

PLANT-BASED SYNTHESIS PRODUCTS

BACKGROUND OF THE INVENTION

[0001] Recombinant protein manufacturing describes the science of producing proteins within another structure other than their original organism. A gene of the protein of interest is inserted into a new organism with the ability of being multiplied. After this expression process, the recombinant proteins are extracted and purified. This technology has been implemented on a commercial scale since the early 1980s and is widely used today to produce a wide range of proteins from enzymes dedicated to the textile industry, to therapeutics, antibodies or growth factors commonly used in science.

[0002] Different expression systems have been developed over the years. Using yeast, bacteria, animal cells or human cells, they rely on similar production steps. Plant molecular farming is the green revolution of recombinant proteins manufacturing, offering an animal-free solution, efficient yield, high flexibility, and easy production scale-up path. Plant technology allows an exceptional flexibility with a production system costing 40 times less than our current cell-ag. competitors, and a scale-up potential meeting the requirements of the clean-meat sector. Plant molecular farming presents a solution to this problem. However, up to 80% of the production cost with plant molecular farming was linked to high particle burden of primary extracts, plant secondary metabolites, pigments and phenols. These additional clarification steps (extraction process) increase costs significantly. Thus, there is a need for more efficient systems for plant molecular farming.

BRIEF SUMMARY

[0003] Provided herein are plant cells comprising a polynucleotide sequence encoding for a heterologous protease inhibitor gene or a functional variant thereof; and a polynucleotide sequence encoding for a mammalian gene or a functional variant thereof. Further provided herein are plant cells, wherein the polynucleotide sequence encoding for a heterologous protease inhibitor gene or a functional variant thereof and the polynucleotide sequence encoding for a mammalian gene or a functional variant thereof are within a bacterial or viral vector. Further provided herein are plant cells, wherein the bacterial vector is an *Agrobacterium* species. Further provided herein are plants cells, wherein the viral vector is Tobacco Mosaic Virus. Further provided herein are plants cells, wherein the mammalian gene is selected from the group consisting of TGF- β , IGF-1, IGF-2, human FGF-2, Activin A, BMP-4 and VEGF. Further provided herein are plants cells, wherein the heterologous protease inhibitor gene is SICYS8. Further provided herein are plants cells, wherein the

polynucleotide sequence encoding for a heterologous protease inhibitor gene or a functional variant thereof and the polynucleotide sequence encoding for a mammalian gene or a functional variant thereof are RNA or DNA.

[0004] Provided herein are plant cells comprising a polynucleotide sequence encoding for a chicken FGF-2 gene or a functional variant thereof. Further provided herein are plants cells, wherein the polynucleotide sequence is within a bacterial or viral vector. Further provided herein are plant cells, wherein the polynucleotide sequence comprises a sequence with at least 75% sequence identity to SEQ ID NO: 21. Further provided herein are plant cells, wherein the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence. Further provided herein are plant cells, wherein the bacterial vector comprises an *Agrobacterium* species. Further provided herein are plant cells, wherein the viral vector is Tobacco Mosaic Virus. Further provided herein are plant cells, wherein the polynucleotide sequence is RNA or DNA.

[0005] Provided herein are plant cells comprising a polynucleotide sequence encoding for an IL-1 β gene or a functional variant thereof. Further provided herein are plant cells, wherein the polynucleotide sequence is within a bacterial or viral vector. Further provided herein are plant cells, wherein the polynucleotide sequence comprises a sequence with at least 75% sequence identity to SEQ ID NO: 27. Further provided herein are plant cells, wherein the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence. Further provided herein are plant cells, wherein the bacterial vector comprises an *Agrobacterium* species. Further provided herein are plant cells, wherein the viral vector is Tobacco Mosaic Virus.

[0006] Provided herein are plant cells comprising a heterologous protease inhibitor protein or a functional variant thereof and a mammalian protein or a functional variant thereof. Further provided herein are plants cells further comprising a bacterial or viral vector. Further provided herein are plant cells, wherein the bacterial vector is an *Agrobacterium* species. Further provided herein are plant cells, wherein the viral vector is Tobacco Mosaic Virus. Further provided herein are plant cells, wherein the mammalian protein is from the group consisting of TGF- β , IGF-1, IGF-2, human FGF-2, Activin A, BMP-4 and VEGF.

[0007] Provided herein are plant cells comprising a chicken FGF-2 protein or a functional variant thereof. Further provided herein are plant cells, wherein the chicken FGF-2 protein comprises a sequence with at least 75% sequence identity to SEQ ID NO: 8. Further provided herein are plant cells, wherein the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence. Further provided herein are plant cells further comprising a bacterial or viral vector. Further provided herein are plant cells, wherein the bacterial vector

comprises a *Agrobacterium* species. Further provided herein are plant cells, wherein the viral vector is Tobacco Mosaic Virus.

[0008] Provided herein are plant cells comprising an IL1- β protein or a functional variant thereof. Further provided herein are plant cells, wherein the IL1- β protein comprises a sequence with at least 75% sequence identity to SEQ ID NO: 9. Further provided herein are plant cells, wherein the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence. Further provided herein are plant cells, further comprising a bacterial or viral vector. Further provided herein are plant cells, wherein the bacterial vector comprises a *Agrobacterium* species. Further provided herein are plant cells, wherein the viral vector is Tobacco Mosaic Virus.

[0009] Provided herein are compositions comprising a mammalian protein or a functional variant thereof; and at least one of the following: flavonoids, rubisco, plant-derived alkaloids, cellulose, lignocellulose, legumalin, phaselin, 11S legumin type, 7s vicilin type, gliadin, zein, hordein, secalin, and glutenins. Further provided herein are compositions, wherein the mammalian protein is at least one of TGF- β , IGF-1, IGF-2, human FGF-2, Activin A, BMP-4 and VEGF. Further provided herein are compositions, wherein the at least one of the following comprises no more than 0.05% of the composition. Further provided herein are compositions, wherein the at least one of the following comprises no more than 0.03% of the composition. Further provided herein are compositions further comprising heparin. In some embodiments, heparin is present at no more than 2 μ g/ml.

[0010] Provided herein are compositions, wherein the composition comprising a chicken FGF-2 protein or a functional variant thereof; and at least one of the following: flavonoids, rubisco, plant-derived alkaloids, cellulose, lignocellulose, legumalin, phaselin, 11S legumin type, 7s vicilin type, gliadin, zein, hordein, secalin, and glutenins. Further provided herein are compositions, wherein the at least one of the following comprises no more than 0.05% of the composition. Further provided herein are compositions, wherein the at least one of the following comprises no more than 0.03% of the composition. Further provided herein are compositions further comprising heparin. In some embodiments, heparin is present at no more than 2 μ g/ml.

[0011] Provided herein are compositions comprising a human interleukin 1 beta (IL1- β) protein or a functional variant thereof and at least one of the following: flavonoids, rubisco, plant-derived alkaloids, cellulose, lignocellulose, legumalin, phaselin, 11S legumin type, 7s vicilin type, gliadin, zein, hordein, secalin, and glutenins. Further provided herein are compositions, wherein the at least one of the following comprises no more than 0.05% of the composition. Further provided herein are compositions, wherein the at least one of the following comprises no more than 0.03% of the

composition. Further provided herein are compositions further comprising heparin. In some embodiments, heparin is present at no more than 2 $\mu\text{g/ml}$.

[0012] Provided herein are methods of manufacturing a mammalian protein comprising culturing the plant cells and extracting a mammalian protein encoded by the mammalian gene or the functional variant thereof to generate an extraction product. Further provided herein are methods, wherein the extraction product comprises the mammalian protein present in an amount of at least 40 μg per gram of biomass. Further provided herein are methods, wherein the mammalian protein is present in an amount of at least 45, 50, or 55 μg per gram of biomass. Further provided herein are methods, wherein the culturing comprises contacting a bacterial or viral vector to the plant cell using a syringe or spray method.

[0013] Provided herein are plant cells comprising a polynucleotide sequence encoding for a mammalian gene or a functional variant thereof, wherein the mammalian gene is selected from the group consisting of TGF- β , IGF-1, IGF-2, FGF-2, BMP-4, and VEGF, and wherein the plant cell is not derived from *Oryza sativa*. Further provided herein are plant cells, wherein the polynucleotide sequence encoding for a mammalian gene or a functional variant thereof are within a bacterial or viral vector. Further provided herein are plant cells, wherein the bacterial vector comprises an *Agrobacterium* species. Further provided herein are plant cells, wherein the viral vector comprises a Tobacco mosaic virus. Further provided herein are plant cells, wherein the polynucleotide sequence encoding for a mammalian gene or a functional variant thereof is RNA or DNA. Further provided herein are plant cells, wherein the functional variant comprises an insertion, a deletion, or a sequence variation compared to the mammalian gene.

[0014] Provided herein are plant cells comprising a mammalian protein or a functional variant thereof, and wherein the plant cell is not derived from *Oryza sativa*. Further provided herein are plant cells comprising a bacterial or viral vector. Further provided herein are plant cells, wherein the bacterial vector comprises an *Agrobacterium* species. Further provided herein are plant cells, wherein the viral vector comprises a Tobacco Mosaic Virus.

[0015] Provided herein are methods of manufacturing a mammalian protein comprising culturing the plant cells and extracting the mammalian protein or the functional variant thereof to generate an extraction product. Further provided herein are methods, wherein the extraction product comprises the mammalian protein present in an amount of at least at least 40 μg per gram of biomass. Further provided herein are methods, wherein the mammalian protein is present in an amount of at least 45,

50, 55 µg per gram of biomass. Further provided herein are methods, wherein the culturing comprises contacting a bacterial or viral vector to the plant cell using a syringe or spray method.

INCORPORATION BY REFERENCE

[0016] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0018] **FIGURE 1** shows a general workflow for the expression of a mammalian protein, exemplifying a plant vector transforming plants into protein-expressing factories. The expressed proteins are extracted and purified to yield purified proteins derived from plants.

[0019] **FIGURE 2** shows a general workflow for a method of introducing heterologous nucleic acids into plants, exemplifying introduction of a bacterial vector or viral vector through agro-infiltration. A gene gun can be used to introduce nucleic acids into a plant without the use of a bacterial or viral vector.

[0020] **FIGURE 3** shows an exemplary plasmid construct used in *Agrobacterium*, showing a human FGF-2 CDS.

[0021] **FIGURE 4** shows a western blot of expressed human FGF-2 obtained from harvested infiltrated tobacco plants.

DETAILED DESCRIPTION OF THE INVENTION

[0022] Provided herein are compositions, systems, devices and methods for plant-based production of non-plant proteins. As will be described in more detail herein are the use of (1) nucleic acid or polynucleotide sequences encoding for heterologous genes, (2) expression of heterologous proteins in plant, (3) large scale purification of such proteins (4) having stability and/or yield exceeding current commercially available options. In some embodiments, plant cells comprise a polynucleotide sequence encoding for a heterologous protease inhibitor gene and a polynucleotide sequence encoding a mammalian gene. In some embodiments, a composition comprises a mammalian protein,

chicken FGF-2 or IL1- β ; and at least one of the following: a flavonoid, rubisco, a plant-derived alkaloid, cellulose, lignocellulose, legumalin, phaselin, 11S legumin type, 7S vicilin type, gliadin, zein, hordein, secalin, and glutenin. In some embodiments, a method of manufacturing mammalian protein comprises extracting the mammalian protein from a plant cell.

[0023] Expression of non-plant proteins can be achieved through the construction of a bacterial or viral vector comprising a sequence encoding for the non-plant protein. The bacterial or viral vector is introduced into the plant, thereby producing a plant capable of producing non-plant proteins. The non-plant proteins will be extracted and purified to yield a purified non-plant protein. As exemplified in **FIGURE 1**, a plant vector transforms plants into protein-expressing factories. The expressed proteins are extracted and purified to yield purified proteins derived from plants.

FIGURE 2 describes methods that can be used to introduce nucleic acids into a plant.

[0024] As used herein, the term “about” or “approximately” can mean within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, e.g., the limitations of the measurement system. For example, “about” can mean plus or minus 10%, per the practice in the art. Alternatively, “about” can mean a range of plus or minus 20%, plus or minus 10%, plus or minus 5%, or plus or minus 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, within 5-fold, or within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term “about” meaning within an acceptable error range for the particular value should be assumed. Also, where ranges and/or subranges of values are provided, the ranges and/or subranges can include the endpoints of the ranges and/or subranges.

[0025] As used herein, the term “comprising” is intended to mean that the compositions and methods include the recited elements, but do not exclude others. “Consisting essentially of” when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination for the intended use. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. “Consisting of” shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this disclosure. Embodiments defined by each of these transition terms are within the scope of this disclosure.

[0026] “Homology” or “identity” or “similarity” can refer to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a

position in each sequence which can be aligned for purposes of comparison. When a position in the compared sequence can be occupied by the same base or amino acid, then the molecules can be homologous at that position. A degree of homology between sequences can be a function of the number of matching or homologous positions shared by the sequences. An “unrelated” or “non-homologous” sequence shares less than 40% identity, or alternatively less than 25% identity, with one of the sequences of the disclosure. Sequence homology can refer to a % identity of a sequence to a reference sequence. As a practical matter, whether any particular sequence can be at least 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to any sequence described herein (which can correspond with a particular nucleic acid sequence described herein), such particular polypeptide sequence can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence, the parameters can be set such that the percentage of identity can be calculated over the full length of the reference sequence and that gaps in sequence homology of up to 5% of the total reference sequence can be allowed.

[0027] Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

Heterologous Nucleic Acid Transfer

[0028] Heterologous protein expression can be performed through the introduction of polynucleotide sequences encoding for heterologous proteins into the leaves of a plant, such as *N. benthamiana*. Introduction of the polynucleotide sequence into the plant with a vector can yield a plant capable of expressing heterologous proteins. Subsequent downstream processing and purification yields a purified protein. In some embodiments, the polynucleotide sequence can be DNA. In some embodiments, the polynucleotide sequence can be RNA.

[0029] An isolated and purified polynucleotide segment can be combined with transcription regulatory sequences using standard molecular biology methods to yield an expression cassette. Typically, these plasmids are constructed to provide for multiple cloning sites having specificity for different restriction enzymes downstream from the promoter. The isolated and purified DNA segment can be subcloned downstream from the promoter using restriction enzymes to ensure that

the DNA is inserted in proper orientation with respect to the promoter so that the DNA can be expressed. Once the isolated and purified DNA segment is operably linked to a promoter, the expression cassette so formed can be subcloned into a plasmid or other vectors.

[0030] Provided herein are polynucleotide sequences for transfer into plant cells. In some embodiments, a polynucleotide sequence encoding the heterologous protein comprises a mammalian protein or a functional variant thereof. In some embodiments, the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence. In some embodiments, the polynucleotide sequence encoding the heterologous protein is a non-host protein. In some embodiments the mammalian protein is TGF- β , IGF-1, IGF-2, human FGF-2, IL1- β , activin A, BMP-4, VEGF, or chicken FGF-2. In some embodiments, the polynucleotide sequence encoding human FGF-2 comprises a sequence with at least 75% sequence identity to SEQ ID NOs: 23, 24, 25, 26, or 28. In some embodiments, the polynucleotide sequence encoding chicken FGF-2 comprises a sequence with at least 75% sequence identity to SEQ ID NO: 21. In some embodiments, the polynucleotide sequence encoding IL1- β comprises a sequence with at least 75% sequence identity to SEQ ID NO: 27.

[0031] In some embodiments, the polynucleotide sequence encoding human FGF-2 comprises a sequence with at least 75% sequence similarity to SEQ ID NOs: 23, 24, 25, 26, or 28. In some embodiments, the polynucleotide sequence encoding chicken FGF-2 comprises a sequence with at least 75% sequence similarity to SEQ ID NO: 21. In some embodiments, the polynucleotide sequence encoding IL1- β comprises a sequence with at least 75% sequence similarity to SEQ ID NO: 27.

[0032] Provided herein are plant cells comprising at least 1 polynucleotide sequence encoding a mammalian gene or functional variant thereof. In some embodiments, the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence. In some embodiments, the plant cell comprises a least 1 polynucleotide sequence encoding at least 1, 2, 3, 4, 5, or 6 mammalian, non-plant genes, or any functional variant thereof. In some embodiments, the plant cell comprises a least 1 polynucleotide sequence encoding at least 1 - 6 mammalian, non-plant genes or any functional variant thereof.

[0033] Provided herein is a plant cell comprising a polynucleotide sequence encoding a protease inhibitor to prevent endogenous degradation of the non-plant protein. In some embodiments, the protease inhibitor is a gene encoding a plant protease inhibitor or a non-plant protease inhibitor. In some embodiments, the protease inhibitor has at least 75% sequence similarity to SEQ ID NO: 22. In some embodiments, the protease inhibitor is SICYS8.

[0034] Provided herein are methods of introduction of a heterologous polynucleotide sequence into a plant using a bacterial or viral vector. In some embodiments, the vector is introduced using agroinfiltration. In some embodiments, agroinfiltration is performed by a syringe-based method. In some embodiments, agroinfiltration is performed using a spray that disperses a solution containing the vector onto the surface of the plant, thereby contacting the vector to the plant. In some embodiments, the solution contains a wetting agent. In some embodiments, the wetting agent comprises Tween 20.

Expression of Heterologous Proteins

[0035] Provided herein are plants comprising any of the protein-encoding polynucleotides described above. The polynucleotides encode non-plant proteins, which can be expressed in the plant. In some embodiments, the non-plant proteins are mammalian proteins. In some embodiments the mammalian protein is TGF- β , IGF-1, IGF-2, human FGF-2, IL1- β , activin A, BMP-4, VEGF, or chicken FGF-2. In some embodiments, the plant cell comprises SICYS8 and/or said mammalian proteins, wherein the mammalian protein is selected from the group consisting of TGF- β , IGF-1, IGF-2, human FGF-2, IL1- β , activin A, BMP-4, VEGF, or chicken FGF-2. In some embodiments, human FGF-2 comprises a sequence with at least 75% sequence identity to SEQ ID NOs 1, 2, 3, 4, 5, or 6. In some embodiments, chicken FGF-2 comprises a sequence with at least 75% sequence identity to SEQ ID NO: 8. In some embodiments, IL1- β comprises a sequence with at least 75% sequence identity to SEQ ID NO: 9. In some embodiments, human FGF-2 comprises a sequence with at least 75% sequence similarity to SEQ ID NOs 1, 2, 3, 4, 5, or 6. In some embodiments, chicken FGF-2 comprises a sequence with at least 75% sequence similarity to SEQ ID NO: 8. In some embodiments, IL1- β comprises a sequence with at least 75% sequence similarity to SEQ ID NO: 9.

[0036] Provided herein are protease inhibitors expressed by a plant. In some embodiments, the plant expresses a protease inhibitor. In some embodiments, the protease inhibitor has 75% sequence identity to SICYS8. In some embodiments, the protease inhibitor has 75% sequence similarity to SEQ ID NO: 7.

[0037] The plant cell can include more than one heterologous protein. In some embodiments, the plant cell comprises at least one protease inhibitor protein or any functional variant thereof and at least one non-plant or mammalian protein or any functional variant thereof. In some embodiments, the plant cell comprises SICYS8 and at least 1 non-plant or mammalian protein. In some embodiments, the plant cell comprises 1) SICYS8 and 2) human FGF-2, chicken FGF-2, IL1- β , or any combination thereof.

Vectors

[0038] Provided herein are vectors for use in transfer of heterologous polynucleotides into plant cells. In some embodiments, the vector is bacterial or viral. Exemplary bacterial vectors can be *Agrobacterium* species. Exemplary viral vectors can be Tobacco mosaic virus (TMV). In some embodiments, the bacterial vector is *Agrobacterium tumefaciens*. In some embodiments, the viral vector is Tobacco mosaic virus (TMV), tobacco rattle virus (TRV), tobacco etch virus (TEV), cowpea mosaic virus (CPMV), potato X virus (PVX), or any variant or strain thereof.

[0039] *Agrobacterium tumefaciens* is a soil-borne pathogen that is widely used to introduce heterologous polynucleotides into plant cells, including plant cells from a plant. *A. tumefaciens* transfers a particular polynucleotide segment of a tumor-inducing (Ti) plasmid into the nucleus of infected host cells. Advantageously, heterologous polynucleotides can be placed between the borders of the Ti plasmid and transferred to plant cells.

[0040] A polynucleotide sequence of interest can be introduced into a competent bacterial strain for nucleic acid transfer (e.g., *Agrobacterium*) via conventional transformation methods. The bacterial strain can be used to introduce the nucleic acid of interest into a plant, plant part, tissue, or cell. Many vectors are available for transformation of *Agrobacterium*. These typically carry at least one T-DNA border sequence and can include vectors such as pCambia, pSim24 or any variant thereof.

[0041] *Agrobacterium* transformation can involve the transfer of a binary vector carrying the foreign nucleic acid of interest to an *Agrobacterium* strain which may depend on the complement of vir genes carried by the host *Agrobacterium* strain either on a co-resident Ti plasmid or chromosomally. The transfer of the recombinant binary vector to *Agrobacterium* can be accomplished by a tri-parental mating procedure using *E. coli* carrying the recombinant binary vector, a helper *E. coli* strain that carries a plasmid and which is able to mobilize the recombinant binary vector to the target *Agrobacterium* strain. Alternatively, the recombinant binary vector can be transferred to *Agrobacterium* by DNA transformation.

[0042] A polynucleotide of interest can be transformed into the *Agrobacterium* strain or other bacterial strain competent for nucleic acid transfer for subsequent transformation of a plant using the methods as disclosed herein. In some embodiments, the *Agrobacterium* is transformed using electroporation. In some embodiments, the nucleic acid is a polynucleotide construct comprising an expression cassette that comprises functional elements that allow for expression of a polynucleotide of interest in a plant following its introduction via the *Agrobacterium*-mediated transformation methods of the disclosure.

[0043] An expression cassette can comprise a nucleic acid encoding a polynucleotide that confers a property that can be used to detect, identify or select for transformed plant cells and tissues (e.g., a marker for the selection of transformed cells). The nucleic acid encoding the marker may be on the same expression cassette as the nucleotide sequence of interest, or may be co-transformed on a separate expression cassette. In some embodiments, the nucleic acid encoding the marker can be the nucleotide sequence of interest. Thus, the nucleic acid of interest comprises an expression cassette that further comprises a nucleotide sequence conferring resistance to a selection agent, and thus, selecting comprises culturing the *Agrobacterium*-inoculated a plant tissue or cell thereof in a medium comprising the selection agent, and selecting a transformed plant tissue or cell thereof comprising the nucleic acid of interest.

Delivery of Nucleic Acids

[0044] Disclosed herein are steps, or the method of manufacturing, directed to introducing an isolated and purified DNA sequence, such as a polynucleotide sequence containing a heterologous protein (i.e. mammalian or non-plant protein), into a plant cell to produce a transformed plant cell. In some embodiments, the transformed plant cell exhibits transient expression of the heterologous protein.

[0045] Cells of the plant tissue source can be embryogenic cells or cell-lines that can regenerate fertile transgenic plants and/or seeds. The cells can be derived from either monocotyledons or dicotyledons. Suitable examples of plants include, but are not limited to, wheat (e.g., *Triticum* species), rice (e.g. *Oryza* species), *Nicotiana* (e.g., *Nicotiana benthamiana*), *Arabidopsis*, tobacco (*Nicotiana* species) , maize (e.g., *Zea* species), soybean (e.g., *Glycine* species), oat (e.g., *Avena*), and the like.

[0046] The choice of plant tissue source for transformation can depend on the nature of the host plant and the transformation protocol. Useful tissue sources include callus, suspension culture cells, protoplasts, leaf segments, stem segments, tassels, pollen, embryos, hypocotyls, tuber segments, meristematic regions, and the like. The tissue source is selected and transformed so that it retains the ability to regenerate whole, fertile plants following transformation, i.e., contains totipotent cells.

[0047] The transformation is carried out under conditions directed to the plant tissue of choice. The plant cells or tissue are exposed to the DNA carrying the isolated and purified DNA sequences for a period of time. This may range from a few minutes to 2-15 days co-cultivation in the presence of plasmid-bearing *Agrobacterium* cells. Buffers and media used can vary with the plant tissue source and transformation protocol.

[0048] After transformation, the plant is grown for a period time in order to allow for accumulation of the heterologous protein within the plant. In some embodiments, the period of time is from about a 1 hour to about 15 days. In some embodiments, the period of time is 1 hour, 2 hours, 6 hours, 12 hours or 1, 5, 6, 7, 8, 9, 10, or 15 days. In some embodiments, the period of time is from 1- 12 hours, 1 hour – 1 day, 1- 5 days, 1-10 days, 1- 15 days, 1 – 5 days, 1-10 days, 1- 15 days, 5 – 6 days, 5-7, days, 5- 8 days, 5-9 days, 5-10 days, or 5- 15 days.

[0049] The method of manufacturing a mammalian protein includes culturing a plant cell in a growth media. Introduction of a nucleic acid sequence into a plant cell provides the plant the ability to express a protein. Following expression of the protein, the mammalian protein is extracted to generate an extraction product. Growth media can include soil or agar-based media supplemented with factors necessary for growth or germination.

[0050] Introduction of a nucleic acid into a plant cell may involve use of a bacterial or viral vector. In some embodiments, the bacterial vector is an *Agrobacterium* species. In some embodiments, the bacterial vector is *Agrobacterium tumefaciens*. In some embodiments, the viral vector is Tobacco mosaic virus (TMV), tobacco rattle virus (TRV), tobacco etch virus (TEV), cowpea mosaic virus (CPMV), potato X virus (PVX), or any variant or strain thereof.

[0051] In some embodiments, the bacterial or viral vector is introduced to the plant cell by contacting the plant cell with the bacterial or viral vector. The contacting may involve mediating physical contact between the plant cell and the bacterial or viral vector. In some embodiments, the physical contact is mediated through use of a syringe-based system. In some embodiments, the contacting is performed on the leaves of a plant, thereby forming an infiltrated leaf.

[0052] Introduction of a nucleic acid can include dispersal of a solution to initiate contact of the nucleic acid into a plant cell. In some embodiments, introduction of a nucleic acid may include using a spray method. The spray method can comprise a solution containing bacterial or viral vector. The bacterial or viral vector can be suspended in a solution and sprayed using a spray-based nozzle or sprinkler system to disperse the solution into a mist that covers a broad area containing plants. In some embodiments, the introduction is performed by contacting a syringe containing the bacterial or viral vector to a plant cell.

[0053] Introduction of a polynucleotide sequence encoding a non-plant protein may use a method of introduction of a polynucleotide instead of using a vector-based system. In some embodiments, introduction of a polynucleotide sequences into a plant cell comprises use of a gene gun. In some embodiments, the polynucleotide sequence is RNA or DNA.

Large Scale Purification

[0054] Purification of large amounts of protein can include purification of large amounts of plant biomass in order to recover substantial amounts of the non-plant or mammalian protein. This may include the use of multiple plants in a vertical farm or large greenhouse that provides a scalable and controlled environment to grow multiple plants that produce the protein of interest.

[0055] After culturing the plant cells, a mammalian protein accumulates in a plant cell and is extracted to generate an extraction product or composition comprising the mammalian protein. The composition may also comprise impurities or non-mammalian components. In some embodiments, the impurities may be present in trace quantities. In some embodiments, the impurities are flavonoids, rubisco protein, proteases, proteins, plant-derived alkaloids, cellulose, lignocellulose, legumalin, phaselins, 11S leghemoglobin type, 7S vicilin type, gliadins, zeins, hordeins, secalins, or glutenins. During purification, the purified protein can contain trace amounts of plant-derived materials or impurities. The impurities may comprise a portion of the composition. In some embodiments, the impurities constitute no more than 0.1% of the composition. In some embodiments, the impurities constitute no more than 0.07%, 0.06%, 0.05%, 0.04%, 0.03%, 0.02%, or 0.01% of the composition. In some embodiments, the impurities constitute 0.0% - 0.1% of the composition.

[0056] In some embodiments, the composition may further comprise an anticoagulant. In some embodiments, the anticoagulant is heparin or a salt thereof. In some embodiments, the anticoagulant is present at 0.1 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$. In some embodiments, the anticoagulant is present at no more than 2 $\mu\text{g/ml}$.

[0057] Purification of the mammalian protein can be performed by purification methods commonly known by one skilled in the art. In some embodiments, the mammalian protein is purified by affinity chromatography, ion-exchange chromatography, size-exclusion chromatography, immobilized metal affinity chromatography, or any combination thereof.

[0058] After purification, post-processing steps can be used to optimize the functionality of the purified protein. In some embodiments, an affinity tag is fused to a non-plant protein to allow for ease of purification using affinity chromatography or purification. The affinity tag can be removed to prevent interference of the tag with protein function. In some embodiments, a protease can be used to cleave an affinity tag used in affinity chromatography, thereby generating a "tag-less" non-plant protein. In some embodiments, the affinity tag can be a poly-His-tag, a Strep-tag, an E-tag, or other epitope tags commonly used in purification. In some embodiments, the protease is an enterokinase.

[0059] In some embodiments, the non-plant or mammalian protein is produced as a proprotein or as a zymogen, thus not functional without additional processing steps. In some embodiments, a protease is used to render the proprotein into a mature protein.

[0060] Extraction of the mammalian protein may exceed yields that are currently available through other methods. Extracting the mammalian protein to generate the extraction product. In some embodiments, the extraction product comprises a mammalian protein is present in an amount of at least 40 μg per gram of biomass. In some embodiments, the extraction product comprises a mammalian protein is present in an amount of at least 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, or 55 μg per gram of biomass. In some embodiments, the extraction product comprises a yield of 40-55 μg per gram of biomass.

Method of Manufacture

[0061] Provided herein are methods of manufacturing a mammalian from a plant cell comprising the polynucleotide sequences encoding the non-plant protein. Plants containing the non-plant protein or mammalian protein can be cultured until a desired amount of protein is produced. The extraction of the plant generates an extraction product comprising a mammalian protein.

[0062] Culturing of plant cells can be performed by growing plants or plant cells in suitable growth media such as soil or agar supplemented with factors needed for growth or seed germination. In some embodiments, the culturing of plant cells or plants occurs in a green house, or other facility that provides a controlled environment that allows for plant growth. Culturing of plants in a scalable manner can provide an increase of potential biomass harvested in the same area used. In some embodiments, vertical farming techniques are used to grow the plants.

[0063] The method of manufacturing the mammalian protein may exceed yields that are currently available through other methods. Extracting the mammalian protein to generate the extraction product. In some embodiments, the extraction product comprises a mammalian protein is present in an amount of at least 40 μg per gram of biomass. In some embodiments, the extraction product comprises a mammalian protein is present in an amount of at least 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, or 55 μg per gram of biomass. In some embodiments, the extraction product comprises a yield of 40-55 μg per gram of biomass.

[0064] Following purification, the non-plant or mammalian protein can be further sterilized. In some embodiments, the sterilization comprises filtering, irradiation, endotoxin purification, or any combination thereof.

[0065] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

Examples

Example 1: Germination of *Nicotiana benthamiana* seedlings

[0066] To prepare *Nicotiana benthamiana* for introduction of a transgene, *N. benthamiana* seeds are incubated for 3 days on moist jiffy pads at 30 degree Celsius in an incubator with a 16-hour light and 8-hour dark cycles. Germinated seeds are transferred into moist soil for further growth.

Example 2: Preparation of the Plant Expression Vector

[0067] *Preparation of Competent E. coli*

[0068] *E. coli* must be competent in order to take up an exogenous nucleic acid such as a plasmid. To do this, 100 μ L of *E. coli* is added to 1 mL of liquid LB or SOC and are incubated overnight at 37 °C, 225 RPM.

[0069] TSS buffer is prepared (30 mM MgCl₂, 5% DMSO, 10% PEG (3350 or 5000)) in LB medium under sterile conditions using aseptic technique and filtered under the laminar flow biochemical hood. The prepared buffer is stored at 4 °C until needed.

[0070] Following the overnight culture, a small amount of the overnight culture is sub-cultured into a larger volume of LB for 2-3 hours until the OD₆₀₀ reaches 0.3-0.4. The culture is then centrifuged at 2700 x g for 10 minutes at 4 °C, the supernatant is removed, and the pelleted cells are resuspended in 5 ml of pre-chilled TSS buffer. The resuspended cells are chilled on ice for 15 minutes. After chilling, the chilled cells will be aliquoted into appropriate freezer tubes and frozen at -80 °C.

Preparation of the Bacterial Vector

[0071] 1 μ L of pCambia2300 (Marker Gene Technologies, PN: M1709) (plasmid (~ 1000 pg)) is added to 100 μ L of competent *E. coli* cells and is mixed well and incubated for 30 minutes at 4 °C. 900 μ L of liquid LB media supplemented with 20 mM glucose is added to the chilled bacteria and incubated at 37 °C at 225 RPM for 1 hour.

[0072] 900 μ L of the incubated bacteria is added to 40 mL of liquid LB media supplemented with 50 μ g/mL kanamycin and incubated overnight at 37 °C at 225 RPM. The plasmid is purified according

to the GenElute plasmid MidiPrep purification kit according to the manufacturer's instructions (Sigma, PLD35).

[0073] The remaining 100 μ L is plated onto LB-agar supplemented with the appropriate antibiotic and stored at 4 °C. Bacterial stocks are prepared for long-term storage by preparing a stock containing a final concentration of 25% glycerol.

Example 3: Transformation of *Agrobacterium tumefaciens*

[0074] *Agrobacterium tumefaciens* LBA4404 (Invitrogen) is electroporated with the nucleic acid vector. Bacterial colonies are verified for uptake of the nucleic acid vector prior to electroporation into *Agrobacterium*.

[0075] *Agrobacterium* LBA4404 is thawed on ice. 1 μ L of the vector is added to 20 μ L of competent cells and mixed gently. Cell:plasmid mixture is added to a 0.1 cm electroporation cuvette (Bio-rad) on ice. The Gene Pulser II is configured with the following parameters: 25 μ F, 300 Ω , and 2 - 2.5 kV. 1 mL of SOC media is added to electroporated cells and transferred to a 1.5 ml tube and incubated for 1 hour at 28 °C, shaking at 100 rpm. Plate 50 - 100 μ l of cells on LB agar plates with 50 μ g/ml kanamycin and 100 μ g/ml streptomycin and incubate for up to 48 hours at 28 °C.

Example 4: Infiltration of *N. benthamiana* via Syringe

[0076] A clonal population of *Agrobacterium* is grown in 5ml of LB with kanamycin and streptomycin. 1 mL of the overnight culture is inoculated into 25 mL LB supplemented with 20 μ M acetosyringone and grown overnight at 28 °C at 100 rpm. Plate 50 - 100 μ l of cells on LB agar plates with 50 μ g/ml kanamycin and 100 μ g/ml streptomycin for up to 48 hours at 28 °C.

[0077] After overnight culture, bacteria is precipitated at 5000 x g for 15 minutes and resuspended such that the OD₆₀₀ is equal to about 0.5 with MM media (10 mM MES, 10 mM MgCl₂, pH 5.6 (KOH). After adjustment, incubate the flask at room temperature for 1-3 hours or overnight.

[0078] Infiltration via syringe is performed by using a 5 ml syringe without a needle. Applying the syringe to the underside of the leaf while exerting counter pressure with your finger on the other side. Successful infiltration is overused as spreading (i.e. wetting) area in the leaf. Infiltration should be performed when fully expanded *N. benthamiana* leaves are present (preferentially in the morning when the stomata are open) with the *A. tumefaciens* suspension at different places to the abaxial part using a 5 ml syringe without needle.

[0079] After 2-5 days, expression of the transgene can be observed to verify successful infiltration.

Example 5: Spray-mediated Infiltration of *N. benthamiana*

[0080] Infiltration of *Agrobacterium* can also be performed using a spray method (**FIGURE 2**) that would be able to be scalable compared to syringe-based infiltration. From the back-up plate or bacteria glycerol stock, take cells and inoculate 5ml of selective LB media (streptomycin/kanamycin). Incubate overnight at 28 °C with 225 rpm shaking. 5 ml of *Agrobacterium* culture is added to 250ml of selective LB-media (streptomycin/kanamycin) and will be incubated for 48 hours to 72 hours at 28 °C with 225 rpm shaking. After incubation, the cells are centrifuged at 4000 x g for 10 minutes. Discard the supernatant. Resuspend the cells in 10 ml of MM medium (10 mM MES, 10 mM MgCl₂, pH 5.6 (KOH)) and incubate at room temperature for one hour in the dark. MM medium is added to adjust the OD at 1.3-1.5 in a total volume of 500ml, add Tween 20 for 0,1% (v/v) final concentration. Spray the solution directly onto the plants in the incubator at 23 °C. Leave the plant for protein expression for 10 to 14 days with daily watering.

Example 6: Protein Extraction

[0081] For purification, plant material is extracted using the buffer containing 20mM citric acid, 20mM Na₂HPO₄, and 30 mM NaCl in a 5:1 (v/w) buffer: biomass ratio. The extraction is carried out at pH 4.

[0082] To prepare 50ml of the extraction buffer, weigh 192 mg of acid citric, 120 mg of NaH₂PO₄, and 88 mg of NaCl. In a beaker, the reagents are diluted in 50 ml in distilled water, and the pH adjusted to pH 4. Store the extraction buffer at 4°C.

[0083] Ground leaf material supplemented with pre-chilled extraction buffer is incubated at room temperature under constant agitation for 30 min followed by centrifugation at 10,000 x g for 15 min. The supernatant is filtered using Miracloth followed by incubation of the filtrate for 20 min at room temperature and centrifugation for 30 min at 10,000 x g at room temperature.

[0084] For poly-histidine-tagged proteins, Ni-NTA Dynabeads are used. For preparation of protein prior to purification via Dynabeads, transfer 50 µL (2 mg) Dynabeads™ magnetic beads to a microcentrifuge tube and is placed on a magnet for 2 minutes. Aspirate and discard the supernatant. The sample (prepared in 1X Binding/Wash Buffer) is added to the beads and is incubated for 5 minutes at room temperature (or colder if the protein is unstable at room temperature). The incubation time may be increased up to 10 minutes. The tube is placed on the magnet for 2 minutes, then discard the supernatant. The beads are washed 4 times with 300 µL 1X Binding/Wash Buffer by placing the tube on a magnet for 2 minutes and discarding the supernatant. The beads are resuspended thoroughly between each washing step. To use bead/protein complexes in other applications, the bead/protein complex is resuspended in a suitable volume of 1X Pull-down Buffer (or other buffer compatible with your downstream application). 100 µL His-Elution Buffer is added

to the suspension and is incubated on a roller for 5 minutes at room temperature (or colder if the protein is unstable at room temperature). A magnet is applied for 2 minutes and the supernatant containing the eluted histidine-tagged protein is transferred to a clean tube. To measure protein concentration, a Bradford assay or protein quantification using a Qubit fluorometer is used.

Enterokinase Cleavage

[0085] *Enterokinase storage buffer preparation 10ml (20mM Tris-HCl, 200mM NaCl, 2mM CaCl₂ and 50% glycerol)*

[0086] Enterokinase reaction:

[0087] The enterokinase cleavage reaction is prepared with the fusion protein mixed into a reaction mixture composed of the reaction buffer (200mM Tris-HCl, 500mM NaCl, 20mM CaCl₂, pH 8) and the enterokinase enzyme. A negative control is generated (no enterokinase) in which 5µl of Enzyme Storage buffer is used in place of Enterokinase. Collect all components by a brief centrifugation. The reaction is incubated at 25 °C and aliquots are removed for analysis at various timepoints, and prepare aliquots for analysis by SDS-PAGE. The optimal time for cleavage analysis is analyzed by comparing the amount of cleaved and uncleaved protein at each time point via SDS PAGE. Another Dynabeads purification is performed to remove any uncleaved protein as well as cleaved poly-His tag. Residual enterokinase is removed using the Enterokinase Removal Kit (Sigma Aldrich Cat No: PRKE). To measure protein concentration of the supernatant, a Bradford assay or protein quantification using a Qubit fluorometer is used.

Example 7: Plant Cultivation Conditions

[0088] For this study, plants were cultivated in a greenhouse or a growth container.

Greenhouse cultivation

[0089] Plants were seeded into Proptek propagation 231 deep cell tray (1020 format) and filled with ProMix fine soil using a Speedy Seeder (Carolina Greenhouses, NC, USA). The conditions for germination and cultivation are described:

[0090] 150+-50 µmol m⁻² s⁻¹ for the germination that takes place into the Percival chambers;

[0091] The temperature was set at 26°C during the day and 25°C during the night. Svenson clothes were always closed on the top. Supplemental lightning was given to the plants with High-Pressure Sodium (HPS) to complete the photoperiod, and light was provided once the external sensor of the greenhouse was below 150 micromoles first and then 200 micromoles; the photoperiod was set at 16

hours of light and 8 hours of darkness; the environmental humidity was set at 50% to reduce microbiological problems.

[0092] The seeds were irrigated with a nutrient solution obtained diluting a stock MiracleGro 24-8-16 + micronutrients with the following characteristics: Electrical conductivity: $1 \text{ dS/m} \pm 0.2$ and pH: 5.7 ± 0.2 .

[0093] The MiracleGro stock was prepared by diluting 200 g of fertilizer/liter of stock. The transplant takes place at 14 days after seeding (DAS 14). The transplant was performed manually from the seeded flat into 3.5 inch x 3.5 inch square pots filled with MiracleGro Potting soil with wetting control agents. The pots were filled the day before the transplant and soaked with water to arrive at the field capacity. In order to provide an irrigation system using 1020 trays, two different 1020 flats were placed on top of each other: one with holes and one with no holes at the bottom. In the top, 1020 flats held 18 pots and, in this way, it was possible to fill and drain the pots all at once.

[0094] The greenhouse was set up with benches that were used only to facilitate the watering operations. Plants were spaced for the first time at DAS 21, placing only 9 plants/flat instead of 18.

[0095] At DAS 28, temperatures were lowered at $22^\circ\text{C} \pm 2$, plants were spaced at 5 or 6 per flat before agroinfection takes place. Plants were harvested after 4 more days of cultivation, the first two days a lower temperature ($22 \pm 2^\circ\text{C}$) was used. The last two days were cultivated with conditions adopted during cultivation ($26 \pm 2^\circ\text{C}$ during the day and $25 \pm 2^\circ\text{C}$ at night).

Growth Container Cultivation

[0096] The container cultivation conditions were provided: $150 \pm 50 \text{ } \mu\text{mol m}^{-2} \text{ s}^{-1}$ (germination that takes into the germination racks with Jiffy 44 seeded, see below), temperature set at 26°C during the day and 25°C during the night; the photoperiod was set at 16 hours of light and 8 hours of darkness; and the environmental humidity was set at 70% during germination and then, for the latter phases, at 50% to reduce the risk of microbiological problems.

[0097] Plants were sown using the Speedy Seeder (Carolina Greenhouses, NC, USA) in a 1020 flat containing Jiffy 7 plugs prehydrated and contained into a plastic net. After the seeding operations, the Jiffys were spaced into QuickPot 45 R which can host 45 sown Jiffys. The Quickpots trays were placed onto the germination racks and after 2-3 days the germination should be evident. After 1 week, environmental humidity was decreased to 50%. After 2 weeks, plants were spaced by placing 5 plants/Quickpot tray and grown into the cultivation racks for another 2 weeks, where they will be agro infected with a solution containing *Agrobacterium tumefaciens*. The harvest took place 4 days later by removing the trays from the irrigation systems and manually cutting the leaves.

[0098] The seeds and plants were fertigated with a nutrient solution described below, which stock is composed the diluted fertilizer prepared as shown in **Table 3**.

Table 3: Recipe of Fertilizer used in Growth Chambers

Tank A

Component	Mass (g)	ml/grams	Volume to Add (L)	% of Final Solution
Jack's B Calcium Nitrate	3900	0.45	1.76	8.8
Iron EDDHA Chelate 6%	200	0.52	0.1	0.5
Water			18.14	90.7
Total Tank A Fertilizer Volume			20	100

Tank B

Component	Mass	ml/grams	Volume to Add (L)	% of Final Solution
Jack's 5-12-26 Part A	3000	0.47	1.41	7.05
Monopotassium phosphate	700	0.47	0.33	1.65
Water volume			18.26	91.3
Total Tank Fertilizer B Volume			20	100

[0099] The stock was further prepared with the following characteristics: Electrical conductivity: 1 dS/m $\pm$ 0.2 during the first week after transplant, then for the latter growing phases 1.5 dS/m $\pm$ 0.2, and pH: 5.7 $\pm$ 0.2.

Example 8: Expression of human FGF-2 in *N. benthamiana* via Agrobacterium transformation

[0100] Plasmid construct pTIA2-hFGF2 was synthesized by Genscript using the backbone of pCAMBIA0380 (**FIGURE 3**). The plasmid construct includes a ubiquitin-10 promoter from *Arabidopsis thaliana* in order to drive expression of the TMV Omega enhancer sequence followed by *N. benthamiana* (tobacco plant) codon optimized human FGF-2 (SEQ ID NO: 24) with a 6x Histidine tag on the N' terminal end of the hFGF-2 polypeptide. The CDS is followed by the NOS terminator. No plant-specific selectable marker was included.

Agrobacterium hFGF-2 stock preparation:

[0101] The plasmid construct pTIA2-hFGF2 was electroporated into *Agrobacterium tumefaciens* strain GV3101 (AbCam) as described in **Example 3** (25 μ F, 300 Ω , and 2 - 2.5 kV). The electroporated *Agrobacterium* was grown for 2 days at 28°C on LB agar plates supplemented with 200mM acetosyringone, 25mg/L rifampicin, 50mg/L gentamicin, and 100mg/L kanamycin.

[0102] A single colony was selected and used to prepare a 25% glycerol stock and be frozen at -80C. The presence of the hFGF-2 was confirmed by PCR.

[0103] Agrobacterium preparation for N. benthamiana infiltration

[0104] Frozen glycerol stocks of transformed *Agrobacterium* containing the hFGF-2 expressing plasmid were streaked on LB agar plates supplemented with 200 mM acetosyringone, 25 mg/L rifampicin, 50 mg/L gentamicin, and 100 mg/L kanamycin and incubated at 28 °C for 2-3 days.

[0105] After incubation, a single colony was selected and used to inoculate a 500ml baffled flask containing 100mL of LB broth supplemented with 200mM acetosyringone, 25mg/L rifampicin, 50mg/L gentamicin, and 100mg/L kanamycin. The culture was incubated in a shaking incubator for 16 – 18 hours at 220 RPM.

[0106] After incubation, 100 mL of the bacterial culture was transferred to two 50 mL falcon tubes. The falcon tubes were centrifuged at 4000 RPM for 30 minutes. The supernatant was discarded.

[0107] 1L of MMA medium containing 10ml 1M MES at pH5.6, 10ml of 1M MgCl₂, 1ml of 200uM acetosyringone was prepared and the bacterial pellet was resuspended in the 1L of MMA medium. The resuspended bacteria was incubated for 2 hours at 22 °C.

[0108] Following incubation, 10 μ M Lipoic acid, 100 mg/L L-cysteine, 125 mg/L STS, 75 mg/L DTT, 0.002% Pluronic F-68 was added to the resuspended culture. Silwet L-77 was added to attain a concentration of 0.01%.

[0109] 4 week old tobacco plants in a vacuum desiccator were vacuum infiltrated by applying 0.02 mPa for 1 minute.

[0110] Incubate *N. benthamiana* plants were vacuum infiltrated in a growth chamber at 22 °C for two days and then 24 °C for a third day.

Example 9: Extraction of total soluble protein (TSP) from tobacco leaves

[0111] An extraction buffer was prepared by mixing a solution containing the following concentrations of reagents: 50mM sodium phosphate pH 7.4, 300mM NaCl, 10mM imidazole, 0.1%

TritonX-100, 40mM ascorbic acid, EDTA-free protease inhibitor cocktail tablets (as per manufacturer instruction).

[0112] The leaves of the infiltrated (3-days post-infiltration) *N. benthamiana* plants from **Example 8** were harvested and frozen at -80 °C.

[0113] 3 grams of the frozen leaf tissue was placed into a 50 mL SPEX tube with two 11 mm metal beads and 10 mL of extraction buffer on ice. The tubes were placed in tube adaptors for the SPEX Geno/Grinder. The adaptors are prechilled at -80 °C.

[0114] The frozen leaves were ground in the SPEX grinder using five 30 seconds bursts at 1500 RPM with 30 seconds of rest time between bursts.

[0115] Following the grinding, the tubes were centrifuged at 4000 RPM for 10 minutes at 4 °C. The pellet contains cellular debris. The leaf material and supernatant was poured through 4 layers cheesecloth into a new falcon tube and centrifuged at ~7000 x g for 30 minutes at 4 °C. The supernatant containing the total soluble protein (TSP) was transferred to a new falcon tube.

[0116] The pH of the TSP was adjusted to a final pH of between 7 and 8 with KOH (potassium hydroxide). The pH adjusted TSP was centrifuged for 15 minutes at ~7000 x g at 4 °C. The supernatant was transferred to a new falcon tube.

Example 10: Detection of human FGF-2

Protein gel:

[0117] A BioRad gel rig loaded with a TGX (Tris Glycine Extended) 4-15% gradient gel prepared with TGS (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.6) running buffer.

[0118] 2.5 µL of 4x Laemmli buffer and 7.5 µL TSP sample was prepared. A positive control was prepared with 10 ng of hFGF2. The samples were denatured by heating the samples at 70 °C for 10 minutes. 10 µL of sample was loaded into the wells and 5 µL of PAGERuler prestained ladder (ThermoFisher) in at least one well. The gel was run at 200V for 30 minutes.

Immunoblot:

[0119] The BioRad Trans-Blot Turbo and Trans-Blot® Turbo™ Mini PVDF Transfer Packs were used to transfer protein from gel to membrane. The gel was transferred to a methanol activate PVDF membrane using the preset 3 minute TGX mini gel transfer protocol. The transferred membrane was placed into a black box containing freshly made 0.75 grams bovine serum albumin and 25 mL of TBS-T blocking buffer and incubated with gentle shaking for 1 hour at room temperature or overnight at 4 °C.

[0120] The blocking buffer was removed. 10 μ L of monoclonal mouse anti-hFGF2 primary antibody (Invitrogen) with 25 mL TBST (1:2500 dilution) was added to the membrane and incubated with gentle rocking for either for 4 hours at room temperature or overnight at 4 °C.

[0121] The primary antibody was removed and washed 3 times with TBST, where each was performed for 10 minutes at room temperature using 50 mL TBS-T.

[0122] The secondary antibody solution was prepared using anti-mouse NIR800 at 1:10000 by mixing 3ul in 30 mL TBST. The solution was added to the membrane and incubated at room temperature for 1 hour with gentle shaking.

[0123] The secondary antibody solution was removed and washed 3 times with 50 mL TBST, as described above.

[0124] The membrane was imaged at 700 nm (ladder) and 800 nm h-FGF2. Human FGF-2 (SEQ ID NO: 2) is approximately 18 kDa. R2-V TSP (Lane 3) and R3-V TSP (Lane 5) show expression of human FGF-2 (**Figure 4**). The expression construct used in Lane 5 is derived from the construct illustrated in **Figure 3** but lacks the TMV Omega enhancer.

[0125] Quantification of hFGF-2 was performed using the hu-FGF basic ELISA kit from Invitrogen and performed according to manufacturer's protocol. ELISA was performed on the total soluble protein (TSP). The TSP was tested at two dilutions: 2x and 20x. Calculated yield based on standard curve was 560-808 pg/ml.

[0126] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

Table 1: Amino Acid sequences of Mammalian Proteins

SEQ ID NO	Name	Sequence
SEQ ID NO 1	Human FGF-2 (full length)	MVGVGGGDVEDVTPRPGGCQISGRGARGCNGIPGA AAWEAALPRRRPRRHPSVNPRSRAAGSPRTRGRRT ERPSGSRLGDRGRGRALPGGRLGGRGRGRAPERVG GRGRGRGTAAPRAAPAARGSRPGPAGTMAAGSITT LPALPEDGGSGAFPPGHFKDPKRLYCKNGGFFLRIHP DGRVDGVREKSDPHIKLQLQAEERGVSIIKGVCAN

		RYLAMKEDGRLLASKCVTDECFFFERLESNNYNTY RSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLP MSAKS
SEQ ID NO 2	Truncated human FGF-2 (18 kDa)	MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKN GGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERG VVSIGKVCANRYLAMKEDGRLLASKCVTDECFFFERLE SNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGPGQ KAILFLPMSAKS
SEQ ID NO 3	>sp P09038 FGF2 _HUMAN Fibroblast growth factor 2 OS=Homo sapiens OX=9606 GN=FGF2 PE=1 SV=3	MVGVGGGDVEDVTPRPGGCQISGRGARGCNGIPGA AAWEAALPRRRPRRHPSVNPRSRAAGSPRTRGRRT ERPSGSRLGDRGRGRALPGGRLGGRGRGRAPER VGRGRGRGTAAPRAAPAARGSRPGPAGTMAAGSIT TLPALPEDGGSGAFPPGHFKDPKRLYCKNNGFFLRIHP DGRVDGVREKSDPHIKLQLQAEERGVSIGKVCAN RYLAMKEDGRLLASKCVTDECFFFERLESNNYNTY RSRKYTSWYVALKRTGQYKLGSKTGPGQKAILFLP MSAKS
SEQ ID NO 4	>sp P09038- 1 FGF2_HUMAN Isoform 2 of Fibroblast growth factor 2 OS=Homo sapiens OX=9606 GN=FGF2	MGDRGRGRALPGGRLGGRGRGRAPERVGGGRGR GTAAPRAAPAARGSRPGPAGTMAAGSITTLPALPED GGSGAFPPGHFKDPKRLYCKNNGFFLRIHPDGRVDG VREKSDPHIKLQLQAEERGVSIGKVCANRYLAMK EDGRLLASKCVTDECFFFERLESNNYNTYRSRKYTS WYVALKRTGQYKLGSKTGPGQKAILFLPMSAKS
SEQ ID NO 5	>sp P09038- 2 FGF2_HUMAN Isoform 3 of Fibroblast growth factor 2 OS=Homo sapiens OX=9606 GN=FGF2	MAAGSITTLPALPEDGGSGAFPPGHFKDPKRLYCKN GGFFLRIHPDGRVDGVREKSDPHIKLQLQAEERG VVSIGKVCANRYLAMKEDGRLLASKCVTDECFFFERLE SNNYNTYRSRKYTSWYVALKRTGQYKLGSKTGPGQ KAILFLPMSAKS
SEQ ID NO 6	>sp P09038- 3 FGF2_HUMAN Isoform 4 of Fibroblast growth factor 2 OS=Homo sapiens OX=9606 GN=FGF2	MGGRGRGRAPERVGGGRGRGRGTAAPRAAPAARG SRPGPAGTMAAGSITTLPALPEDGGSGAFPPGHFKDP KRLYCKNNGFFLRIHPDGRVDGVREKSDPHIKLQLQ AEERGVSIGKVCANRYLAMKEDGRLLASKCVTDE CFFFERLESNNYNTYRSRKYTSWYVALKRTGQYKL GSKTGPGQKAILFLPMSAKS
SEQ ID NO 7	SICYS8	AHLEYVENLNVKEQLVAGTLYYITLVATDAGKKKI YETKIWVKEWEDFKKVVEFKLVGDDSPNPGGITNV PFPNLPQFKDLARFAVQDYNKKENAHLEFVENLNV KEQVVAGIIYYITLVATDAGKKKIYETKILVKGWEN FKEVQEFKLVGDATK

SEQ ID NO 8	Chicken FGF-2 (NP_990764.1)	MAAGAAGSITTLPALPDDGGGGAFFPGHFKDPKRLY CKNGGFFLRINPDGRVDGVREKSDPHIKLQLQAEER GVVSIKGVSANRFLAMKEDGRLLALKCATEECFFFE RLESNNYNTYRSRKYSWYVALKRTGQYKPGPKTG PGQKAILFLPMSAKS
SEQ ID NO 9	Human IL-1beta	MHHHHHHHHHHHHHDDDDKAPVRSNLNCTLRDSQQK SLVMSGPYELKALHLQGQDMEQQVVFMSFVQGEE SNDKIPVALGLKEKNLYLSCVLKDDKPTLQLESVDP KNYPKKKMEKRFVFNKIEINNKLFEESAQFPNWYIS TSQAENMPVFLGGTKGGQDITDFTMQFVSS
SEQ ID NO 10	TGF-1 beta human/bovine/por cine Active form	ALDTNYCFSSSTEKNCCVRQLYIDFRKDLGWKWIHEP KGYHANFCLGPCPYIWSLDTQYSKVLALYNQHNPG ASAAPCCVPQALEPLIVYYVGRKPKVEQLSNMIVR SCKCS
SEQ ID NO 11	TGF-1 beta Chicken Active form	DLDTDYCFGPGTDEKNCCVRPLYIDFRKDLQW KWIHEPKGYMANFCMGPYIWSADTQYTKVLALY NQHNPGASAAPCCVPQTLDPPIIYYVGRNVRVEQL SNMVVRACKCS
SEQ ID NO 12	IGF-1 human/bovine/por cine Active form	GPETLCGAELVDALQFVCGDRGFYFNKPTG YGSSRRAPQTGIVDECCFRSCDLRRLEMY CAPLKPAKSA
SEQ ID NO 13	IGF-1 Chicken Active form	GPETLCGAELVDALQFVCGDRGFYFSKPTG YGSSRRRLHHKGIVDECCFQSCDLRRLEMY CAPIKPPKSA
SEQ ID NO 14	IGF-2 human Mature form	MGIPMGKSMVLVLLTFLAFASCCIAAYRPSE TLCGGELVDTLQFVCGDRGFYFSRPASRV RRSRGIVEECCFRSCDLALLETYCATPAKS ERDVSTPPTVLPDNFPRYPVGKFFQYDTWK QSTQRLRRGLPALLRARRGHVLAKELEAFR EAKRHRPLIALPTQDPAHGGAPPEMASNRK
SEQ ID NO 15	IGF-2 bovine Mature form	MGITAGKSVLVLLAFLAFASCCYAYRPSE TLCGGELVDTLQFVCGDRGFYFSRPSSRIN RRSRGIVEECCFRSCDLALLETYCATPAKS ERDVSASTTVLPDDVTAYPVGKFFQYDIWK QSTQRLRRGLPAFLRARRGRTLAKELEALR EAKSHRPLIALPTQDPATHGGASSKASSD
SEQ ID NO 16	IGF-2 porcine Mature form	MGIPMRKPLLVLVFLALASCCYAYRPSETLCGGE LVDTLQFVCGDRGFYFSRPASRVNRRSRGIVEECCF RSCDLALLETYCATPAKSERDVSTPPTVLPDNFPRYP VGKFFRYDTWKQSAQRLRRGLPALLRARRGRTLAK ELEAVREAKRHRPLTARPTRDPAAHGGASPEASGHR K
SEQ ID NO 17	IGF-2 chicken Mature form	MCAARQILLLLAFLAYALDSAAAYGTAETLCGGEL VDTLQFVCGDRGFYFSRPVGRNNRRINRGIVEECCF RSCDLALLETYCAKSVKSERDLSATSLAGLPALNKE SFQKPSHAKYSKYNVWQKKSSQRLQREVPGILRAR RYRWQAEGQLAAEEARAMHRPLISLPSQRPPAPRAS PEATGPQE

SEQ ID NO 18	Activin A human	MPLLWLRGFLASCWIIVRSSPTPGSEGHSAAPDCPS CALAALPKDVPNSQPEMVEAVKKHILNMLHLKKRP DVTQVPVKAALLNAIRKLHVGVGENGYVEIEDDIG RRAEMNELMEQTSEIITFAESGTARKTLHFEISKEGS DLSVVERAEVWFLKVPKANRTRTKVTIRLFQQQK HPQGLDGTGEEAEEVGLKGERSELLSEKVVDARKS TWHVFPVSSSIQRLLDQKSSLDVRIACEQCQESGAS LVLLGKKKKKEEGEGKKKGGGEGGAGADEEKEQ SHRPFLMLQARQSEDHPHRRRRRGLECDGKVNICK KQFFVSKDIGWNDWHIAPSGYHANYCEGECPSHIA GTSGSSLSFHSTVINHYRMRGHSPFANLKSCCVPTKL RPM SMLYYDDGQNIKKDIQNMIVEECGCS
SEQ ID NO 19	BMP-4 human	MIPGNRMLMVVLLCQVLLGGASHASLIPETGKKKV AEIQGHAGGRRSGQSHELLRDFEATLLQMFGLRRRP QPSKSAVIPDYMRDLYRLQSGEEEEQIHSTGLEYPE RPASRANTVRSFHHHEHLENIPGTSENSAFRFLNLS IPENEVISSAELRLFREQVDQGPDWERGFHRINIYEV MKPPAEVVPGHLITRLLDTRLVHHNVTRWETFVSP AVLRWTREKQPNYGLAIEVTHLHQTRTHQGQHVRI SRSLPQGSNGWAQLRPLLVTFGHDGRGHALTRRRR AKRSPKHHSQRARKKNKNCRRHSLYVDFSDVGWN DWIVAPPGYQAFYCHGDCPFPLADHLNSTNHAIVQT LVNSVNSSIPKACCVPTELSAISMLYLDEYDKVVLK NYQEMVVEGCGCR
SEQ ID NO 20	VEGF-A	MTDRQTDTPSPSYHLLPGRRRTVDAAASRGQGPEP APGGGVEGVGARGVALKLFVQLLGC SRFGGAVVRA GEAEPSGAARSASSGREEPQPEEGEEEEKEEERGPQ WRLGARKPGSWTGEAAVCADSAPAAAPQALARA SGRGGRVARRGAEESGPPHSPSRSGSASRAGPGRAS ETMNFLLSWVHWSLALLYLHHAKWSQAAPMAEG GGQNHHEVVKFMDVYQRSYCHPIETLVDIFQEYPDE IEYIFKPSCVPLMRCGGCCNDEGLECVPTESNITMQ IMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQEK KSVRGKGKG QKRKRKKSRYKSWSVYVGARCLMPWSLPGPHPC GPCSERRKHLFVQDPQTCKCSCKNTDSRCKARQLEL NERTCRCDKPRR

Table 2: Nucleotide Sequences of Mammalian Proteins

SEQ ID NO 21	Chicken FGF-2	GAGGCTGGACGGCCGCGGCAGGGGGCGAGCCCGC CCGGCGCTGGCGGCGGCGGCCGCGGGGGCCCGG GGCGGCGGGGAGCCGCCGGGGCCCGGCGCATGGC GGCGGGGGCGGCGGGGAGCATCACCACGCTGCCG GCGCTGCCCCGACGACGGGGGCGGCGGCGCTTTTC CCCCGGGCACTTCAAGGACCCCAAGCGGCTCTA CTGCAAGAACGGCGGCTTCTTCTGCGCATCAACC CCGACGGCAGGGTGGACGGCGTCCGCGAGAAGAG CGATCCGCACATCAAACCTGCAGCTTCAAGCAGAA
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		GAAAGAGGAGTAGTATCAATCAAAGGCGTAAGTG CAAACCGCTTTCTGGCTATGAAGGAGGATGGCAG ATTGCTGGCACTGAAATGTGCAACAGAGGAATGT TTCTTTTTCGAGCGCTTGAATCTAATAACTATAA CACTTACCGGTCACGGAAGTACTCTGATTGGTATG TGGCACTGAAAAGGACTGGACAGTACAAGCCCGG ACCAAAAACCTGGACCTGGACAGAAAAGCTATCCTT TTCTTTCCAATGTCTGCTAAAAGCTGA
SEQ ID NO 22	SICYS8	ATGGCGCATCTGGAATATGTGGAACCTGAACG TGAAAGAACAGCTGGTGGCGGGCACCCTGTATTA TATTACCCTGGTGGCGACCGATGCGGGCAAAAAA AAAATTTATGAAACCAAAATTTGGGTGAAAGAAT GGGAAGATTTTAAAAAAGTGGTGAATTTAACT GGTGGGCGATGATAGCCCGAACCCGGGCGGCATT ACCAACGTGCCGTTTCCGAACCTGCCGCAGTTTAA AGATCTGGCGCGCTTTGCGGTGCAGGATTATAAC AAAAAAGAAAACGCGCATCTGGAATTTGTGAAA ACCTGAACGTGAAAGAACAGGTGGTGGCGGGCAT TATTTATTATATTACCCTGGTGGCGACCGATGCGG GCAAAAAAAAAAATTTATGAAACCAAAATTCTGGT GAAAGGCTGGGAAAACCTTTAAAGAAGTGCAGGAA TTTAACTGGTGGGCGATGCGACCAAATAG
SEQ ID NO 23	Human FGF-2 (full length)	CTGGTGGGTGTGGGGGGTGGAGATGTAGAAGATG TGACGCCGCGGCCCGGCGGGTGCCAGATTAGCGG ACGCGGTGCCCCGCGGTTGCAACGGGATCCCGGGC GCTGCAGCTTGGGAGGCGGCTCTCCCCAGGCGGC GTCCGCGGAGACACCCATCCGTGAACCCAGGTC CCGGGCCGCGGCTCGCCGCGCACCAAGGGCCCGG CGGACAGAAGAGCGGCCGAGCGGCTCGAGGCTGG GGGACCGCGGGCGCGGCCGCGCGCTGCCGGGCGG GAGGCTGGGGGGCCGGGGCCGGGGCCGTGCCCCG GAGCGGGTCGGAGGCGGGGCCGGGGCCGGGGG ACGGCGGCTCCCCGCGCGGCTCCAGCGGCTCGGG GATCCCGGCCGGGCCCCGAGGGACCATGGCAGC CGGGAGCATCACACGCTGCCCCGCTTGCCCCGAG GATGGCGGCAGCGGCGCCTTCCCGCCCGGCCACT TCAAGGACCCCAAGCGGCTGTACTGCAAAAACGG GGGCTTCTTCTGCGCATCCACCCCGACGGCCGAG TTGACGGGGTCCGGGAGAAGAGCGACCCTCACAT CAAGCTACAACCTCAAGCAGAAGAGAGAGGAGTT GTGTCTATCAAAGGAGTGTGTGCTAACCGTTACCT GGCTATGAAGGAAGATGGAAGATTACTGGCTTCT AAATGTGTTACGGATGAGTGTTCCTTTTTTGAACG ATTGGAATCTAATAACTACAATACTTACCGGTCAA GGAAATACACCAGTTGGTATGTGGCACTGAAACG AACTGGGCAGTATAAACTTGGATCCAAAACAGGA CCTGGGCAGAAAGCTATACTTTTCTTCCAATGTC TGCTAAGAGCTGA

SEQ ID NO 24	Truncated human FGF-2 (18 kDa)	CGCGTGGATGGCGTGCGCGAAAAAAGCGATCCGC ATATTAAACTGCAGCTGCAGGCGGAAGAACGCGG CGTGGTGAGCATTAAAGGCGTGTGCGCGAACC GC TATCTGGCGATGAAAGAAGATGGCCGCCTGCTGG CGAGCAAATGCGTGACCGATGAATGCTTTTTTTT GAACGCCTGGAAAGCAACAATAACACCTATC GCAGCCGCAAATATACCAGCTGGTATGTGGCGCT GAAACGCACCGGCCAGTATAAACTGGGCAGCAAA ACCGGCCCGGCCAGAAAGCGATTCTGTTTCTGCC GATGAGCGCGAAAAAGC
SEQ ID NO 25	>sp P09038 FGF2_HUMAN Fibroblast growth factor 2 OS=Homo sapiens OX=9606 GN=FGF2 PE=1 SV=3	CTGGTGGGTGTGGGGGGTGGAGATGTAGAAGATG TGACGCCGCGGCCCGCGGGTGCCAGATTAGCGG ACGCGGTGCCCGCGGTTGCAACGGGATCCCGGGC GCTGCAGCTTGGGAGGCGGCTCTCCCCAGGCGGC GTCCGCGGAGACACCCATCCGTGAACCCAGGTC CCGGGCCGCGGGCTCGCCGCGCACCAAGGGGCCG CGGACAGAAGAGCGGCCGAGCGGCTCGAGGCTGG GGGACCGCGGGCGCGGCCGCGCTGCCGGGCGG GAGGCTGGGGGGCCGGGGCCGGGGCCGTGCCCCG GAGCGGGTCGGAGGCGGGGCCGGGGCCGGGGG ACGGCGGCTCCCCGCGCGGCTCCAGCGGCTCGGG GATCCCGGCCGGGCCCGCAGGGACCATGGCAGC CGGGAGCATCACACGCTGCCCGCCTTGCCCGAG GATGGCGGCAGCGGCGCCTTCCCGCCGGCCACT TCAAGGACCCCAAGCGGCTGTACTGCAAAAACGG GGGCTTCTTCTGCGCATCCACCCGACGGCCGAG TTGACGGGGTCCGGGAGAAGAGCGACCCTCACAT CAAGCTACAACCTCAAGCAGAAGAGAGAGGAGTT GTGTCTATCAAAGGAGTGTGTGCTAACCGTTACCT GGCTATGAAGGAAGATGGAAGATTACTGGCTTCT AAATGTGTTACGGATGAGTGTCTTTTTTTGAACG ATTGGAATCTAATAACTACAATACTTACCGGTCAA GGAAATACACCAGTTGGTATGTGGCACTGAAACG AACTGGGCAGTATAAACTTGGATCCAAAACAGGA CCTGGGCAGAAAGCTATACTTTTCTTCCAATGTC TGCTAAGAGCTGA
SEQ ID NO 26	>sp P09038-1 FGF2_HUMAN Isoform 2 of Fibroblast growth factor 2 OS=Homo sapiens OX=9606 GN=FGF2	ATGGCAGCCGGGAGCATCACACGCTGCCCGCCT TGCCCGAGGATGGCGGCAGCGGCGCCTTCCCGCC CGGCCACTTCAAGGACCCCAAGCGGCTGTACTGC AAAAACGGGGGCTTCTTCTGCGCATCCACCCCG ACGGCCGAGTTGACGGGGTCCGGGAGAAGAGCGA CCCTCACATCAAGCTACAACCTCAAGCAGAAGAG AGAGGAGTTGTGTCTATCAAAGGAGTGTGTGCTA ACCGTTACCTGGCTATGAAGGAAGATGGAAGATT ACTGGCTTCTAAATGTGTTACGGATGAGTGTCT TTTTTGAACGATTGGAATCTAATACTACAATACT TACCGGTCAAGGAAATACACCAGTTGGTATGTGG CACTGAAACGAACTGGGCAGTATAAACTTGGATC

		CAAAACAGGACCTGGGCAGAAAGCTATACTTTTT CTTCCAATGTCTGCTAAGAGCTGA
SEQ ID NO 27	IL1-beta	CTCGAGATGCATCATCATCATCATCATCATCA TCATCATCATGATGATGATGATAAAGCACCTGTAC GATCACTGAACTGCACGCTCCGGGACTCACAGCA AAAAAGCTTGGTGATGTCTGGTCCATATGAACTG AAAGCTCTCCACCTCCAGGGACAGGATATGGAGC AACAAGTGGTGTTCTCCATGTCCTTTGTACAAGGA GAAGAAAGTAATGACAAAATACCTGTGGCCTTGG GCCTCAAGGAAAAGAATCTGTACCTGTCCTGCGT GTTGAAAGATGATAAGCCCACTCTACAGATGGAG AGTGTAGATCCCCAAAATTACCCAAAGAAGAAGA TGGAAAAGCGATTTGTCTTCAACAAGATAGAAAT CAATAACAAGCTGGAATTTGAGTCTGCCCAGTTCC CCAAGTGGTACATCAGCACCTCTCAAGCAGAAAA CATGCCCCGTCTTCCTGGGAGGGACCAAAGGCGGC CAGGATATAACTGACTTCACCATGCAATTTGTGTC TTCCTAAACGCGT
SEQ ID NO 28	FGF-2 stable mutant (human)	CGCGTGGATGGCGTGCGCGAAAAAAGCGATCCGC ATATTAACTGCAGCTGCAGGCGGAAGAACGCGG CGTGGTGAGCATTAAAGGCGTGTGCGCGAACC TATCTGGCGATGAAAGAAGATGGCCGCCTGCTGG CGAGCAAATGCGTGACCGATGAATGCTTTTTTTTT GAACGCCTGGAAAGCAACAACCTATAACACCTATC GCAGCCGCAAATATACCAGCTGGTATGTGGCGCT GAAACGCACCGGCCAGTATAAACTGGGCAGCAAA ACCGGCCCGGGCCAGAAAGCGATTCTGTTTCTGCC GATGAGCGCGAAAAAGC

CLAIMS

WHAT IS CLAIMED IS:

1. A plant cell, wherein the plant cell comprises:
 - a polynucleotide sequence encoding for a heterologous protease inhibitor gene or a functional variant thereof;
 - a polynucleotide sequence encoding for a mammalian gene or a functional variant thereof.
2. The plant cell of claim 1, wherein the polynucleotide sequence encoding for a heterologous protease inhibitor gene or a functional variant thereof and the polynucleotide sequence encoding for a mammalian gene or a functional variant thereof are within a bacterial or viral vector.
3. The plant cell of claim 2, wherein the bacterial vector is an *Agrobacterium* species.
4. The plant cell of claim 2, wherein the viral vector is Tobacco mosaic virus.
5. The plant cell of claim 1, wherein the mammalian gene is selected from the group consisting of TGF- β , IGF-1, IGF-2, human FGF-2, Activin A, BMP-4 and VEGF.
6. The plant cell of claim 1, wherein the heterologous protease inhibitor gene is SICYS8.
7. The plant cell of claim 1, wherein the polynucleotide sequence encoding for a heterologous protease inhibitor gene or a functional variant thereof and polynucleotide sequence encoding for a mammalian gene or a functional variant thereof are RNA or DNA.
8. A plant cell, wherein the plant cell comprises:
 - a polynucleotide sequence encoding for a chicken FGF-2 gene or a functional variant thereof.
9. The plant cell of claim 8, wherein the polynucleotide sequence is within a bacterial or viral vector.
10. The plant cell of claim 9, wherein the polynucleotide sequence comprises a sequence with at least 75% sequence identity to SEQ ID NO: 21.
11. The plant cell of claim 8, wherein the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence.
12. The plant cell of claim 9, wherein the bacterial vector comprises a *Agrobacterium* species.
13. The plant cell of claim 8, wherein the viral vector is Tobacco mosaic virus.

14. The plant cell of claim 8, wherein the polynucleotide sequence is RNA or DNA.
15. A plant cell, wherein the plant cell comprises:
 - a polynucleotide sequence encoding for a IL-1 β gene or a functional variant thereof.
16. The plant cell of claim 15, wherein the polynucleotide sequence is within a bacterial or viral vector.
17. The plant cell of claim 8, wherein the polynucleotide sequence comprises a sequence with at least 75% sequence identity to SEQ ID NO: 27.
18. The plant cell of claim 8, wherein the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence.
19. The plant cell of claim 16, wherein the bacterial vector comprises a *Agrobacterium* species.
20. The plant cell of claim 16, wherein the viral vector is Tobacco mosaic virus.
21. A plant cell, wherein the plant cell comprises:
 - a heterologous protease inhibitor protein or a functional variant thereof;
 - and a mammalian protein or a functional variant thereof.
22. The plant cell of claim 21, further comprising a bacterial or viral vector.
23. The plant cell of claim 22, wherein the bacterial vector is an *Agrobacterium* species.
24. The plant cell of claim 22 wherein the viral vector is Tobacco Mosaic Virus.
25. The plant cell of claim 21, wherein the mammalian protein is from the group consisting of TGF- β , IGF-1, IGF-2, human FGF-2, Activin A, BMP-4 and VEGF.
26. A plant cell, wherein the plant cell comprises:
 - a chicken FGF-2 protein or a functional variant thereof.
27. The plant cell of claim 26, where the chicken FGF-2 protein comprises a sequence with at least 75% sequence identity to SEQ ID NO: 8.
28. The plant cell of claim 26, wherein the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence.
29. The plant cell of claim 26, further comprising a bacterial or viral vector.
30. The plant cell of claim 29, wherein the bacterial vector comprises a *Agrobacterium* species.
31. The plant cell of claim 29, wherein the viral vector is Tobacco mosaic virus.
32. A plant cell, wherein the plant cell comprises:
 - a IL1- β protein or a functional variant thereof.

33. The plant cell of claim 32, where the IL1- β protein comprises a sequence with at least 75% sequence identity to SEQ ID NOs: 9.
34. The plant cell of claim 32, wherein the functional variant comprises an insertion, deletion, or sequence variation compared to the full-length sequence.
35. The plant cell of claim 32, further comprising a bacterial or viral vector.
36. The plant cell of claim 35, wherein the bacterial vector comprises a *Agrobacterium* species.
37. The plant cell of claim 35, wherein the viral vector is Tobacco mosaic virus.
38. A composition, wherein the composition comprises:
 - a mammalian protein or a functional variant thereof; and
 - at least one of the following: flavonoids, rubisco, plant-derived alkaloids, cellulose, lignocellulose, legumalin, phaselin, 11S legumin type, 7S vicilin type, gliadin, zein, hordein, secalin, and glutenins.
39. The composition of claim 36, wherein the mammalian protein is at least one of TGF- β , IGF-1, IGF-2, human FGF-2, Activin A, BMP-4 and VEGF.
40. The composition of claim 38, wherein the at least one of the following comprises no more than 0.05% of the composition.
41. The composition of claim 39, wherein the at least one of the following comprises no more than 0.03% of the composition.
42. The composition of claim 38, further comprising heparin.
43. The composition of claim 42, wherein heparin is present at no more than 2 $\mu\text{g/ml}$.
44. A composition, wherein the composition comprises:
 - a chicken FGF-2 protein or a functional variant thereof; and
 - at least one of the following: flavonoids, rubisco, plant-derived alkaloids, cellulose, lignocellulose, legumalin, phaselin, 11S legumin type, 7S vicilin type, gliadin, zein, hordein, secalin, and glutenins.
45. The composition of claim 44, wherein the at least one of the following comprises no more than 0.05% of the composition.
46. The composition of claim 45, wherein the at least one of the following comprises no more than 0.03% of the composition.
47. The composition of claim 44, further comprising heparin.
48. The composition of claim 47, wherein heparin is present at no more than 2 $\mu\text{g/ml}$.
49. A composition, wherein the composition comprises:

a human interleukin 1 beta (IL1- β) protein or a functional variant thereof and at least one of the following: flavonoids, rubisco, plant-derived alkaloids, cellulose, lignocellulose, legumalin, phaselin, 11S legumin type, 7s vicilin type, gliadin, zein, hordein, secalin, and glutenins.

50. The composition of claim 49, wherein the at least one of the following comprises no more than 0.05% of the composition.
51. The composition of claim 49, wherein the at least one of the following comprises no more than 0.03% of the composition.
52. A method of manufacturing a mammalian protein, comprising:
 - culturing the plant cells of any of claims 1-20; and
 - extracting a mammalian protein encoded by the mammalian gene or the functional variant thereof to generate an extraction product.
53. The method of claim 52, wherein the extraction product comprises the mammalian protein present in an amount of at least 40 μg per gram of biomass.
54. The method of claim 53, wherein the mammalian protein is present in an amount of at least 45, 50, 55 μg per gram of biomass.
55. The method of claim 52, wherein the culturing comprises contacting a bacterial or viral vector to the plant cell using a syringe or spray method.
56. A method of manufacturing a mammalian protein, comprising:
 - culturing the plant cells of any of claims 21-37; and
 - extracting the mammalian protein or the functional variant thereof to generate an extraction product.
57. The method of claim 56, wherein the extraction product comprises the mammalian protein present in an amount of at least at least 40 μg per gram of biomass.
58. The method of claim 57, wherein the mammalian protein is present in an amount of at least 45, 50, 55 μg per gram of biomass.
59. The method of claim 56, wherein the culturing comprises contacting a bacterial or viral vector to the plant cell using a syringe or spray method.
60. A plant cell comprising a polynucleotide sequence encoding for a mammalian gene or a functional variant thereof, wherein the mammalian gene is selected from the group consisting of TGF- β , IGF-1, IGF-2, FGF-2, BMP-4, and VEGF, and wherein the plant cell is not derived from *Oryza sativa*.

61. The plant cell of claim 60, wherein the polynucleotide sequence encoding for a mammalian gene or a functional variant thereof are within a bacterial or viral vector.
62. The plant cell of claim 61, wherein the bacterial vector comprises an *Agrobacterium* species.
63. The plant cell of claim 61, wherein the viral vector comprises a Tobacco mosaic virus.
64. The plant cell of any one of claims 60-63, wherein the polynucleotide sequence encoding for a mammalian gene or a functional variant thereof is RNA or DNA.
65. The plant cell of any one of claims 60-64, wherein the functional variant comprises an insertion, a deletion, or a sequence variation compared to the mammalian gene.
66. A plant cell, wherein the plant cell comprises a mammalian protein or a functional variant thereof, and wherein the plant cell is not derived from *Oryza sativa*.
67. The plant cell of claim 66, further comprising a bacterial or viral vector.
68. The plant cell of claim 67, wherein the bacterial vector comprises an *Agrobacterium* species.
69. The plant cell of claim 67, wherein the viral vector comprises a Tobacco Mosaic Virus.
70. A method of manufacturing a mammalian protein, comprising:
culturing the plant cells of any of claims 60-65; and
extracting a mammalian protein encoded by the mammalian gene or the functional variant thereof to generate an extraction product.
71. The method of claim 70, wherein the extraction product comprises the mammalian protein present in an amount of at least 40 μg per gram of biomass.
72. The method of claim 71, wherein the mammalian protein is present in an amount of at least 45, 50, 55 μg per gram of biomass.
73. The method of any one of claims 70-72, wherein the culturing comprises contacting a bacterial or viral vector to the plant cell using a syringe or spray method.
74. A method of manufacturing a mammalian protein, comprising:
culturing the plant cells of any of claims 66-69; and

extracting the mammalian protein or the functional variant thereof to generate an extraction product.

75. The method of claim 74, wherein the extraction product comprises the mammalian protein present in an amount of at least at least 40 μg per gram of biomass.

76. The method of claim 75, wherein the mammalian protein is present in an amount of at least 45, 50, 55 μg per gram of biomass.

77. The method of any one of claims 74-76, wherein the culturing comprises contacting a bacterial or viral vector to the plant cell using a syringe or spray method.