenhouse for seed production.

[0107] In the example of the in vivo formation of micelles in soybean as an embodiment, immunogold labeling techniques can be used to identify the location and morphology of the casein micelles formed in vivo. As it relates to this example for the in vivo formation of micelles as an embodiment, tissue can be obtained from soybean plants that have been transformed with a plasmid including TUS_1, TUS_2, and optionally TUS_3, and optionally TUS_4, shown in FIG. 6, FIG. 7, FIG. 8, and FIG. 9, respectively. The tissue can be treated with casein-specific antibodies using standard immunogold labeling techniques, and imaged with transmission electron microscopy to identify the location and morphology of the micelles formed in vivo. In tissue obtained from the transformed soybean plants, the casein micelles are visualized as gold-antibody labeled subcellular structures that range in size from 50nm to 600nm, which is similar to the size of bovine casein micelles. As a control, no casein micelles are visualized using immunogold labeling techniques in tissue obtained from untransformed soybean plants.

[0108] Continuing this example of the in vivo formation of micelles in soybean as an embodiment, protein extraction and polyacrylamide gel electrophoresis analysis can be used to evaluate the protein composition of the casein micelles formed in vivo. For this example for the in vivo formation of micelles as an embodiment, tissue can be obtained from soybean plants that have been transformed with a plasmid including TUS_1, TUS_2, and optionally TUS_3, and optionally TUS_4, shown in FIG. 6, FIG. 7, FIG. 8, and FIG. 9, respectively. Proteins extracted from the transformed soybean plant tissue and subjected to polyacrylamide gel electrophoresis analysis show bands on the polyacrylamide gel corresponding in size to each of the four casein proteins found in a casein micelle, including αS_1 casein, αS_2 casein, β casein, and κ casein. As a control, proteins extracted from untransformed soybean plant tissue and subjected to polyacrylamide gel electrophoresis analysis do not show bands on the polyacrylamide gel corresponding to the four casein proteins.

[0109] Further continuing this example of the in vivo formation of micelles in soybean as an embodiment, protein extraction and HPLC analysis can be used to evaluate the protein composition of the casein micelles formed in vivo. For this example for the in vivo formation of micelles as an embodiment, tissue can be obtained from soybean plants that have been transformed with a plasmid including TUS_1, TUS_2, and optionally TUS_3, and optionally TUS_4, shown in FIG. 6, FIG. 7, FIG. 8, and FIG. 9, respectively. Proteins extracted from the transformed soybean plant tissue can be separated using HPLC and detected by ultraviolet absorbance. Proteins extracted from the transformed soybean plant tissue and subjected to HPLC analysis show peaks associated with each four proteins found in a casein micelle, including αS_1 casein, αS_2 casein, β casein, and κ casein, that display retention times similar to those reported by Bordin et al. for each of the four casein proteins found in bovine casein micelles. As a control, proteins extracted from the untransformed soybean plant tissue and subjected to HPLC analysis do not show peaks associated with the four casein proteins.

[0110] Further continuing this example of the in vivo formation of micelles in soybean as an embodiment, the amount of each casein protein found in micelles formed in vivo can be quantified by measuring the area under the peaks produced upon HPLC analysis. Quantification of the peaks produced upon HPLC analysis of proteins extracted from transformed soybean plant tissue produces measurements showing that αS_1 casein is the most abundant, followed by β casein as the next most abundant, then αS_2 casein and κ casein as the least abundant casein proteins, which correlates to the relative abundances of each of the four casein proteins in bovine casein micelles as previously reported in the Handbook of Dairy Foods and Nutrition, Table 1.1.

[0111] Further continuing this example of the in vivo formation of micelles in soybean as an embodiment, RNA analysis can be used to evaluate the suppression of native soybean seed genes during the formation of casein micelles in vivo. For this example for the in vivo formation of micelles as an embodiment, soybean plants that have been transformed with a plasmid including TUS_1, TUS_2, TUS_3, and optionally TUS_4, shown in FIG. 6, FIG. 7, FIG. 8, and FIG. 9, respectively, can be grown to the flowering stage in a greenhouse and soybean embryos removed from the flowering seed pods at 35 days using standard dissection techniques. The expression levels of native soybean seed genes can be analyzed using standard techniques for RNA extraction and sequencing. RNA analysis of the embryos from transformed soybean plants show at least a 10% reduction in the expression of one or more of the native soybean seed genes, including genes in the glycinin family (Glyma.03g163500, Glyma.19g164900, Glyma.10g037100, Glyma.13g123500, Glyma.19g164800) and genes in the β-conglycinin family (Glyma.10g246300, Glyma.20g148400, Glyma.20g148300, Glyma.20g146200,

Glyma.20g148200, Glyma.10g246500, Glyma.10g028300, Glyma.02g145700). As a control, RNA analysis of embryos from untransformed soybean plants do not show a reduction in the expression of native soybean seed genes.

[0112] Further continuing this example of the in vivo formation of micelles in soybean as an embodiment, commercially available assays and X-ray fluorescence techniques can be used to evaluate calcium oxalate levels during the formation of casein micelles in vivo. As it relates to this example for the in vivo formation of micelles as an embodiment, soybean plants that have been transformed with a plasmid including TUS_1, TUS_2, and optionally TUS_3, and TUS_4, shown in FIG. 6, FIG. 7, FIG. 8, and FIG. 9, respectively, can be grown to the flowering stage in a greenhouse and soybean embryos removed from the flowering seed pods at 27 days using standard dissection techniques. The oxalate concentration can be measured using commercially available assays, and the calcium concentration can be measured using X-ray fluorescence. Embryos from transformed soybean plants show at least a 5% reduction in oxalate concentration and at least a 4% increase in calcium concentration as compared to control embryos from untransformed soybean plants, indicating that embryos from transformed soybean plants have at least 4% more available calcium compared to embryos from untransformed soybean plants.

[0113] Further continuing this example of the in vivo formation of micelles in soybean as an embodiment, commercially available assays can be used to evaluate phosphate levels during the formation of casein micelles in vivo. As it relates to this example for the in vivo formation of micelles as an embodiment, soybean plants that have been transformed with a plasmid including TUS_1, TUS_2, and optionally TUS_3, and TUS_4, shown in FIG. 6, FIG. 7, FIG. 8, and FIG 9., respectively, can be grown to the flowering stage in a greenhouse and soybean embryos removed from the flowering seed pods at 27 days using standard dissection techniques. Embryos can be ground with a mortar and pestle, sonicated and centrifuged to produce a supernatant that can be tested for phosphatase levels using commercially available assays. Embryos from transformed soybean plants show at least a 5% increase in phosphatase levels as compared to control embryos from untransformed soybean plants, indicating that embryos from transformed soybean plants have at least 5% more available phosphate compared to embryos from untransformed soybean plants.

[0114] Aspects of the disclosure can be further illustrated by a specific embodiment in which micelles produced in vivo are purified as further described in FIG. 10.

[0115] Referring now to FIG. 10, therein is shown an example of a flow for the purification of micelles formed in vivo in soybean. Also, the flow in FIG. 10 is an example of isolating a recombinant micelle. Further in this example, FIG. 10 depicts a process where casein micelles

produced in soybeans are purified from the plant tissue in a way that the micelles are still functional after the purification. The input material for the purification process is dried soybeans harvested from plants that have been transformed with a plasmid containing all four transcription unit sets, TUS_1, TUS_2, TUS_3, and TUS_4, described in FIG. 6, FIG. 7, FIG. 8, and FIG. 9, respectively. The input material for the purification process is shown in FIG. 10 and depicted as a rectangle enclosing the word "SOYBEAN".

[0116] Continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, the hulls are removed from the dried soybeans in a series of steps including cleaning, cracking, and aspiration, shown in FIG. 10 and depicted as rectangles enclosing the words "CLEANING", "CRACKING" and "ASPIRATION". In this embodiment, the hulls do not contain useful amounts of casein micelles and are discarded, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the word "HULLS".

[0117] Further continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, the remaining material is flaked to increase the surface area and allow for faster aqueous or solvent infiltrations. The resulting flaked material is shown in FIG. 10 and depicted as a rectangle enclosing the words "FULL FAT FLAKES".

[0118] Further continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, the flaked material is then defatted with hexane using standard defatting equipment and solvent extraction techniques, shown in FIG. 10 and depicted as a rectangle enclosing the words "SOLVENT EXTRACTION". Defatting can occur using any standard hexane based solvent, followed by desolventizing using flash or vapor-based processes. The resulting oil is removed, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the words "CRUDE OIL", leaving behind the defatted flakes, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the words "DEFATTED SOY FLAKES".

[0119] Further continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, the defatted flakes are then mixed with water and wet milled, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the words "H2O WET MILLING". The milling process pulverizes the defatted flakes which releases the casein micelles and allows the micelles to come into contact with an aqueous medium. In addition to the milling process, the defatted flakes are also vigorously agitated to assist in the release of casein micelles into the water, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the word "AGITATION". The milling process and vigorous agitation of the defatted flakes yields a slurry where soybean material has been finely ground and many of the casein micelles have been released into suspension in the water (not shown). Additionally, many other proteins and

carbohydrates are also dissolved in the water (not shown). In some embodiments, wet milling is done using perforated disc or colloid continuous flow mills.

[0120] Further continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, the slurry is fed through a series of mesh screens to remove larger particles from the casein micelles, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the word "FILTRATION". In this embodiment, the slurry is first passed through a screen with 5mm sieve openings (not shown), and then is passed through a screen with 0.5mm sieve openings (not shown). The material trapped by the screens is discarded, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the words "ORGANIC MATERIAL".

[0121] Further continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, the remaining material in the slurry that passed through both screens is then sonicated to break up aggregates of casein micelles such that the majority of micelles are not contacting other micelles, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the word "SONICATION". In some embodiments, continuous flow sonication with multiple sonicators in parallel are used to maximize flow rates.

[0122] Further continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, after sonication the slurry is passed through a 2μm microfiltration unit to eliminate larger particles while allowing casein micelles to pass through, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the word "MICROFILTRATION". The material trapped by the microfiltration unit is discarded, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the words "LARGE PARTICLES". The remaining material that passed through the microfiltration unit is largely composed of casein micelles as well as dissolved proteins, salts and carbohydrates (not shown).

[0123] Further continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, the material that passed through the microfiltration unit is then processed with an ultrafiltration unit that allows dissolved molecules lower than 100nm in diameter to pass through while retaining casein micelles, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the words "ULTRAFILTRATION". In some embodiments, continuous flow ultrafiltration with multiple filters in parallel are used to maximize flow rates. The soluble proteins, salts and minerals that passed through the ultrafiltration unit are discarded, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the words "SOL. PROTEINS, SALTS, MINERALS".

[0124] Further continuing this example of the purification of micelles formed in vivo in soybean as an embodiment, the final output from this process is an aqueous liquid where the most

common component after water is casein micelles, shown in FIG. 10 and depicted as an arrow pointing to a rectangle enclosing the words "CONCENTRATED MICELLES". These micelles (not shown) retain their shape and function such that they can be used in downstream processes such as in making synthetic milk or cheese.

[0125] As additional examples for FIG. 10, a method of isolating recombinant micelles from a seed of a plant produced can include cleaning, and deshelling or dehulling seeds, flaking cleaned seeds to 0.005 – 0.02 inch thickness, solvent extraction of oil from the flake, desolventizing the flake without cooking and collecting the defatted, clean flake, separating micelles into an aqueous slurry by hydrating, agitating and wet milling the flake, passing the slurry through a series of mesh screens to remove particulate above 0.5mm in size and collecting the permeate, sonication of the permeate from previous step, microfiltration of the product from previous step to remove particulate above 2um in size, ultrafiltration of the permeate from previous step using a device that allows particles > 100nm in diameter to pass through in the ultrafiltration permeate, collecting the retentate of previous step which contains concentrated recombinant micelles.

[0126] Continuing with this example, the method of isolating recombinant micelles from a seed further includes centrifuging the retentate of a previous step to separate the micelles from the remainder of the retentate. Also the method continues from the ultrafiltration step to passing the slurry through an ultrafiltration device and collecting a permeate containing protein and other molecules and a retentate containing micelles and thereafter adding a diafiltration fluid to the retentate at substantially the same rate that the permeate is collected and passing said retentate through the ultrafiltration device. Yet further the method continues where the seed is milled from at least one plant selected from the group of plants consisting of maize, rice, sorghum, cowpeas, soybeans, cassava, coyam, sesame, peanuts, peas, cotton and yams.

[0127] The resulting method, process, apparatus, device, product, and system is cost-effective, highly versatile, and accurate, and can be implemented by adapting components for ready, efficient, and economical manufacturing, application, production, and utilization. Another important aspect of an embodiment of the present disclosure is that it valuably supports and services the historical trend of reducing costs, simplifying systems, and increasing yield.

[0128] These and other valuable aspects of the embodiments of the present disclosure consequently further the state of the technology to at least the next level. While the disclosure has been described in conjunction with a specific best mode, it is to be understood that many alternatives, modifications, and variations will be apparent to those skilled in the art in light of the descriptions herein. Accordingly, it is intended to embrace all such alternatives, modifications, and variations that fall within the scope of the included claims. All matters set

forth herein or shown in the accompanying drawings are to be interpreted in an illustrative and non-limiting sense.

What is claimed is:

1. A method of in vivo assembly of a recombinant micelle comprising:
introducing a plasmid into a plant cell, wherein:
the plasmid includes a segment of deoxyribonucleic acid (DNA) for encoding a ribonucleic acid (RNA) for a protein in a casein micelle,
the segment of DNA is transcribed and translated;
forming recombinant casein proteins in the plant cell, wherein:
the recombinant casein proteins include a κ-casein and at least one of an αS1-casein, an αS2-casein, a β-casein; and
assembling in vivo a recombinant micelle within the plant cell, wherein:
an outer layer of the recombinant micelle is enriched with the κ-casein,
an inner matrix of the recombinant micelle include at least one of the αS1-casein, the αS2-casein, the β-casein.

2. The method as claimed in claim 1 wherein the plasmid includes a further segment of DNA encoding a N-terminal signal peptide that targets the recombinant casein proteins to a vacuole in the plant cell.

3. The method as claimed in claim 1 wherein the plasmid includes a further segment of DNA encoding a selectable marker or a screenable marker.

4. The method as claimed in claim 1 wherein the plasmid includes a further segment of DNA encoding interference RNA to suppress expression of a native protein or a native peptide in the plant cell.

5. The method as claimed in claim 1 wherein the plasmid includes a further segment of DNA encoding a protein capable of altering an intracellular environment of the plant cell.

6. The method as claimed in claim 1 further comprising:
introducing a further plasmid into the plant cell;
wherein:
the further plasmid includes a further segment of DNA for encoding a further RNA for a further protein in the casein micelle;
the further segment of DNA is transcribed and translated; and
the further segment of DNA is at least one of the encoding:

a N-terminal signal peptide that targets the recombinant casein proteins to an endoplasmic reticulum in the plant cell,

a further N-terminal signal peptide that targets the recombinant casein proteins to a vacuole in the plant cell,

a selectable marker or a screenable marker,

a protein capable of altering an intracellular environment of the plant cell.

7. The method as claimed in claim 1 wherein the plasmid includes a further segment of DNA including one or more nucleotide sequences selected from SEQ ID NO:1 to SEQ ID NO:8.

8. A recombinant micelle comprising:
an outer layer enriched with a κ-casein; and
an inner matrix including at least one of a αS1-casein, a αS2-casein, a β-casein.

9. The recombinant micelle as claimed in claim 8 wherein the inner matrix includes a calcium and a phosphate.

10. A plasmid comprising a segment of deoxyribonucleic acid (DNA) for encoding a protein in a casein micelle wherein the segment of DNA includes a promoter and a N-terminal signal peptide.

11. The plasmid as claimed in claim 10 wherein the plasmid includes a further segment of DNA encoding a N-terminal signal peptide that targets the recombinant casein proteins to a vacuole in a plant cell.

12. The plasmid as claimed in claim 10 wherein the plasmid includes a further segment of DNA encoding a selectable marker or a screenable marker.

13. The plasmid as claimed in claim 10 wherein the plasmid includes a further segment of DNA encoding interference RNA to suppress expression of a native protein or a native peptide in a plant cell.

14. The plasmid as claimed in claim 10 wherein the plasmid includes the plasmid includes a further segment of DNA encoding a protein capable of altering an intracellular environment of a plant cell.

15. The plasmid as claimed in claim 10 wherein the plasmid includes a further segment of DNA including one or more nucleotide sequences selected from SEQ ID NO:1 to SEQ ID NO:8.

16. A method of isolating a recombinant micelle comprising:
processing a seed including a cytoplasm with the recombinant micelle;
microfiltering the cytoplasm to remove a particulate above 2um;
ultrafiltering the cytoplasm microfiltered to a further particulate greater than 100 nm; and
collecting the recombinant micelle from the cytoplasm ultrafiltered.

17. The method as claimed in claim 16 wherein:
processing the seed includes:
cleaning, and deshelling or dehulling the seed,
flaking the seed cleaned to 0.005 – 0.02 inch thickness,
extracting with a solvent of oil from the seed flaked,
desolventizing the seed flaked without cooking and collecting the de-oiled,
cleaned
separating the recombinant micelle into a slurry by hydrating, agitating and wet
milling the seed flaked,
passing the slurry through a mesh screen to remove a particulate above 0.5mm in
size and collecting a permeate; and
microfiltering the cytoplasm includes microfiltering the permeate.

18. The method as claimed in claim 16 wherein:
microfiltering the cytoplasm includes microfiltering a permeate;
ultrafiltering the cytoplasm microfiltered includes ultrafiltering the permeate
microfiltered; and
collecting the recombinant micelle from the cytoplasm ultrafiltered includes collecting a
retentate from the permeate ultrafiltered.

19. The method as claimed in claim 16 wherein:
microfiltering the cytoplasm includes microfiltering a permeate;
ultrafiltering the cytoplasm microfiltered includes ultrafiltering the permeate
microfiltered;

collecting the recombinant micelle from the cytoplasm ultrafiltered includes collecting a retentate from the permeate ultrafiltered; and

further comprising:

diafiltering the retentate at a rate that the permeate is collected and passing the retentate through the ultrafiltering.

20. The method as claimed in claim 16 wherein processing the seed includes processing the seed milled from a maize, a rice, a sorghum, a cowpea, a soybean, a cassava, a coyam, a sesame, a peanut, a pea, a cotton, a yam, or a combination thereof.

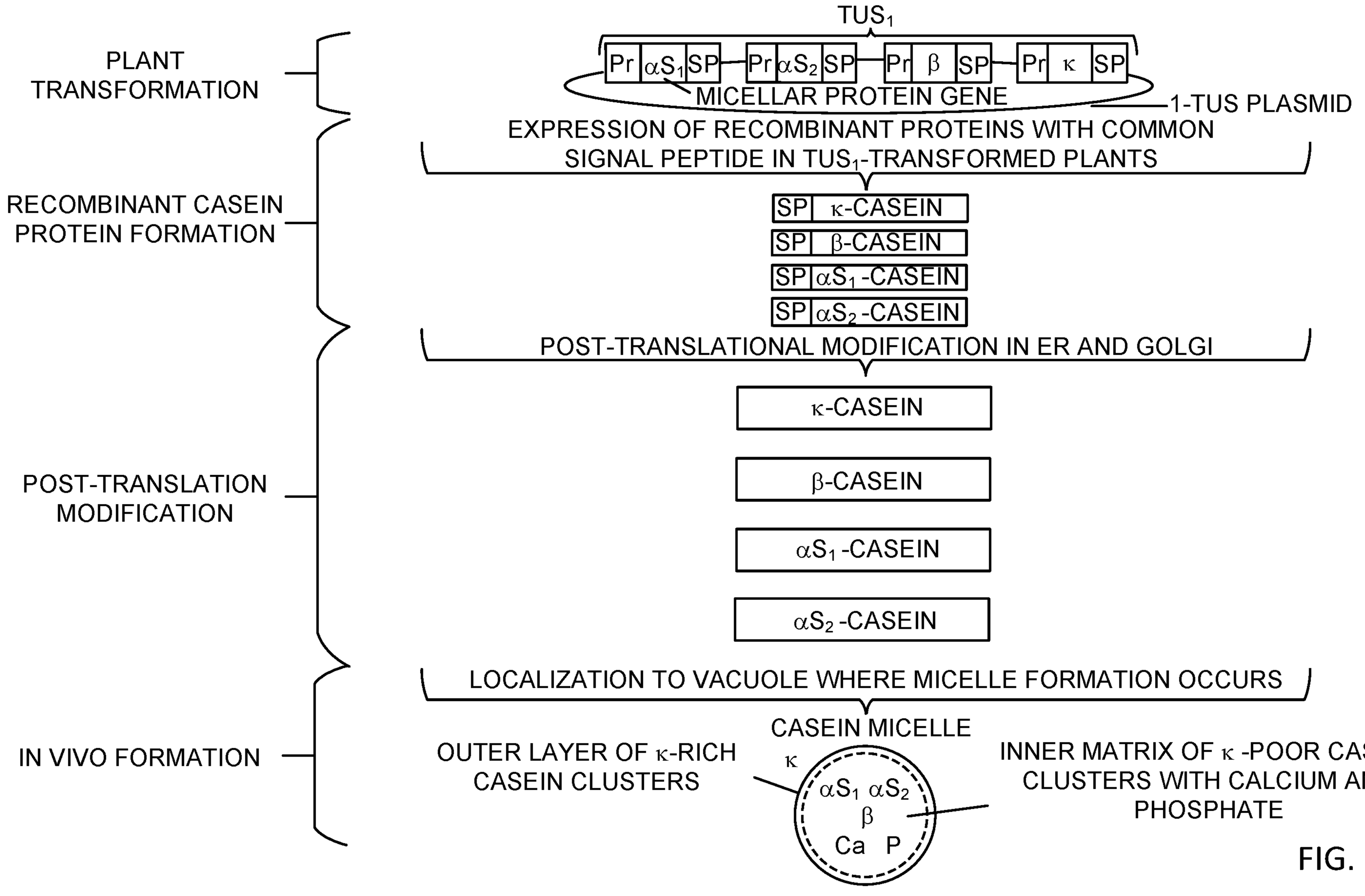

PLANT TRANSFORMATION
RECOMBINANT CASEIN PROTEIN FORMATION
POST-TRANSLATION MODIFICATION
IN VIVO FORMATION
TUS1
Pr αS1 SP
Pr αS2 SP
Pr β SP
Pr κ SP
MICELLAR PROTEIN GENE
1-TUS PLASMID
EXPRESSION OF RECOMBINANT PROTEINS WITH COMMON SIGNAL PEPTIDE IN TUS1-TRANSFORMED PLANTS
SP κ-CASEIN
SP β-CASEIN
SP αS1 -CASEIN
SP αS2 -CASEIN
POST-TRANSLATIONAL MODIFICATION IN ER AND GOLGI
κ-CASEIN
β-CASEIN
αS1 -CASEIN
αS2 -CASEIN
LOCALIZATION TO VACUOLE WHERE MICELLE FORMATION OCCURS
CASEIN MICELLE
OUTER LAYER OF κ-RICH CASEIN CLUSTERS
κ
αS1 αS2
β
Ca P
INNER MATRIX OF κ -POOR CASEIN CLUSTERS WITH CALCIUM AND PHOSPHATE

FIG. 1

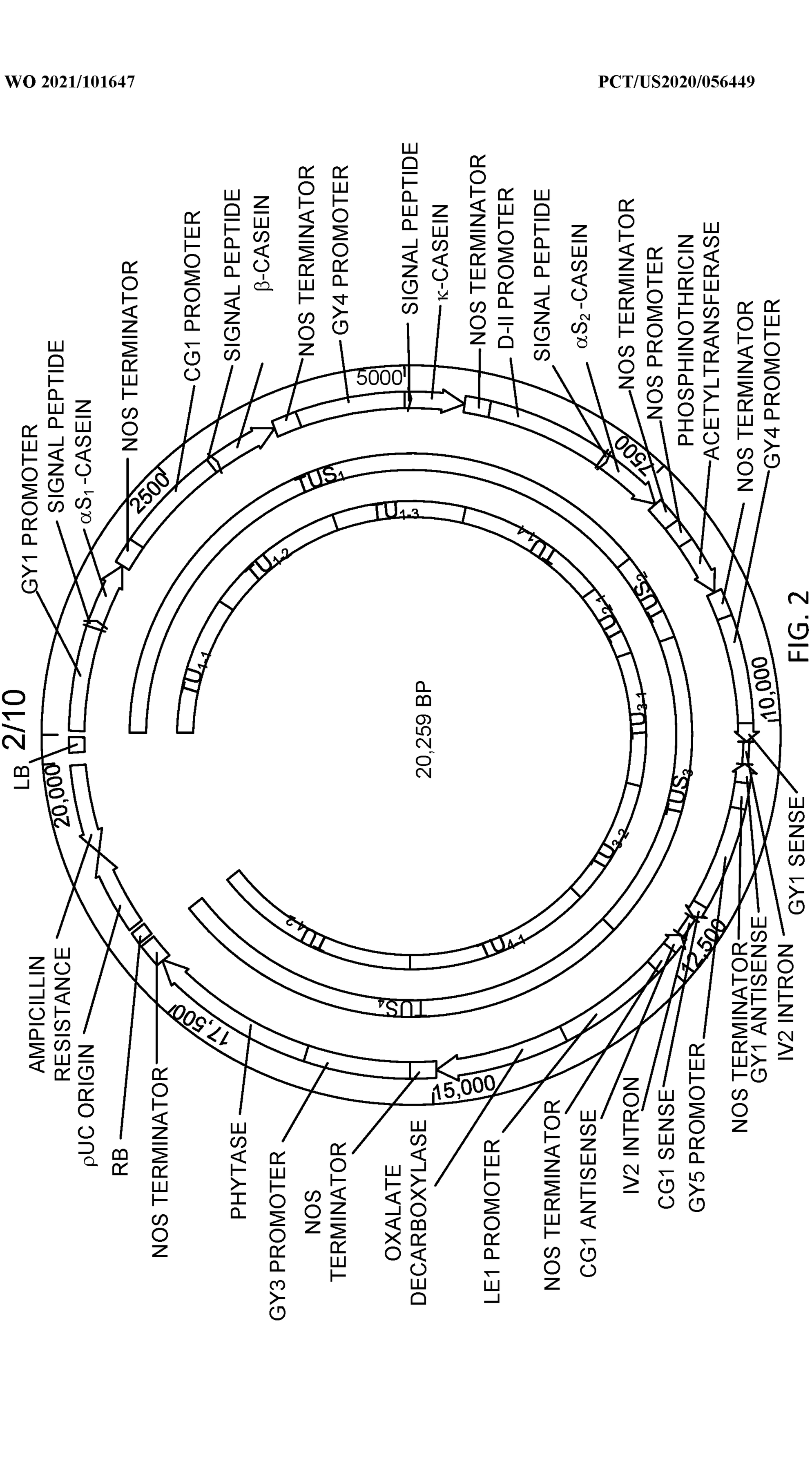

2/10
20,259 BP
5000
2500
7500
10,000
12,500
15,000
17,500
20,000
GY1 PROMOTER
SIGNAL PEPTIDE
αS1-CASEIN
NOS TERMINATOR
CG1 PROMOTER
SIGNAL PEPTIDE
β-CASEIN
NOS TERMINATOR
GY4 PROMOTER
SIGNAL PEPTIDE
κ-CASEIN
NOS TERMINATOR
D-II PROMOTER
SIGNAL PEPTIDE
αS2-CASEIN
NOS TERMINATOR
NOS PROMOTER
PHOSPHINOTHRICIN
ACETYLTRANSFERASE
NOS TERMINATOR
GY4 PROMOTER
GY1 SENSE
IV2 INTRON
GY1 ANTISENSE
NOS TERMINATOR
GY5 PROMOTER
CG1 SENSE
CG1 ANTISENSE
IV2 INTRON
NOS TERMINATOR
NOS PROMOTER
LE1 PROMOTER
NOS TERMINATOR
OXALATE DECARBOXYLASE
GY3 PROMOTER
PHYTASE
NOS TERMINATOR
RB
ρUC ORIGIN
AMPICILLIN RESISTANCE
LB
TUS1
TU1-1
TU1-2
TU1-3
TUL1
TUL2
TUS2
TU3-1
TUS3
TU3-2
TU4-1
TU4-2
TUS4

FIG. 2

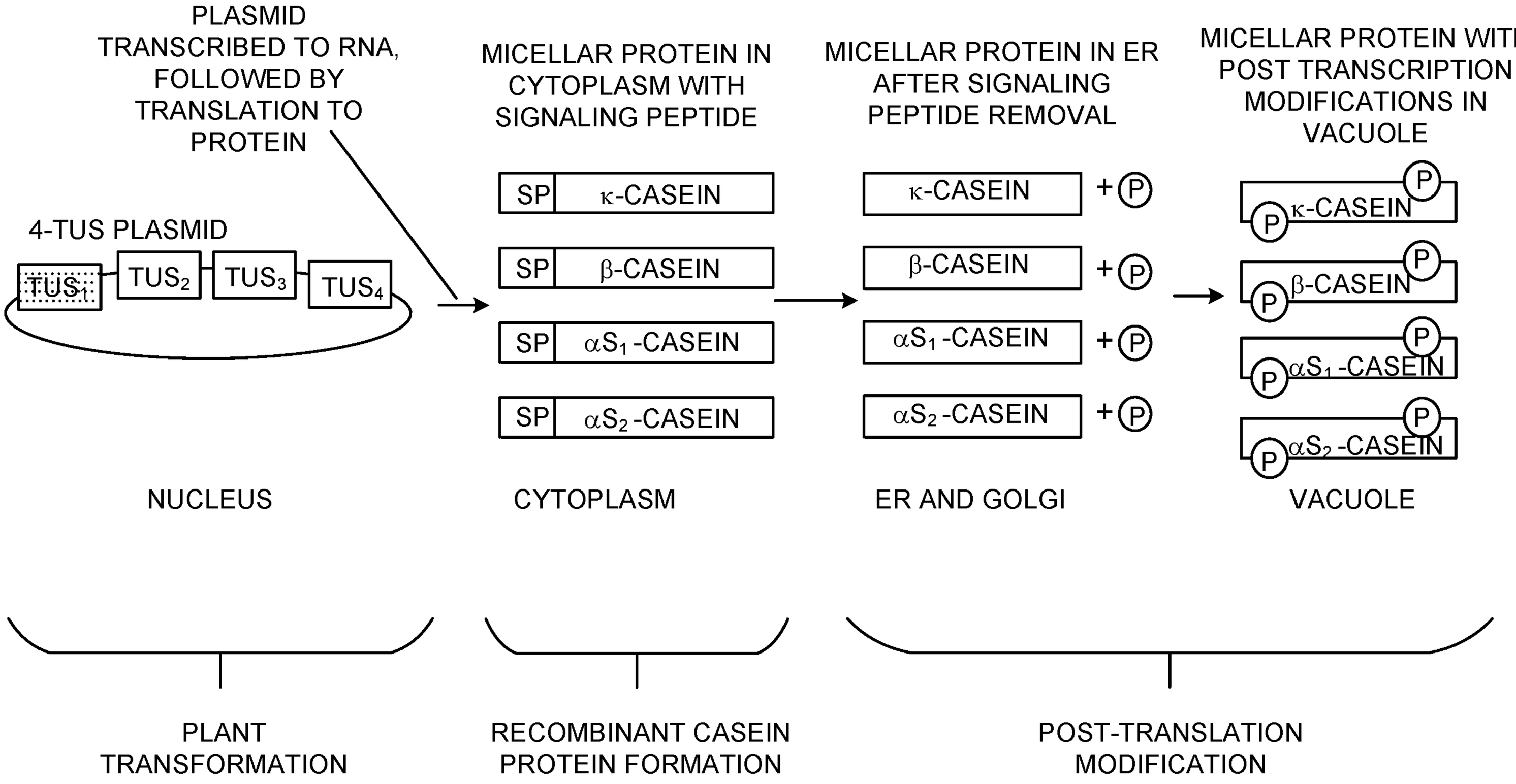

PLASMID TRANSCRIBED TO RNA, FOLLOWED BY TRANSLATION TO PROTEIN
4-TUS PLASMID
TUS1
TUS2
TUS3
TUS4
MICELLAR PROTEIN IN CYTOPLASM WITH SIGNALING PEPTIDE
SP κ-CASEIN
SP β-CASEIN
SP αS1 -CASEIN
SP αS2 -CASEIN
MICELLAR PROTEIN IN ER AFTER SIGNALING PEPTIDE REMOVAL
κ-CASEIN + P
β-CASEIN + P
αS1 -CASEIN + P
αS2 -CASEIN + P
MICELLAR PROTEIN WITH POST TRANSCRIPTION MODIFICATIONS IN VACUOLE
P κ-CASEIN P
P β-CASEIN P
P αS1 -CASEIN P
P αS2 -CASEIN P
NUCLEUS
CYTOPLASM
ER AND GOLGI
VACUOLE
PLANT TRANSFORMATION
RECOMBINANT CASEIN PROTEIN FORMATION
POST-TRANSLATION MODIFICATION

FIG. 3A

FIG. 3B

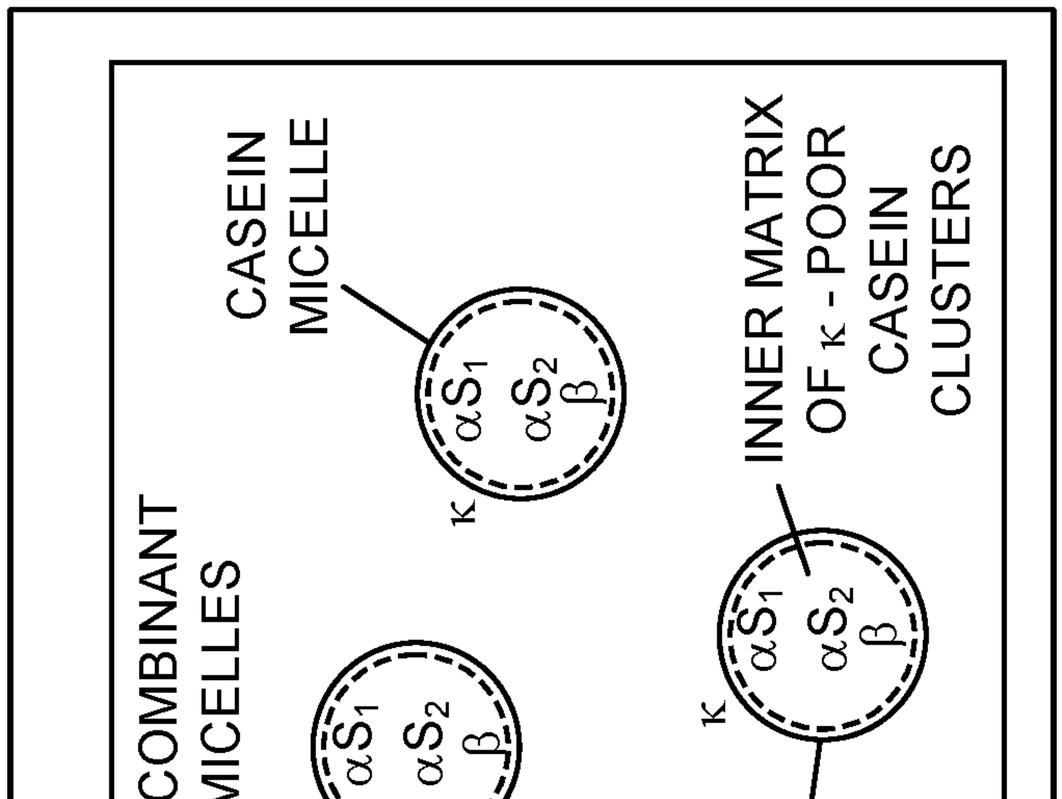
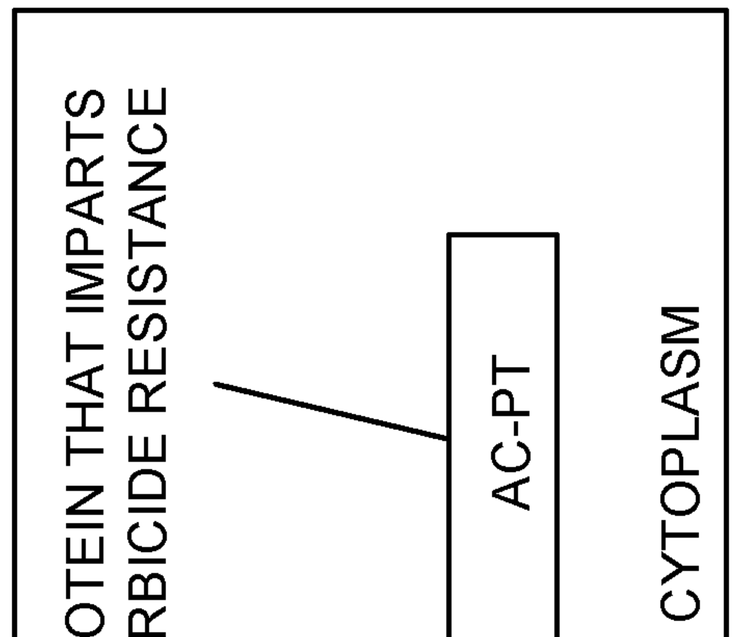

4/10
CYTOPLASM
INDIVIDUAL MICELLAR PROTEINS FROM TUS1
κ-CASEIN
β-CASEIN
αS1-CASEIN
αS2-CASEIN
VACUOLE
RECOMBINANT MICELLES
CASEIN MICELLE
κ
αS1
αS2
β
OUTER LAYER OF κ- RICH CASEIN CLUSTERS
INNER MATRIX OF κ - POOR CASEIN CLUSTERS
P

FIG. 3C


PROTEIN THAT IMPARTS HERBICIDE RESISTANCE
AC-PT
CYTOPLASM
NUCLEUS
PLASMID TRANSCRIBED TO RNA, FOLLOWED BY TRANSLATION TO PROTEIN
4-TUS PLASMID
TUS1
TUS2
TUS3
TUS4

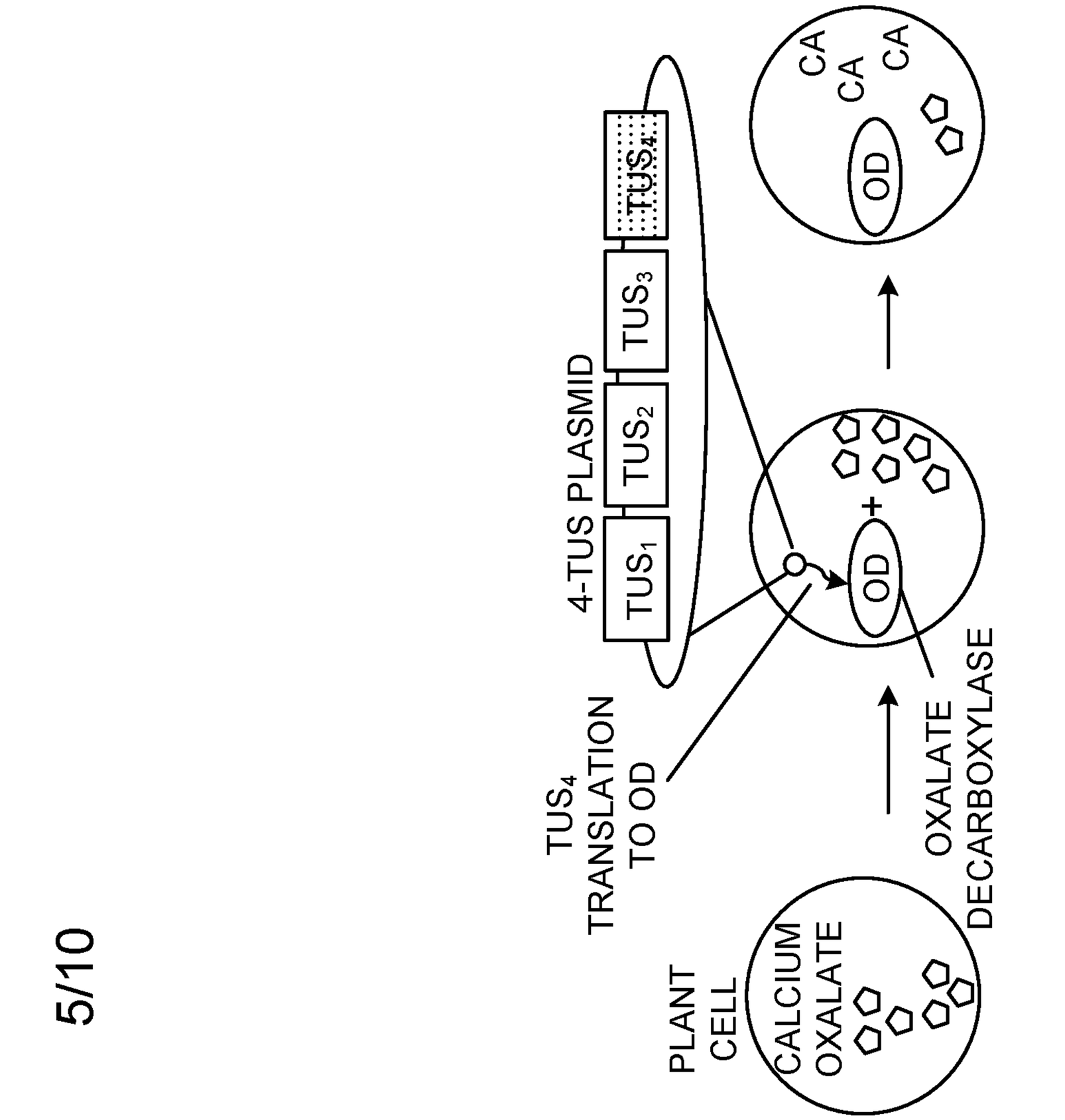

4-TUS PLASMID
TUS1
TUS2
TUS3
TUS4
TUS4 TRANSLATION TO OD
PLANT CELL
CALCIUM OXALATE
OXALATE DECARBOXYLASE
OD
CA
CA
CA
+

FIG. 3E

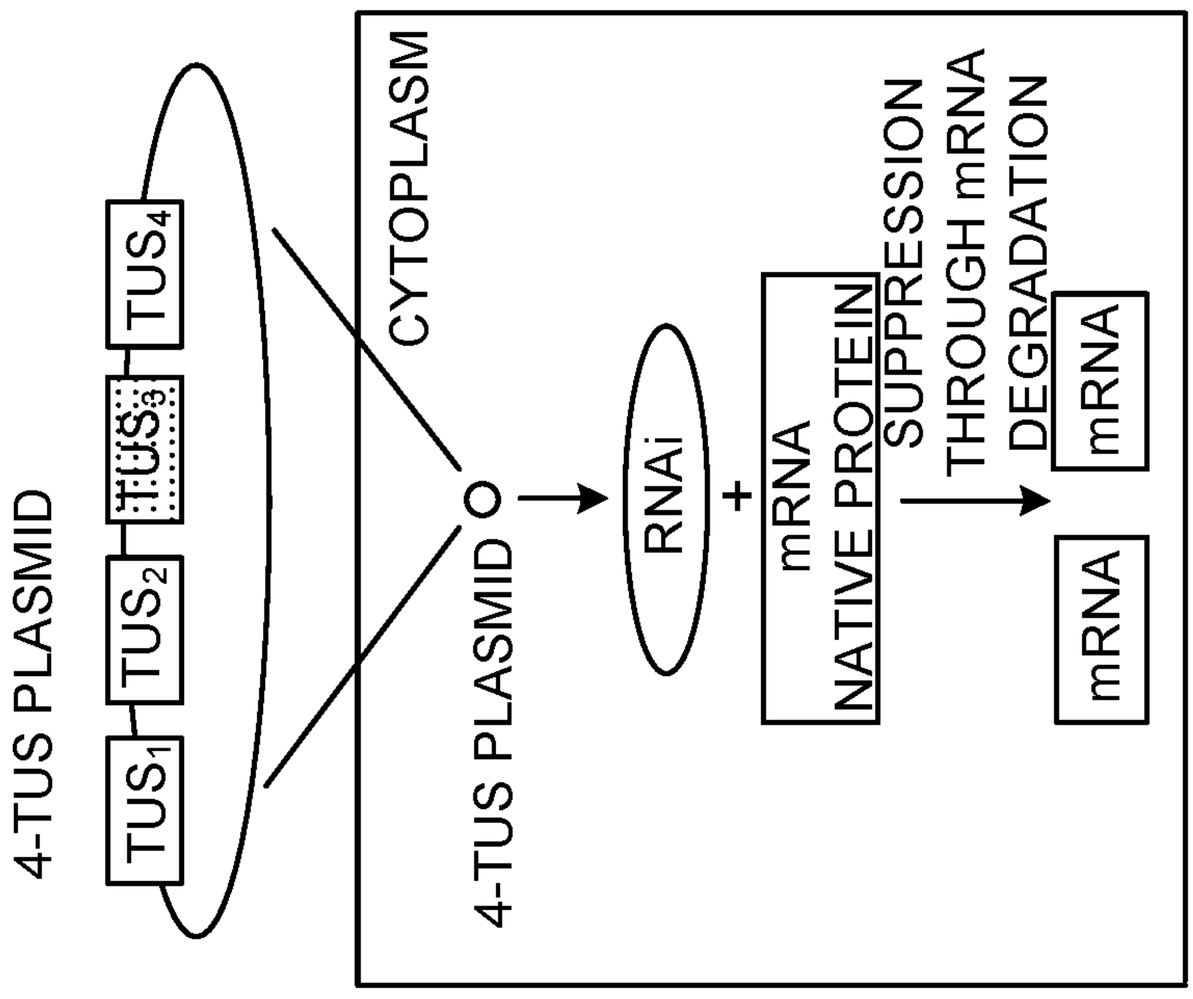

4-TUS PLASMID
TUS1
TUS2
TUS3
TUS4
CYTOPLASM
4-TUS PLASMID
RNAi
+
mRNA
NATIVE PROTEIN
SUPPRESSION THROUGH mRNA DEGRADATION
mRNA
mRNA

FIG. 3D

FIG. 3F

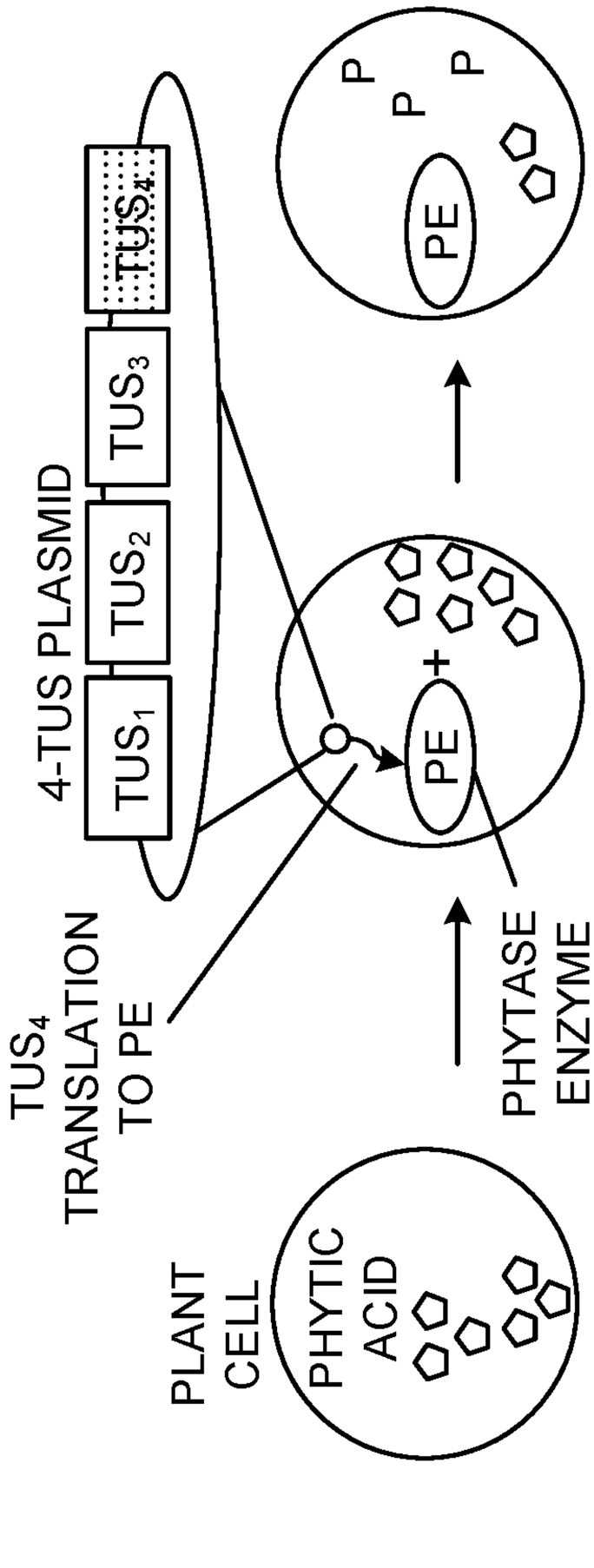
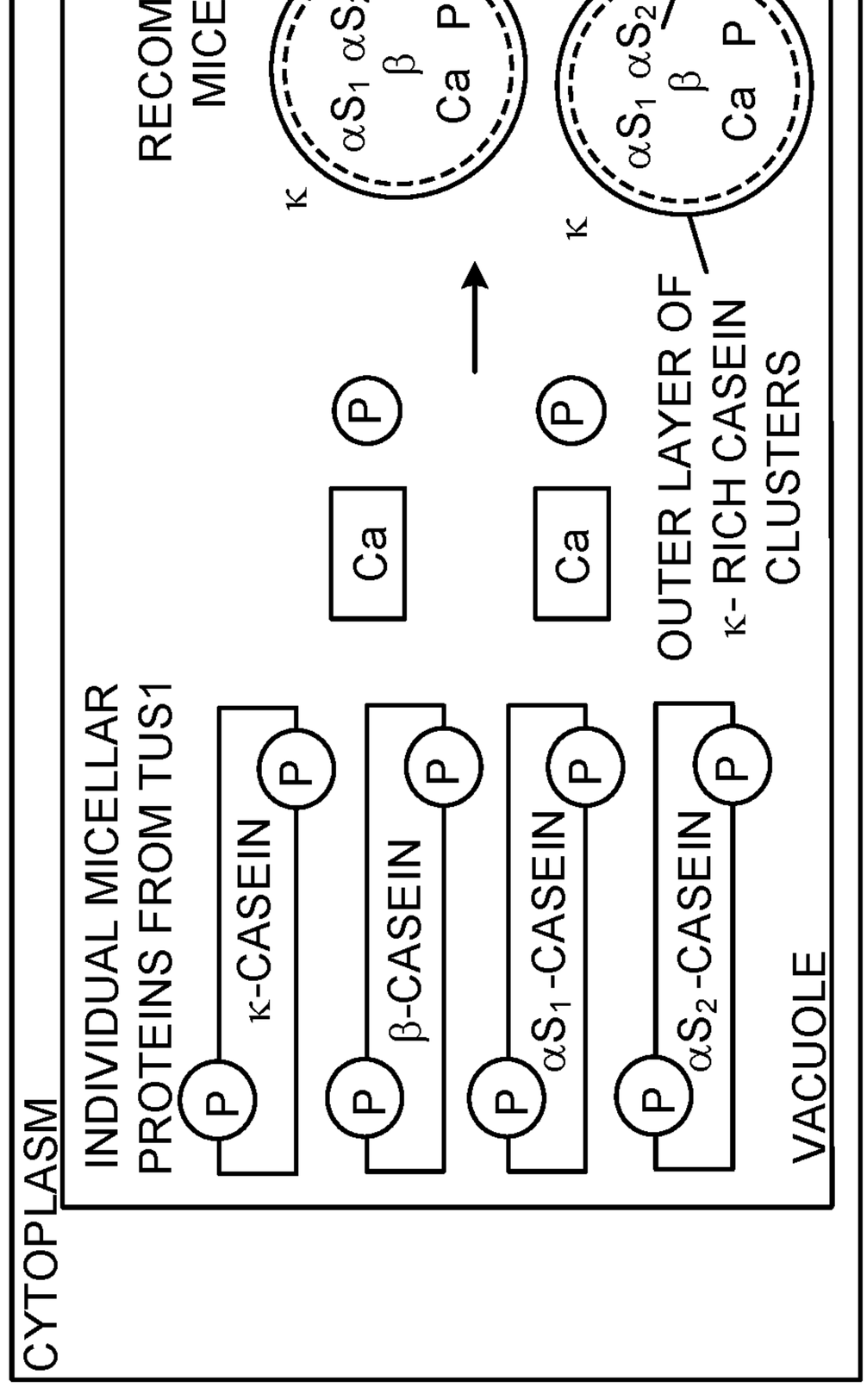

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4-TUS PLASMID
TUS1
TUS2
TUS3
TUS4
TUS4 TRANSLATION TO PE
PE
P
P
P
PE
PHYTASE ENZYME
PLANT CELL
PHYTIC ACID

FIG. 3G


RECOMBINANT MICELLES
CASEIN MICELLE
κ
αS1 αS2
β
Ca P
INNER MATRIX OF κ-POOR CASEIN CLUSTERS WTIH CALCIUM AND PHOSPHATE
OUTER LAYER OF κ- RICH CASEIN CLUSTERS
Ca
P
CYTOPLASM
INDIVIDUAL MICELLAR PROTEINS FROM TUS1
κ-CASEIN
β-CASEIN
αS1 -CASEIN
αS2 -CASEIN
VACUOLE

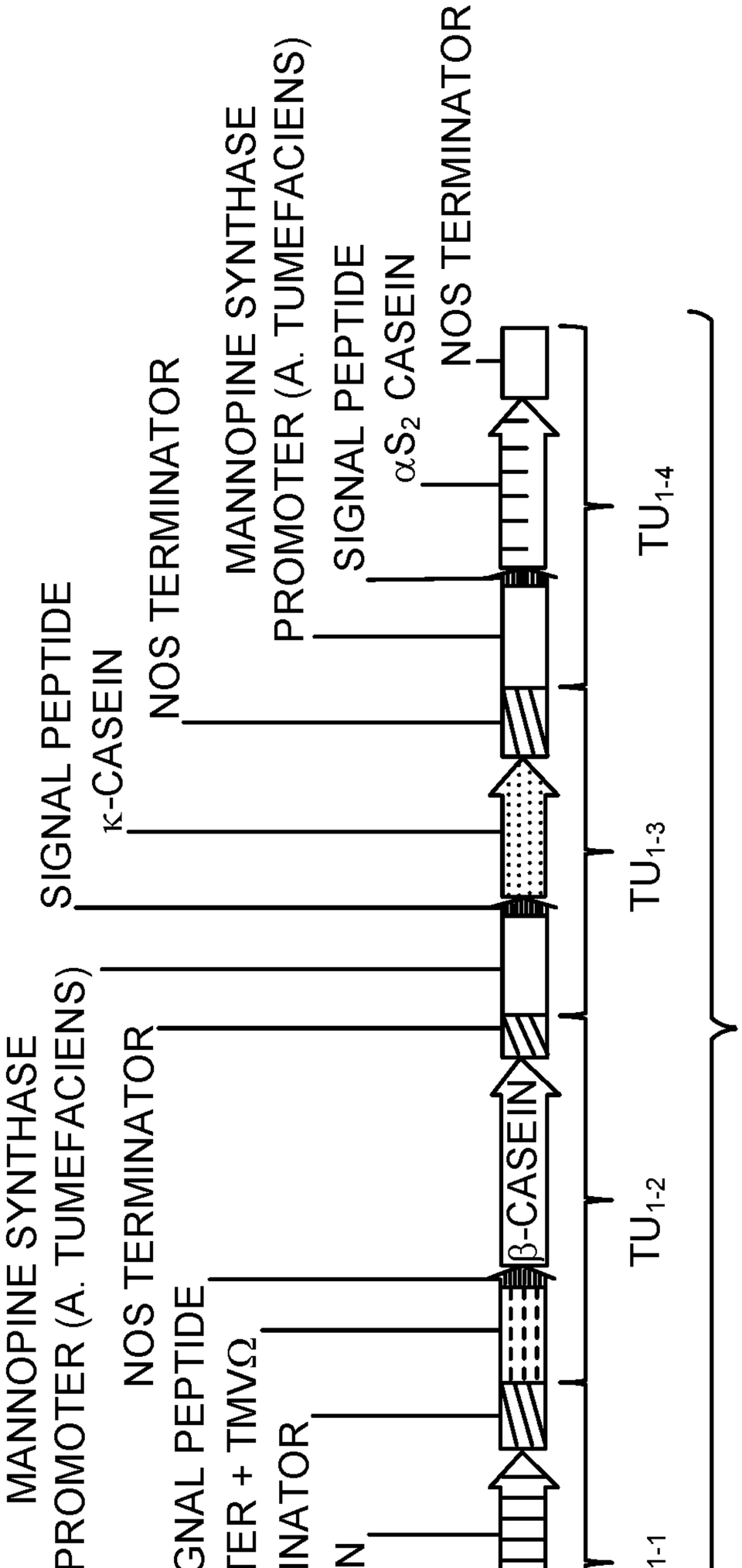

35S SHORT PROMOTER + TMVΩ
SIGNAL PEPTIDE
2X35S PROMOTER + TMVΩ
αS1 CASEIN
NOS TERMINATOR
SIGNAL PEPTIDE
β-CASEIN
MANNOPINE SYNTHASE
PROMOTER (A. TUMEFACIENS)
NOS TERMINATOR
TU1-1
TU1-2
TU1-3
TUS1

FIG. 4

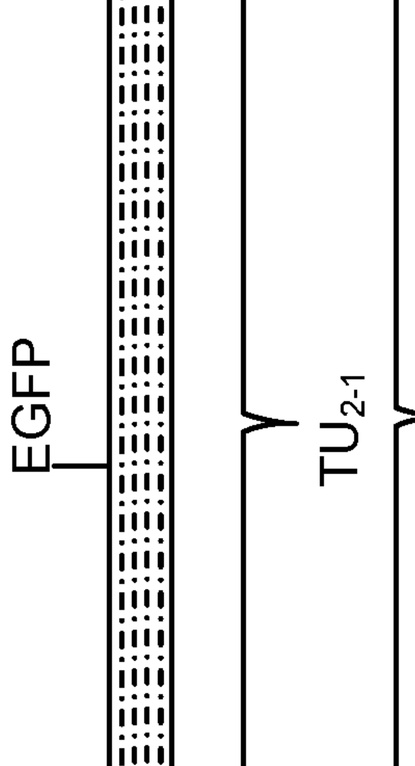

NOS PROMOTER
EGFP
NOS TERMINATOR
TU2-1
TUS2

FIG. 5

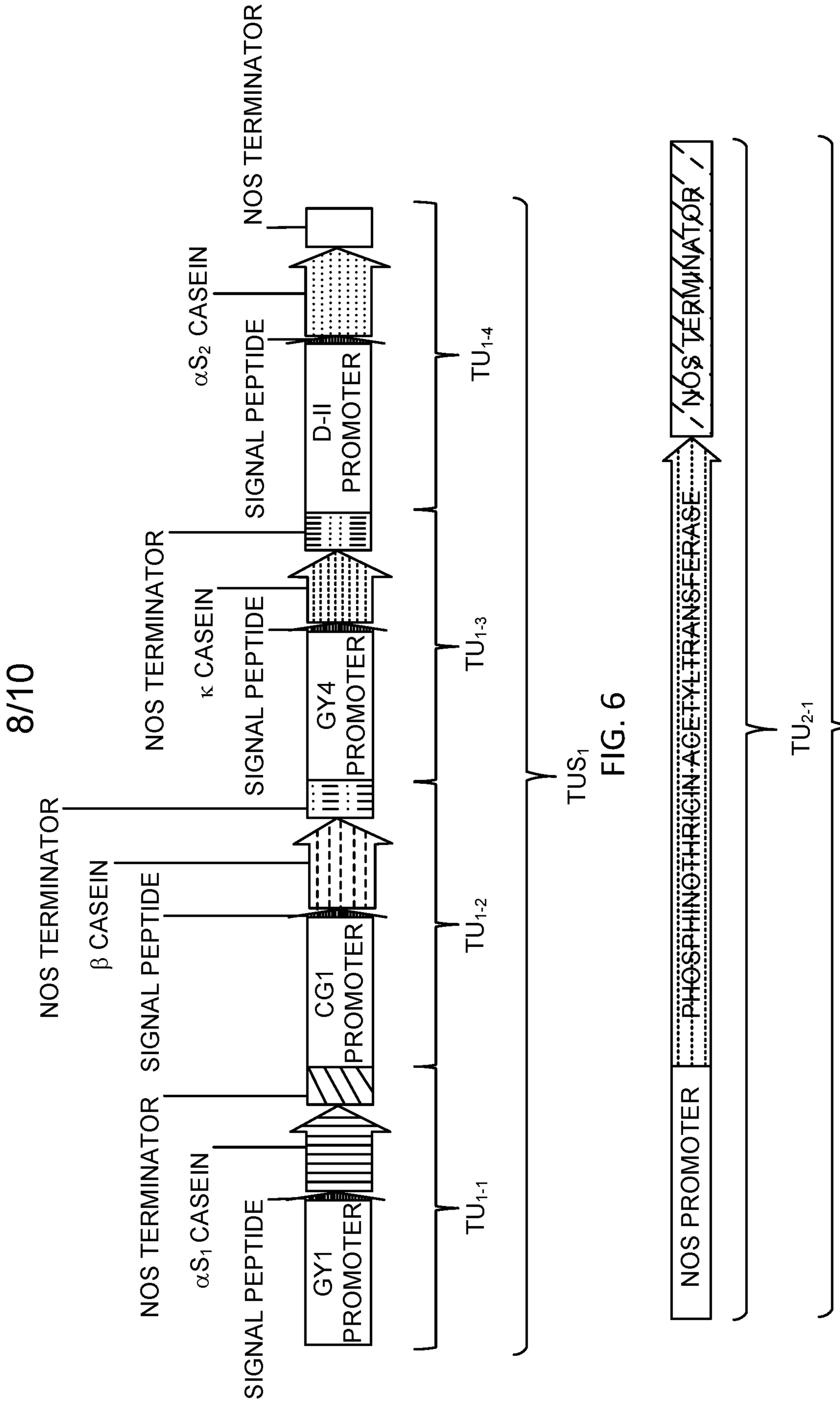

8/10
NOS TERMINATOR
β CASEIN
SIGNAL PEPTIDE
CG1
PROMOTER
NOS TERMINATOR
αS1 CASEIN
SIGNAL PEPTIDE
GY1
PROMOTER
TU1-1
TU1-2
NOS TERMINATOR
κ CASEIN
SIGNAL PEPTIDE
GY4
PROMOTER
NOS TERMINATOR
αS2 CASEIN
SIGNAL PEPTIDE
D-II
PROMOTER
TU1-3
TU1-4
TUS1
NOS PROMOTER
PHOSPHINOTHRICIN ACETYLTRANSFERASE
NOS TERMINATOR
TU2-1
TUS2

FIG. 6

FIG. 7

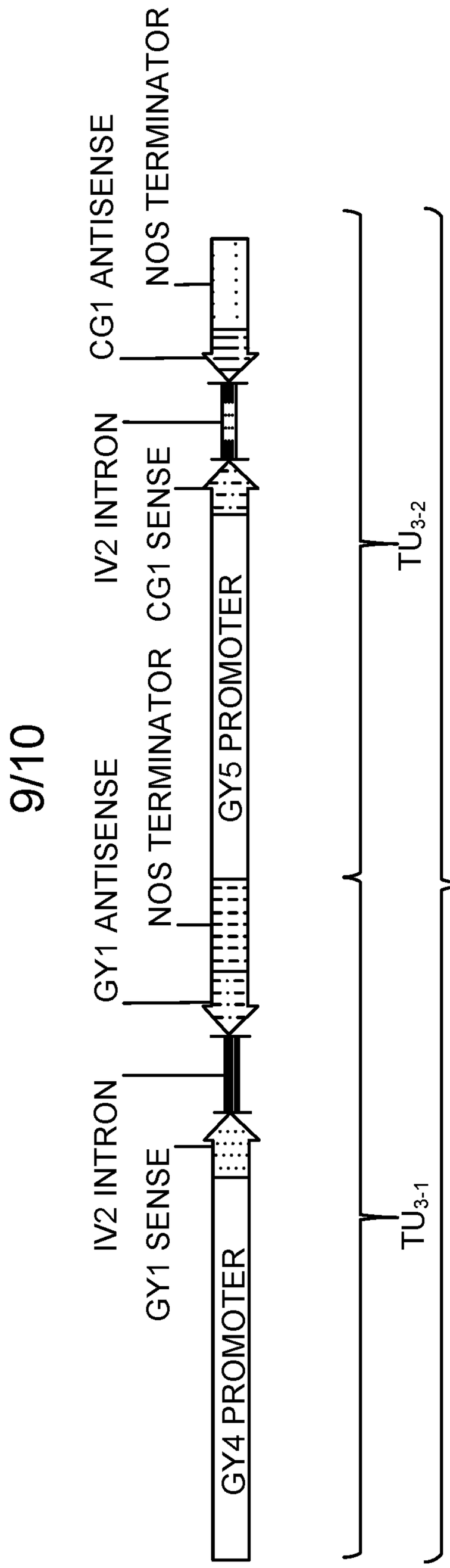


FIG. 8

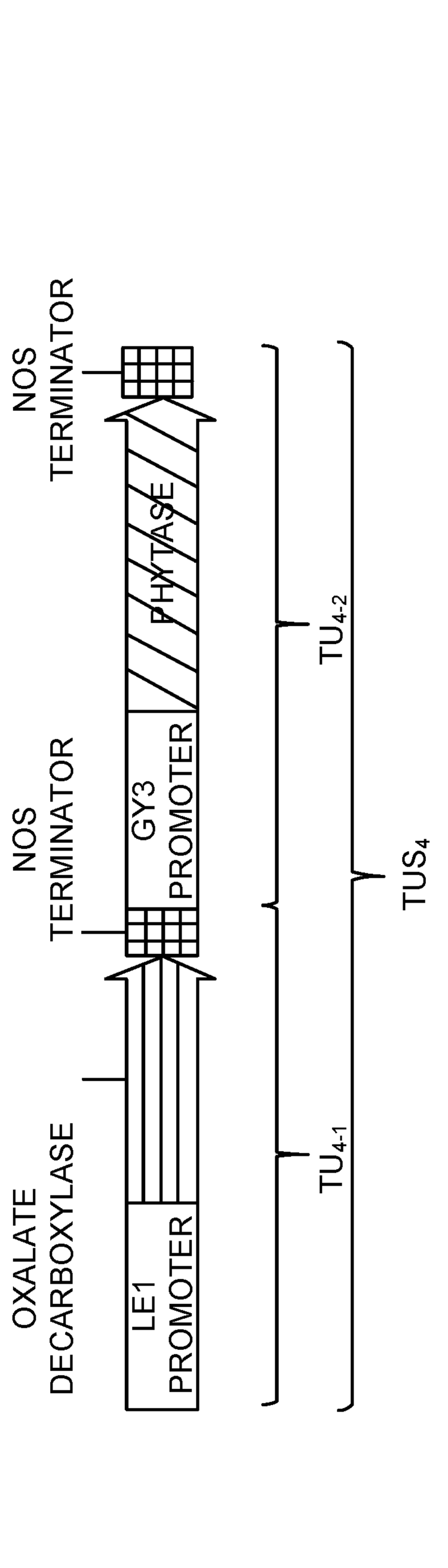


FIG. 9

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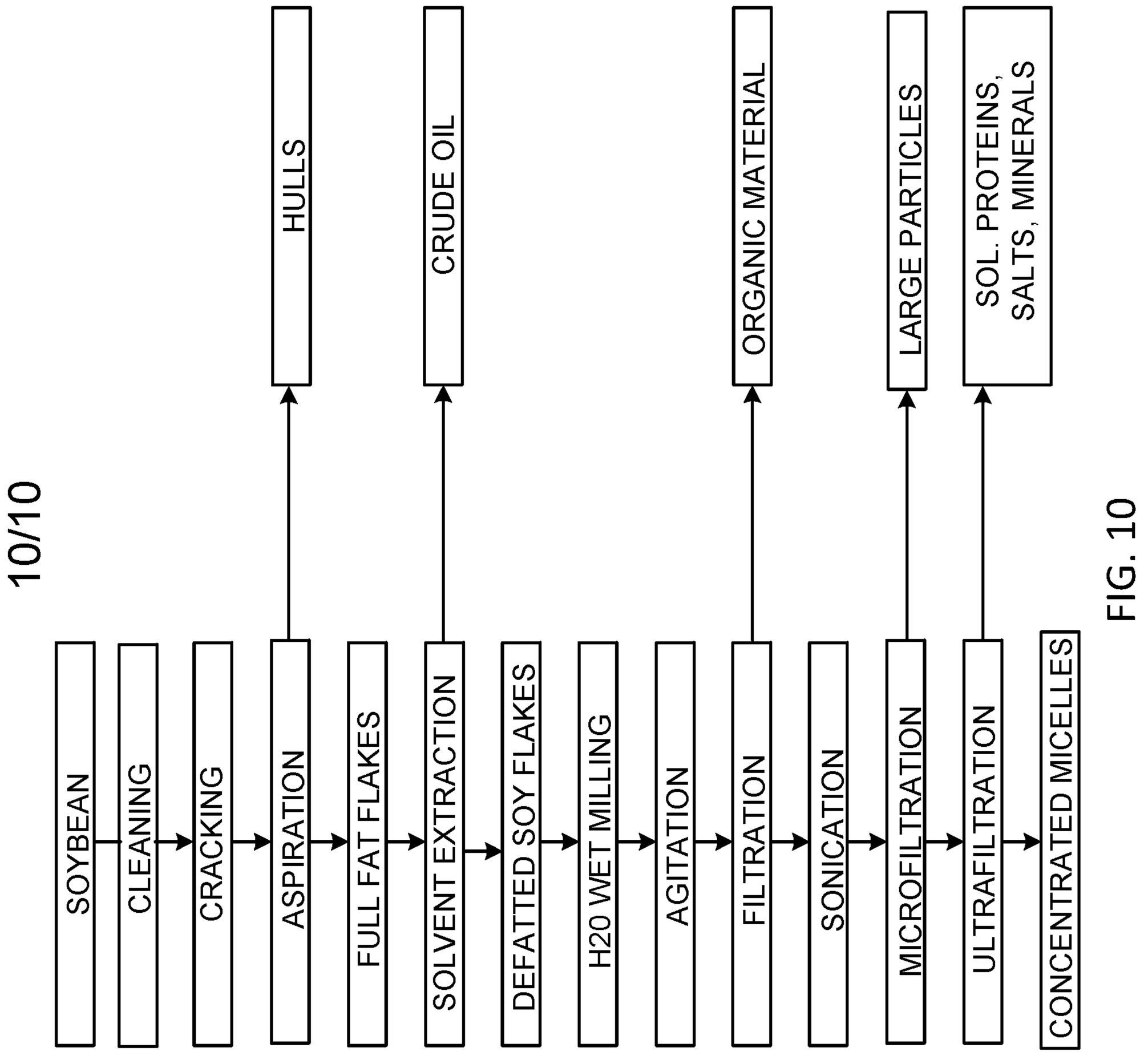

10/10
SOYBEAN
CLEANING
CRACKING
ASPIRATION
FULL FAT FLAKES
SOLVENT EXTRACTION
DEFATTED SOY FLAKES
H20 WET MILLING
AGITATION
FILTRATION
SONICATION
MICROFILTRATION
ULTRAFILTRATION
CONCENTRATED MICELLES
HULLS
CRUDE OIL
ORGANIC MATERIAL
LARGE PARTICLES
SOL. PROTEINS, SALTS, MINERALS

FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2020/056449

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/09; A61K 38/24; C07K 7/23; C07K 14/575; C07K 14/59; G01N 33/53 (2021.01)
CPC - A61K 38/00; A61K 38/09; A61K 38/24; C07K 7/23; C07K 14/59; G01N 33/76 (2021.01)

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)
see Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
see Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
see Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0273328 A1 (PERFECT DAY, INC.) 28 September 2017 (28.09.2017) entire document	8-10, 12
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Y		11, 13, 14
Y	US 2005/0172356 A1 (CHRISTELLER et al) 04 August 2005 (04.08.2005) entire document	11
Y	US 2005/0166289 A1 (CHUAN CHIANG et al) 28 July 2005 (28.07.2005) entire document	13
Y	US 5,547,870 A (DATTA et al) 20 August 1996 (20.08.1996) entire document	14
Y	US 2018/0291392 A1 (ALPINE ROADS, INC.) 11 October 2018 (11.10.2018) entire document	1-20
A	US 2016/0168198 A1 (BIOCON LIMITED) 16 June 2016 (16.06.2016) entire document	1-20
A	US 2013/0295646 A1 (LEJARS et al) 07 November 2013 (07.11.2013) entire document	1-20
A		

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"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
07 January 2021	03 FEB 2021
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver Telephone No. PCT Helpdesk: 571-272-4300