product by weight, and the connective tissue replica accounts for 1-30% of the product by weight.

In some embodiments, the meat substitute product comprises 60-90% water; 5-30% protein content; and 1-20% of a fat; wherein the protein content comprises one or more isolated and purified plant proteins.

In some embodiments the consumable contains components to replicate the components of meat. The main component of meat is typically skeletal muscle. Skeletal muscle typically consists of roughly 75 percent water, 19 percent protein, 2.5 percent intramuscular fat, 1.2 percent carbohydrates and 2.3 percent other soluble non-protein substances. These include organic acids, sulfur compounds, nitrogenous compounds, such as amino acids and nucleotides, and inorganic substances such as minerals. Accordingly, some embodiments of the present invention provide for replicating approximations of this composition for the consumable. For example, in some embodiments, the consumable is a plant-based meat replica comprising roughly 75% water, 19% protein, 2.5% fat, 1.2% carbohydrates; and 2.3 percent other soluble non-protein substances. In some embodiments the consumable is a plant-based meat replica comprising between 60-90% water, 10-30% protein, 1-20% fat, 0.1-5% carbohydrates; and 1-10 percent other soluble non-protein substances. In some embodiments the consumable is a plant-based meat replica comprising between 60-90% water, 5-10% protein, 1-20% fat, 0.1-5% carbohydrates; and 1-10 percent other soluble non-protein substances. In some embodiments the consumable is a plant-based meat replica comprising between 0-50% water, 5-30% protein, 20-80%% fat, 0.1-5% carbohydrates; and 1-10 percent other soluble non-protein substances.

In some embodiments, a meat replica contains between 0.01% and 5% by weight of a heme containing protein. In some embodiments, the replica contains between 0.01% and 5% by weight of leghemoglobin. Some meat also contains myoglobin, a heme containing protein, which accounts for most of the red color and iron content of some meat. It is understood that these percentages can vary in meat and the meat replicas can be produced to approximate the natural variation in meat. In embodiments that include a heme-containing protein and optional flavors, k-carrageenan can be used absorb some of

the liquid contributed from the flavor and heme solution so the ground tissue is not excessively wet. During addition of flavor heme mix solution and the k-carrageenan powder are distributed evenly over the tissue mixture to ensure homogeneity in the final ground product.

It will be appreciated that when proteins are supplied as a solution, water removal techniques such as freeze-drying or spray drying can optionally be used to concentrate the protein. The proteins may then be reconstituted in an amount of liquid that prevents the ground tissue from being too moist.

Additionally, in some instances, the present invention provides for improved meat replicas, which comprise these components in unnatural percentages. The concentration of heme containing protein is an important determinant of meat flavor and aroma. Thus for example a meat replica could have a higher heme protein content than typical beef. For example a meat replica can be produced with a higher than typical average fat content. The percentages of these components may also be altered to increase other desirable properties.

In some instances a meat replica is designed so that, when cooked, the percentages of components are similar to cooked meat. So, in some embodiments, the uncooked consumable has different percentages of components than uncooked meat, but when cooked, the consumable is similar to cooked meat. For example, a meat replica may be made with a higher than typical water content for raw meat, but when cooked in a microwave, the resulting product has non-starch polysaccharides such as arabinoxylans, cellulose, and many other plant components such as resistant starch, resistant dextrins, inulin, lignin, waxes, chitins, pectins, beta-glucans, and oligosaccharide percentages of components similar to meat cooked over a fire.

In some embodiments, the consumable is a meat replica with a lower that typical water content for meat. In some embodiments the inventions provides for methods for hydrating a meat replica to cause the meat replica to have a water content similar to meat. For example a meat replica with a water content that would be low for meat, for example 1%, 10%, 20%, 30%, 40% or 50% water, can be hydrated to roughly 75% water. Once hydrated, in some embodiments, the meat replica is then cooked for human consumption.

The consumable can have a protein component. In some embodiments the protein content of the consumable is 10%, 20%, 30%, or 40%. In some embodiments the protein content of the consumable is similar to meat. In some embodiments the protein content in the consumable is greater than that of meat. In some embodiments the consumable has less protein than meat.

The protein in the consumable can come from a variety or combination of sources. Non-animal sources can provide some or all of the protein in the consumable. Non-animal sources can include vegetables, non food biomass such as carrot tops and *Miscanthus*, seaweed, fruits, nuts, grains, algae, bacteria, or fungi. See, e.g, Sections III A and B. The protein can be isolated or concentrated from one or more of these sources. In some embodiments the consumable is a meat replica comprising protein only obtained from non-animal sources.

In some embodiments protein is formed into asymmetric fibers for incorporation into the consumable. In some embodiments these fibers replicate muscle fibers. In some embodiments the protein are spun fibers. Accordingly, the present invention provides for methods for producing asymmetric or spun protein fibers. In some embodiments the consumable contains a protein or proteins that have all of the amino acids found in proteins that are essential for human nutrition. In some embodiments the proteins added to the consumable are supplemented with amino acids.

The physical organization can be a determinant of the response of the meat substitute to cooking. For example, the flavor of meat is modified by the size of the particles. Ground meat that has been reduced to a paste provides different flavors than more crudely ground beef upon cooking. The ability to control the relative size and orientation of individual tissue replicas enables the flavor and aroma profile of consumables to be modified during cooking. For example, muscle tissue replicas and adipose tissue replicas provide different flavor profiles when cooked independently or when mixed. Further changes in the flavor profile are observed based on the method by which the different tissue replicas are mingled.

The physical organization of the meat substitute product can be manipulated by controlling the localization, organization, assembly, or orientation of the muscle, fat,

and/or connective tissue replicas described herein. In some embodiments the product is designed in such a way that the replicas described herein are associated with one another as in meat. In some embodiments the consumable is designed so that after cooking the replicas described herein are associated with one another as in cooked meat.

Characteristic flavor and fragrance components of meat are mostly produced during the cooking process by chemical reactions, the substrates for which are amino acids, fats and sugars which are found in plants as well as meat. Therefore in some embodiments the consumable is tested for similarity to meat during or after cooking. In some embodiments human ratings, human evaluation, olfactometer readings, or GCMS measurements, or combinations thereof, are used to create an olfactory map of cooked meat. Similarly, an olfactory map of the consumable, for instance a meat replica, can be created. These maps can be compared to assess how similar the cooked consumable it so meat. In some embodiments the olfactory map of the consumable during or after cooking is similar to or indistinguishable from that of cooked or cooking meat. In some embodiments the differences are sufficiently small as to be below the detection threshold of human perception.

In some embodiments the individual tissue replicas are assembled in layers, sheets, blocks and strings in defined positions and orientations.

In some embodiments, the replicas are combined in the process of passing through the plates of a meat grinder with the holes set at less than 1/2 inch (e.g., at ¼ inch). The grinder provides multiple functions of reducing the particle size, providing additional mixing or working, and forming the material into cylindrical portions like what is typically done for ground beef. During the assembly, grinding, and forming, it is important to keep the replica tissues cold (e.g., 4 – 15°C) to control microbial growth, limit flavor reactions, and also to maintain the adipose in a solid state so discrete pieces of adipose will be maintained through the grinding process.

Prior to grinding, the replica tissues are usually broken down in some manner to a defined particle size. For example, in some embodiments, the individual tissue replicas can be formed into small pieces less than 1 cm in diameter or less than 5 mm in diameter before combination with the other tissue replicas. Adipose tissue can be crumbled into

about 3 – 7 mm particles. This is important to both the appearance of the final material and the behavior of fat leakage during cooking. This size range allows for a natural appearance of adipose flecks in the final raw product. If the adipose tissues are too small (e.g., less than 2 mm), there will be an insufficient amount of fat leaked from the product when cooked.

Soft connective tissue replicas can be broken down into pieces of about 1 – 3 mm in length with ragged edges. If the pieces are too large (e.g., greater than about 4 mm), the texture of the final product can be too beady.

Sticky tissue or noodles tissue replicas, composed of amorphous or long noodle like tissue replica pieces, respectively, and raw tissue replicas can be manually broken down into pieces about 1 - 3 cm in diameter. Achieving particles in this size range allows for adequate mixing and suitable homogeneity in the final ground material.

In some embodiments, hard connective tissue replicas can be chopped to three levels, (e.g., coarse, intermediate, and fine). Chopping to three levels provides a greater amount of heterogeneity than a single step chopping process, and makes the mouthfeel of the final product more similar to ground beef.

In formulations containing gluten an additional function of the food grinder is to work the gluten and develop a gluten network of aligned gluten molecules. For gluten containing formulations it is important to minimize interaction of the adipose with the gluten network. This is done by prechilling the adipose replicas and ground tissue replicas prior to combining and also by minimizing the amount of manipulation after the adipose is added. Overworking of adipose replicas into the gluten will break down or "shorten" the gluten network.

Finally for gluten containing formulations the patties are allowed to rest at room temperature for 30 min or overnight at 4C prior to cooking. This allows time for the gluten network to relax, giving an overall better texture.

In some embodiments, the connective tissue replica is incorporated into the protein solution before formation of the muscle tissue replica.

In some embodiments, the connective tissue replica is directly incorporated into the emulsion before formation of the adipose tissue replica.

In some embodiments, the adipose tissue replica is added to the muscle tissue replica in strands and sheets to replicate the effect of "marbling," or streaky bacon.

Mixed meat tissue replicas can increase the sense of flavors, such flavors including, but not limited to, multiple aromatic compounds associated with fruity/grean bean/metallic, nutty/green, peanut butter/musty, raw potato/roasted/earthy, vinegary, spicy/caramel/almond, creamy, sweet, fruity/stale beer, musty/nutty/coumarin/licorice/walnut/bread, coconut/woody/sweet, penetrating/sickening, minty, or toasty caramel aromas.

In some embodiments the mixed meat replicas increase the presence of volatile odorants, such as 2-pentyl-furan; 4-methylthiazole; ethyl pyrazine,; 2,3-dimethylpyrazine, acetic acid; 5-methyl-2-furancarboxaldehyde; butyrolactone; 2,5-dimethyl-3-(3-methyl butyl) pyrazine; 2-cyclopentene-1-one, 2-hydroxy-3-methyl; 3-acetyl-1h-pyrolline; pantolactone; 1-methyl 1(H)-pyrrole-2-2carboxaldehyde,; caprolactam; 2,3-dihydro-3,5-dihydroxy-6-methyl-4(H)-pyran-4-one. in some embodiments, the undesired flavors including but not limited to gasoline-like, petroleum, sour/putrid/fish-like, bland/woody/yogurt, fatty/honey/citrus, pungent/sweet/caramelic and nutty/burnt green aromas, form only in individual tissue replicas, but do not accumulate in mixed meat replicas. In some embodiments, the individual tissue replicas increase the presence of volatile odorants including but not limited to nonane, 2,6-dimethyl, 3-methyl 3-hexene,; pyridine; acetoin; octanal; 1-hydroxy-2-propanone; and/or ethenyl pyrazine. In some embodiments the levels to which all of the above compounds accumulate during cooking depend on the sizes of tissue replica units and how they are mixed (coarse, fine, or blended).

In some embodiments, the mixed meat tissue replicas increase the sense of flavors including but not limited to, multiple aromatic compounds associated with fruity/grean bean/metallic, nutty/green, peanut butter/musty, raw potato/roasted/earthy, vinegary, spicy/caramel/almond, creamy, sweet, fruity/stale beer, musty/nutty/coumarin/licorice/walnut/bread, coconut/woody/sweet, penetrating/sickening, minty, or toasty caramel aromas. In some embodiments, the mixed meat replicas increase the presence of volatile odorants including but not limited

to phenylacetaldehyde, 1-octen-3-one, 2-n-heptylfuran, 2-thiophenecarboxaldehyde, 3-thiophenecarboxaldehyde, butyrolactone, 2-undecenal, methyl-pyrazine, furfural, 2-decanone, pyrrole, 1-octen-3-ol, 2-acetylthiazole, (E)-2-octenal, decanal, benzaldehyde, (E)-2-nonenal, pyrazine, 1-hexanol, 1-heptanol, dimethyl trisulfide, 2-nonanone, 2-pentanone, 2-heptanone, 2,3-butanedione, heptanal, nonanal, 2-octanone, 1-octanol, 3-ethylcyclopentanone, 3-octen-2-one, (E,E)-2,4-heptadienal, (Z)-2-heptenal, 2-heptanone, 6-methyl-, (Z)-4-heptenal, (E,Z)-2,6-nonadienal, 3-methyl-2-butenal, 2-pentyl-furan, thiazole, (E, E)-2,4-decadienal,, hexanoic acid, 1-ethyl-5-methylcyclopentene, (E,E)-2,4-nonadienal, (Z)-2-decenal, dihydro-5-pentyl-2(3H)-furanone, trans-3-nonen-2-one, (E,E)-3,5-octadien-2-one, (Z)-2-octen-1-ol, 5-ethyldihydro-2(3H)-furanone, 2-butenal, 1-penten-3-ol, (E)-2-hexenal, formic acid, heptyl ester, 2-pentyl-thiophene, (Z)-2-nonenal, 2-hexyl-thiophene, (E)-2-decenal,, 2-ethyl-5-methyl-pyrazine, 3-ethyl-2,5-dimethyl-pyrazine, 2-ethyl-1-hexanol, thiophene, 2-methyl-furan, pyridine, butanal, 2-ethyl-furan, 3-methyl-butanal, trichloromethane, 2-methyl-butanal, methacrolein, 2-methyl-propanal, propanal, acetaldehyde, 2-propyl-furan, dihydro-5-propyl-2(3H)- furanone, 1,3-hexadiene, 4-decyne, pentanal, 1-propanol, heptanoic acid, trimethyl-ethanethiol, , 1-butanol, 1-penten-3-one, dimethyl sulfide, 2-ethyl furan, 2-pentyl-thiophene, 2-propenal, 2-tridecen-1-ol, 4-octene, 2-methyl thiazole,, methyl-pyrazine, 2-butanone, 2-pentyl-furan, 2-methyl-propanal, butyrolactone,, 3-methyl-butanal, methyl-thiirane, 2-hexyl-furan, butanal,, 2-methyl-butanal, 2-methyl-furan, furan, octanal, 2-heptenal, 1-octene, formic acid heptyl ester, 3-pentyl-furan, and 4-penten-2-one. In some embodiments the levels to which all of the above compounds accumulate during cooking depend on the sizes of tissue units and how they are mixed (coarse, fine, or blended).

The production of volatile odorants can be enhanced when the adipose, muscle and connective tissue replicas are contacting one another. In some embodiments, the production of volatile odorants is enhanced when the adipose, muscle and connective tissues are intimately mixed with a mean size of the individual tissue replicas of 5 mm. In some embodiments, the production of volatile odorants is enhanced when the fat, muscle and connective tissues are intimately mixed with mean size of the individual tissue replicas of 2 mm. In some embodiments, said production of volatile odorants is enhanced

when the fat, muscle and connective tissue replicas are intimately mixed with mean size of the individual tissue replicas of 1 mm.

In some embodiments, the meat substitute is optimized for particular cooking methods (optimized for cooking in a microwave oven, or optimized for cooking in a stew).

In some embodiments, the meat substitute is optimized for dehydration.

In some embodiments, said meat substitute is optimized for fast rehydration upon exposure of the dehydrated meat replica to water.

In some embodiments, said meat substitute is optimized for use as emergency, camping or astronaut food.

The methods described herein can be used to provide a meat replica with defined cooking characteristics to allow the production of meat replicas that are optimized for particular cooking techniques. For example, stews require slow cooking to gelatinize the connective tissue in the meat, whereas meat replicas can be designed wherein the connective tissue replica is more easily gelatinized thus allowing stews to be prepared quickly.

B. INDICATORS OF COOKING MEAT

The consumable can include compositions which can indicate that the consumable is cooking or has cooked. The release of odorants upon cooking is an important aspect of meat consumption. In some embodiments, the consumable is a meat replica entirely composed of non-animal products that when cooked generates an aroma recognizable by humans as typical of cooking beef. In some embodiments, the consumable when cooked generates an aroma recognizable by humans as typical of cooking pork, bacon, chicken, lamb, fish, or turkey. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, with an odorant that is released upon cooking or that is produced by chemical reactions that take place upon cooking. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, containing mixtures of proteins, peptides, amino acids, nucleotides, sugars and polysaccharides and fats in

combinations and spatial arrangements that enable these compounds to undergo chemical reactions during cooking to produce odorants and flavor-producing compounds.

In some embodiments, the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, with a volatile or labile odorant that is released upon cooking.

In some embodiments, the indicator is a visual indicator that accurately mimics the color transition of a meat product during the cooking progression. The color transition can be, for example, from red to brown, from pink to white or tan, or from a translucent to opaque color during the cooking progression.

In some embodiments, the indicator is an olfactory indicator that indicates cooking progression. In one embodiment, the olfactory indicator is one or more volatile odorants released during cooking.

In some embodiments, the indicator comprises one or more isolated, purified iron-containing proteins. In some embodiments, the one or more isolated, purified iron-containing proteins (e.g., a heme-containing protein, see section III B) is in a reduced state before cooking. In some embodiments, the one or more isolated and purified iron carrying proteins in a reduced or oxidized state has a similar UV-VIS profile to a myoglobin protein derived from an animal source when in an equivalent reduced or oxidized state. The *Aquifex aeolicus* hemoglobin has a peak absorbance wavelength at 413 nm; the *Methylacidiphilum infernorum* hemoglobin has a peak absorbance wavelength at 412 nm; the *Glycine max* leghemoglobin has a peak absorbance wavelength at 415 nm; the *Hordeum vulgare* and *Vigna radiata* non-symbiotic hemoglobins each have a peak absorbance wavelength at 412 nm. The *Bos taurus* myoglobin has a peak absorbance wavelength at 415 nm.

In some embodiments, the difference between the peak absorbance wavelength of the one or more isolated and purified iron-containing proteins and the peak absorbance wavelength of myoglobin derived from an animal source is less than 5%.

Odorants released during cooking of meat are generated by reactions that can involve as reactants fats, protein, amino acids, peptides, nucleotides, organic acids, sulfur compounds, sugars and other carbohydrates. In some embodiments the odorants that

combine during the cooking of meat are identified and located near one another in the consumable, such that upon cooking of the consumable the odorants combine. So, in some embodiments, the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions involving amino acids, fats and sugars found in plants as well as meat. So, in some embodiments, the characteristic flavor and fragrance components are mostly produced during the cooking process by chemical reactions involving one or more amino acids, fats, peptides, nucleotides, organic acids, sulfur compounds, sugars and other carbohydrates found in plants as well as meat.

Some reactions that generate odorants released during cooking of meat can be catalyzed by iron, in particular the heme iron of myoglobin. Thus in some embodiments, some of the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions catalyzed by iron. In some embodiments, some of the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions catalyzed by heme. In some embodiments, some of the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions catalyzed by the heme iron in leghemoglobin. In some embodiments, some of the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions catalyzed by the heme iron in a heme protein. For example, hemeproteins (e.g., from *Aquifex aeolicusm*, *Methylacidiphilum infernorum*, *Glycine max*, *Hordeum vulgare*, or *Vigna radiate*) provide a significantly different profile of volatile odorants when heated in the presence of cysteine and glucose than any subset of the three components when analysed by GC-MS. Volatile flavor components that are increased under these conditions include but are not limited to furan, acetone, thiazole, furfural, benzaldehyde, 2-pyridinecarboxaldehyde, 5-methyl-2-thiophenecarboxaldehyde, 3-methyl-2-thiophenecarboxaldehyde, 3-thiophenmethanol and decanol. Under these conditions, cysteine and glucose alone or in the presence of iron salts such as ferrous glucanate produced a sulfurous, odor but addition of heme proteins reduced the sulfurous odor and replaced it with flavors including but not limited to chicken broth, burnt mushroom, molasses, and bread.

Additionally, a hemeprotein (e.g., from *Aquifex aeolicus*, *Methylacidiphilum infernorum*, *Glycine max*, *Hordeum vulgare*, or *Vigna radiata*) when heated in the presence of ground chicken increased specific volatile odorants that are elevated in beef compared to chicken when analyzed by GC-MS. Volatile flavor components that are increased under these conditions include but are not limited to propanal, butanal, 2-ethyl-furan, heptanal, octanal, trans-2-(2-pentenyl)furan, (Z)-2-heptenal (E)-2-octenal pyrrole, 2,4-dodecadienal, 1-octanal, or (Z)-2-decenal 2-undecenal.

C. COLOR INDICATORS

The color of meat is an important part the experience of cooking and eating meat. For instance, cuts of beef are of a characteristic red color in a raw state and gradually transition to a brown color during cooking. As another example, white meats such as chicken or pork have a characteristic pink color in their raw state and gradually transition to a white or brownish color during cooking. The amount of the color transition is used to indicate the cooking progression of beef and titrate the cooking time and temperature to produce the desired state of done-ness. In some aspects, the invention provides a non-meat based meat substitute product that provides a visual indicator of cooking progression. In some embodiments, the visual indicator is a color indicator that undergoes a color transition during cooking. In some embodiments, the color indicator recapitulates the color transition of a cut of meat as the meat progresses from a raw to a cooked state. In more embodiments, the color indicator colors the meat substitute product a red color before cooking to indicate a raw state and causes the meat substitute product to transition to a brown color during cooking progression. In other embodiments, the color indicator colors the meat substitute product a pink color before cooking to indicate a raw state and causes the meat substitute product to transition to a white or brown color during cooking progression.

The main determinant of the nutritional definition of the color of meat is the concentration of iron carrying proteins in the meat. In the skeletal muscle component of meat products, one of the main iron-carrying proteins is myoglobin. As described above, the myoglobin content of varies from under 0.05% in the white meat of chicken to 1.5-

2.0% in old beef. So, in some embodiments, the consumable is a meat replica which comprises an iron-carrying protein (e.g., a heme-containing protein). In some embodiments, the meat replica comprises about 0.05%, about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1%, about 1.1%, about 1.2%, about 1.3%, about 1.4%, about 1.5%, about 1.6%, about 1.7%, about 1.8%, about 1.9%, about 2%, or more than about 2% of an iron-carrying protein (e.g., a heme-containing protein) by dry weight or total weight. In some cases, the iron carrying protein has been isolated and purified from a source. In other cases, the iron carrying protein has not been isolated and purified. In some cases, the source of the iron-carrying protein is an animal source, or a non-animal source such as a plant, fungus, or genetically modified organisms such as, e.g., plant, algae, bacteria or fungus. In some cases, the iron-carrying protein is myoglobin. In some embodiments the consumable is a plant based meat replica that has animal myoglobin added. So, for example a replica of young beef can have about 0.4-1% myoglobin. In some embodiments the consumable is a plant based meat replica that has a leghemoglobin or a cytochrome added. So, for example, a replica of young beef can have about 0.4-1% leghemoglobin or cytochrome.

Another example of iron-carrying proteins is hemoglobin, the iron-containing oxygen-binding protein in the red blood cells of vertebrates. Hemoglobin is similar in color to myoglobin. In some embodiments the invention provides methods of saving and recycling blood from animal farming to supplement the color of a consumable. For example, blood is saved from a slaughter house, and hemoglobin from the blood is used to enhance the color of a consumable. In some aspects the consumable is a plant-based meat replica containing hemoglobin.

Additional iron containing proteins exist in nature. In some embodiments the consumable comprises an iron containing protein that is not myoglobin. In some embodiments the consumable does not contain myoglobin. In some embodiments the consumable does not contain hemoglobin. In some embodiments the consumable is a meat replica that comprises an iron containing protein other than myoglobin or hemoglobin. See, for example, Section III B for examples of heme-containing proteins, as well as FIG. 3. For example, in some embodiments the consumable comprises a

hemoprotein (e.g., a hemoglobin, myoglobin, neuroglobin, cytoglobin, leghemoglobin, non-symbiotic hemoglobin, Hell's gate globin I, a bacterial hemoglobin, a ciliate myoglobin, or a flavohemoglobin).

Leghemoglobin, similar in structure and physical properties to myoglobin, is readily available as an unused by-product of commodity legume crops (e.g., soybean or pea). The leghemoglobin in the roots of these crops in the US exceeds the myoglobin content of all the red meat consumed in the US.

In some embodiments, the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, and containing a heme protein (e.g., a leghemoglobin or member of the globin protein family). For example, a meat replica can be principally or entirely composed of ingredients derived from non-animal sources, including a muscle tissue replica, an adipose tissue replica, a connective tissue replica, and a heme protein. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, with a high iron content from a heme protein. In some embodiments the iron content is similar to meat. In some embodiments the consumable has the distinctive red color of meat, such color provided by leghemoglobin.

A heme protein (e.g., a heme-containing protein described in Section III B) can be used as an indicator that the consumable is finished cooking. So, one embodiment of the invention is a method for cooking a consumable comprising detecting leghemoglobin, which has migrated from the interior of the consumable to the surface when the product is cooked. Another embodiment of the invention is a method for cooking a consumable comprising detecting the change in color of from red to brown when the product is cooked.

In some embodiments, the increased shelf life is provided by an extension of the lifetime of the desired red color of food products (e.g., a non-meat based meat substitute).

In one embodiment, this invention provides hemoproteins that provide a desired color to non-meat meat substitutes. In some embodiments, the hemoproteins are derived from a non-animal source such as a plant, fungus, or genetically modified organisms such

as, e.g., plant, algae, bacteria or fungus. See, e.g., section III B. In some embodiments the life time of the hemoproteins is extended by treatment with meat shelf life extenders.

In some embodiments, the meat shelf life extenders are selected from a groups consisting of carbon monoxide, nitrites, sodium metabisulfite, Bombal, rosemary extract, green tea extract, catechins and other anti-oxidants.

In one embodiment, this invention provides hemoproteins that provide a desired flavor profiles to food products (e.g., non-meat meat substitutes). In some embodiments, the ability of the hemoproteins to generate the desired flavor profile is similar to that of myoglobin

In some embodiments, the life time of the ability of the hemoproteins to generate the desired flavor profile is 10%, 20%, 30% 50%, or 100% or more greater than that of myoglobin.

D. Food products comprising isolated, purified heme proteins

In some embodiments, heme proteins described herein are added to meat or a consumable described herein to enhance the properties of the meat or consumable. For example, a heme protein containing solution can be injected into raw (e.g., raw white meat) or cooked meat to improve the organoleptic properties of the meat during cooking adding a "beefy" flavor (e.g., to white meats such as chicken).

In another example, a heme protein solution can be dripped over meat or a consumable of the invention to enhance appearance. In one embodiment, advertising, photography, or videography of food products such as meat or a meat substitute can be enhanced with a heme protein.

In another embodiment, a heme protein is added to the consumable as an iron supplement.

In one application of the invention, hemeproteins may be used as food dyes. In one embodiment, the heme proteins may be used as a safe, digestible replacement for FD&C Red No. 40 – Allura Red AC, E129 (red shade) in a variety of applications. A non-limiting list of such potential uses would include making pictures, especially in forms such as body-painting or as theatrical blood.

In some embodiments, the present invention provides methods for obtaining hemeproteins (e.g., leghemoglobin) from a plant. Leghemoglobin can be obtained from a variety of plants. Various legumes species and their varieties (e.g., soybean, fava bean, lima bean, cowpeas, English peas, yellow peas, Lupine, kidney beans, garbanzo beans, peanuts, Alfalfa, Vetch hay, clover, Lespedeza, or pinto bean) contain nitrogen-fixing root nodules in which leghemoglobin has a key role in controlling oxygen concentrations (for example root nodules from a pea plant). In one embodiment leghemoglobin protein is purified from root nodules of legume plants (e.g., soybeans, favabeans, or peas) using ion-exchange chromatography. In an embodiment, leghemoglobin is purified from soybean, favabean or sweet pea root nodules.

Plants can be grown using standard agricultural methods, with the exception that, in some instances, fertilizer is not applied and soil is enriched in natural nitrogen-fixing bacteria from the *Rhizobium* genus. Either whole roots or root nodules can be harvested and lysed, for example in 20mM potassium phosphate pH 7.4, 100mM potassium chloride and 5mM EDTA using grinder-blender. During this process, leghemoglobin is released into the buffer. Root-nodule lysate containing leghemoglobin can be cleared from cell debris by filtration through 5μm filter. In some embodiments, filtration is followed by centrifugation (7000g, 20min). Clarified lysate containing leghemoglobin is then filtered through 200nm filter and applied onto anion-exchange chromatography column (High Prep Q; High Prep DEAE, GE Healthtcare) on fast protein liquid chromatography machine (GE Healthcare). Leghemoglobin is collected in the flowthrough fraction and concentrated over 3kDa filtration membrane to a desired concentration. Purity (partial abundance) of purified leghemoglobin is analyzed by SDS-PAGE gel: in lysate leghemoglobin is present at 20-40%, while after anion-exchange purification it is present at 70-80%. In another embodiment, soybean leghemoglobin flowthrough from anion-exchange chromatography is applied onto size-exclusion chromatography (Sephacryl S-100 HR, GE Healthcare). Soybean leghemoglobin is eluted as two fractions corresponding to dimer and monomer species. Purity (partial abundance) of leghemoglobin was analyzed by SDS-PAGE and determined to be ~ 90-100%.

Proteins in legume root-nodule lysate can be transferred into 10 mM sodium carbonate pH 9.5, 50mM sodium chloride buffer, filtered through 200nm filter and applied onto anion-exchange chromatography column on fast protein liquid chromatography instrument (GE Healthcare). Leghemoglobin can be bound to anion-exchange chromatography matrix and eluted using sodium chloride gradient. Purity (partial abundance) of leghemoglobin can be analyzed by SDS-PAGE and determined to be ~ 60-80%.

Undesired small molecules from legume roots can be removed from purified leghemoglobin by passing leghemoglobin in solution over anion-exchange resin. These small molecules imbue varying shades of brown color to root-root nodule lysates, thus decreasing the color quality of leghemoglobin solution. In one embodiment, anion-exchange resin is FFQ, DEAE, Amberlite IRA900, Dowex 22, or Dowex 1x4. Leghemoglobin purified either by ammonium sulfate fractionation (60% wt/v and 90% wt/v ammonium sulfate) or by anion-exchange chromatography was buffer exchanged into 20mM potassium phosphate pH 7.4, 100mM sodium chloride and solution passed over one of the above mentioned anion-exchange resins. Flowthrough can be collected and its colored compared to the color of the solution before passage over anion-exchange resins. Color improvement to purified leghemoglobin solution as evaluated by visual inspection can be observed (from yellow/brown to more apparent red), however to different extent of removal of yellow-brown tinge.

Alternatively, the heme-containing protein can be recombinantly produced as described in section III B. For example, a non-symbiotic hemoglobin from moong bean can be recombinantly expressed in *E.coli* and purified using anion-exchange chromatography or cation-exchange chromatography. A cell lysate can be loaded over FF-Q resin on fast protein liquid chromatography instrument (GE Healthcare). Moong bean non-symbiotic hemoglobin eluted in the flowthrough fractions. Purity (partial abundance) of Moong bean non-symbiotic hemoglobin was analyzed by SDS-PAGE and determined to be as a fraction of total protein: 12 % in *E.coli* lysate, and 31% after purification on FFQ. UV-Vis analysis of purified protein showed spectra characteristic of heme bound protein.

Alternatively, the cell lysate can be loaded over a FF-S resin on a fast protein liquid chromatography instrument (GE Healthcare). Moong bean non-symbiotic hemoglobin can be bound to FF-S column and eluted using sodium chloride gradient (50mM- 1000mM). Purity (partial abundance) of Moong bean non-symbiotic hemoglobin can be analyzed by SDS-PAGE and determined to be: *E.coli* lysate 13 %, after purification on FFQ 35%. UV-Vis analysis of purified protein can show spectra characteristic of heme bound protein.

In some embodiments, the heme proteins are utilized as ingredients in food products where the flavor of blood is desired. Heme-containing proteins of the invention were tasted by a panel of volunteers and in each case described as tasting like blood.

Heme proteins, for example leghemoglobin, can be combined with other plant based meat replica components. In some embodiments the heme proteins are captured in a gel which contains other components, for example lipids and or other proteins. In some aspects, multiple gels are combined with non-gel based heme proteins. In some embodiments, the combination of the heme proteins and the other compounds of the consumable are done to insure that the heme proteins are able to diffuse through the consumable. In some embodiments the consumable is soaked in a heme-protein containing solution, for instance a leghemoglobin solution, e.g., for 1, 5, 10, 15, 30, or 45 minutes or for 1, 5, 10, 15, 20 or 30 hours.

Given the usefulness of heme proteins for coloring consumables, it is useful to detect whether a product contains a particular heme protein. Accordingly the present invention includes in some embodiments methods to determine whether a product contains a heme protein. For example, an ELISA, a proximity-ligation assay, a luminex assay, or western blot analysis can be performed to determine whether leghemoglobin or other heme-containing protein is present in a food product such as meat or a meat replica. In one embodiment the detection methods are performed to determine whether meat has been altered with leghemoglobin or other heme-containing protein.

E. MAYONNAISE SPREAD REPLICA.

Mayonnaise is thick, creamy sauce. Traditional mayonnaise is a stable emulsion of oil and egg yolk. It is thought that lecithin and proteins from egg yolk stabilize the emulsion. Traditional commercial mayonnaise typically contains 70-80% (wt/wt) fat and 5% (wt/wt) of egg yolk. Lower fat commercial products can contain ~ 20% wt/wt fat. The consumable can comprise a composition that has comparable properties to mayonnaise.

In one embodiment, purified plant proteins can be used as substitute for egg proteins to make stable, creamy protein-fat emulsions whose visual and mouth-feel appearance resemble traditional mayonnaise. Fat (~ 20- 80% wt/wt) can be from a single source or from multiple sources as described herein. Non-traditional mayonnaise products can be used for all the culinary applications that traditional mayonnaise is used for. In one embodiment, vinegar and/or lemon and/or lime juice are added as flavor additives. In one embodiment, the purified plant proteins are not soy proteins. In one embodiment, the flavor can be modified by addition of mustard, spices, herbs, and/or pickles.

A mayonnaise replica can comprise a mixture of non-animal proteins. In one embodiment, a mayonnaise replica is a mixture of 50% (wt/v) rice bran oil and 7% (wt/v) Moong bean 8S protein. In one embodiment, a mayonnaise replica is a mixture of 70% (wt/v) sunflower oil or cocoa butter, 2.4% (wt/v) RuBisCo,0.29% (wt/wt) soybean lecithin, and optionally 8 μM oleosin.

The mixture can be emulsified, and the stability of the emulsion can be controlled by modifying the size of the oil-water-protein particles by high pressure homogenization or sonication. Oil can be added as liquid. Protein can be added as solution in buffer. Soybean lecithin can be resuspended in water and sonicated prior to mixing with an oil and protein solution. A resulting oil, protein and lecithin solution can be homogenized, for example, first at 5000psi and then at 8000psi, or can be sonicated at 40% duty cycle for 2 minutes at maximum setting. Thickness, texture, creaminess and visual appearance of resulting products are similar to one of traditional mayonnaise. In some instances (e.g. using Moong bean 8S protein and rice bran oil), a product is a light off-white in color.

F. CREAM LIQUOR REPLICA

Traditionally, cream liquors contain dairy cream and liquor as their base. Examples of liquor include whiskey, Irish whiskey, Scotch whiskey, rum, vodka, grappa, or fermented fruits (e.g., cherry liquor), plum brandy, tequila, or herbal bitters. A cream liquour replica can be produced by substituting the dairy cream in cream liquor with a non-dairy cream fraction from plant sources. In one embodiment, dairy cream in a cream liquor can be substituted by a stable emulsion of plant fats and isolated or purified proteins of a consistency similar to dairy cream. In one embodiment, purified plant proteins and/or plant fats can be from single or multiple sources as described herein. For example, a cream liquor can include a sunflower cream fraction, RuBsiCo and whiskey, and one or more optional flavorings (e.g., vanilla, chocolate, and or coffee).

G. PROTEIN ENRICHED ALCOHOLIC BEVERAGE

Traditionally, alcoholic beverages contain negligible to low amounts of protein. Addition of plant proteins to various alcoholic beverages would positively modify their flavor, mouthfeel, physical state and increase their nutritional protein content. In addition, the presence of protein in various alcoholic beverages used in cocktails would positively modify the cocktail's flavor, mouthfeel, physical state and increase their nutritional protein content. Different classes of alcoholic beverages contain different amounts of alcohols. For example, wine coolers contain about 4-7% alcohol, beer contains about ~ 3-10% alcohol, wine contains about 8-14% v/v alcohol, dessert wines contain about 17-20% alcohol, whiskey contains about ~ 40% alcohol, and vodka contains about 35-50% alcohol. In addition, some traditional alcoholic beverages include sugars (for example, Bacardi Razz at 10% wt/v).

Accordingly beverages containing alcohol can be supplemented by addition of purified plant proteins at for example 0.1-5% wt/v and optionally sugar (1-15% wt/v). The sugar can be, for example, cane sugar, brown sugar, sucrose, or glucose. For example, purified Rubisco at 180mg/ml in 20mM K-phosphate pH 7.0, 150mM NaCl can be added to a whiskey. Jameson whiskey supplemented with 5% wt/v Rubsico formed a soft gel, with a consistency similar to traditional Jello shots.

For example, Rubsico, moong bean 8S, and pa Globulin enriched alcoholic beverages were made by adding purified Rubsico, Moong bean 8S and pea globulin proteins at final protein concentrations of 0.5%, 1% and 5% wt/v, respectively, to Corona beer, Pinot grigio wine and Jameson whiskey. Zein was added at 0.5%, 1% and 5% wt/v to a 60% ethanol, 5% sucrose solution in water.

Pea proteins were extracted from pea flour by resuspending the flour in 5%, 20% or40% ethanol, 5% sucrose solutions in water, followed by incubation for 1hr at room temperature. Any undissolved solids were removed by centrifugation at 5000g for 10min. The resultant supernatant solution was clear in appearance. The 5% ethanol solution was particularly useful.

A sensory panel evaluated all protein enriched alcoholic beverages as having aromas and flavors different from beverages not-enriched in protein. In some cases, generated aromas and flavors were judged as neutral, in some cases as more appealing and in some cases as less appealing than controls. In particular example, addition of both 0.5% wt/v and 1% wt/v of Moong bean 8S protein to Jameson whiskey softened the aroma and mouthfeel of Jameson. Addition of 0.5% wt/v of moong bean to Jameson whisky added a slightly creamy flavor to Jameson, with an aroma similar to a traditional White Russian cocktail. Addition of 5% wt/v zein to Jameson whiskey generated aromas and flavor characterized as moldy beans and raw potato.

In another example where Corona beer was enriched with 0.5% wt/v pea Globulin, the aroma changed to hoppy and resembled one of Indian Pale Ale, and the flavor changed to carrying pea notes. Addition of 0.5% wt/v and 5% wt/v of moong bean 8S protein changed the Corona aroma towards sweet peony flower with an intensified hop aroma. The flavor was neutral in the case of 0.5% wt/v moong bean 8S and carried planty-nutty notes in the case of 5% wt/v moong bean 8S.

In another example where Pinot Grigio wine was enriched with 1% wt/v moong bean 8S protein, additional aroma notes of sweet and citrus were detected, and the flavor changed to that of carrying notes of peanut-butter. Addition of 1% wt/v of Pea Globulins modified the aroma to that of strong moldy oak and wet leaves. Flavor was modified to

carry notes of mud. Addition of 5% wt/v of Rubisco generated aroma and flavor of wet hay.

A zein enriched 60% ethanol, 5% sucrose solution carrried burnt tortilla chip aroma notes compared to a corresponding solution without zein. There was no difference in flavor.

Pea proteins enriched 5%, 20% and 40% ethanol, 5% sucrose solutions all developed earthy aroma and flavor compared to protein free controls. In addition, flavor of peas was detected and bitter flavor increased at higher alcohol content.

H. CHOCOLATE SPREAD

Chocolate spread is a chocolate flavored spread whose traditional main ingredients are cocoa powder, dairy milk, plant oil, and sugar. Traditional chocolate spreads are either firm or soft solids at ambient room temperature and melt at temperatures below that of cocoa butter. The product can be used as a spread on bread, crepes, pancakes, icing for cakes and cookies, filling for chocolate confectionary, or non-dairy chocolate cake filling.

In one embodiment, dairy milk and milk products such as ice cream, whey, cream, yoghurt, sour cream or butter fat are substituted by a non-dairy cream fraction made as described herein. In one embodiment, a non-dairy cream fraction comes from a single source or multiple sources described herein. In one embodiment, dairy milk and milk products are substituted by any non-dairy milk described herein. In one embodiment, dairy milk and milk products are substituted by purified plant proteins described herein. In one embodiment, dairy milk and milk products are substituted by soft solid stable emulsions made from single or multiple plant oil and single or multiple purified plant proteins.

I. OTHER APPLICATIONS:

In one embodiment, non-dairy plant cream fraction can be used as a substitute for dairy milk and dairy milk products to make non-dairy milk chocolate bars or non-dairy milk chocolate confectionary.

In one embodiment, non-dairy plant cream fraction and purified plant proteins can be used as substitute for dairy milk and dairy milk products to make non-dairy milk chocolate bars or a non-dairy milk chocolate confectionary.

In one embodiment, non-dairy plant cream fraction and plant proteins can be used to make chocolate mousse. Traditional main chocolate mousse ingredients are bittersweet or semisweet chocolate, dairy butter and eggs. In one embodiment, dairy butter can be substituted by a non-dairy plant cream fraction. In one embodiment, dairy butter and eggs can be substituted by a non-dairy plant cream fraction and a foam stabilizing plant seed storage proteins such as pea albumin.

In one embodiment, a vegan consumable such as pate analog can be made. Vegan pate analog can be made by finely chopping 10 g of fat replica and heating it on a frying pan with finely chopped shallots for 2-3 min. Muscle replica (20 g) made without connective tissue replica fibers can be chopped into ½-inch cubes and browned in the fat and shallots mix for another 3-5 min. The mixture can be forced through a sieve until homogeneous. The pan, while still warm, can be rinsed with a tablespoon of madeira without allowing it to evaporate fully. The liquid from the pan is added to the homogenized mix, spices (salt, pepper) are added to taste, and the mix is forced through a sieve again. After chilling in a refrigerator (e.g., for 15 minutes), the pate is ready to be served.

In some embodiments, other fat-to-muscle replica ratios are used to create leaner or richer pates. For example, pate can contain 0.5-10%, about 5%-40%, about 10%-60%, or about 30-70%, or >70% of an adipose tissue replica.

In some embodiments, a muscle tissue replica with a higher iron content can be used for pate to make it a closer imitation of pig or bird liver pate. For example, muscle tissue replica can contain about 1%, about 1.5%, about 2%, or >2% of a heme protein.

In some embodiments, muscle tissue replica with lower iron content can be used for pate to make it closer imitation of bird meat or fish pate. For example, muscle tissue replica can contain about 1%, about 0.5%, about 0.2%, or <0.2% of a heme protein.

In one embodiment, a vegan consumable such as a blood sausage analog can be made. Vegan blood sausage is made from a blood analog created by mixing solutions of

heme protein and purified plant protein. For example, 35 ml of a mixed solution of leghemoglobin (120 mg/ml) and pea albumin (100 mg/ml), which approximates the composition of blood, can be carefully mixed with a slurry of corn flour in salt water (6:5 w/v flour to water ratio). A tablespoon of chopped onion can be fried with 10 g of chopped adipose tissue replica, mixed with a few raisins and cooled to room temperature before mixing with blood/flour mix. The mixture can be seasoned to taste (for example, using salt, pepper, parsley and/or cinnamon), loaded into vegetarian sausage casings, and poached in near-boiling water for about 45 min. After cooling, the sausage can be consumed as is or further cooked, for example smoked, crisped in an oven or roasted.

In some embodiments, muscle replica can be included in the recipe to imitate meat/blood sausages. In some embodiments, barley, buckwheat, oat, rice, rye, sorghum, wheat or other grains can be used in the blood sausage. In some embodiments, bread, chestnuts, potato, sweet potato, starch or other fillers can be added to, or substitute for, grains in blood sausage.

EXAMPLES

EXAMPLE 1: PROTEIN ISOLATION.

All steps were carried out at 4˚C or room temperature. Centrifugation steps were at 8000 g for 20 mins, 4˚C or room temperature. The flour is suspended in a specific buffer, the suspension is centrifuged and the supernatant is microfiltered through a 0.2 micron PES membrane and then concentrated by ultrafiltration on a 3 kDa, 5 kDa, or 10 kDa molecular weight cutoff PES membrane on a Spectrum Labs KrosFlo hollow fiber tangential flow filtration system.

Once fractionated, all ammonium sulfate precipitate fractions of interest were stored at -20˚C until further use. Prior to their use in experiments, the precipitates were resuspended in 10 volumes of 50 mM K Phosphate buffer, pH 7.4, + 0.5 M NaCl. The suspensions were centrifuged and the supernatants microfiltered through a 0.2 micron PES membrane and then concentrated by ultrafiltration on a 3 kDa, 5 kDa, or 10 kDa molecular weight cutoff PES membrane on a Spectrum Labs KrosFlo hollow fiber tangential flow filtration system. Protein composition at individual fractionation steps

was monitored by SDS-PAGE and protein concentrations were measured by standard UV-Vis methods.

(i) Pea-albumins: Dry green or yellow pea flour was used as a source of pea albumins. The flour was suspended in 10 volumes of 50 mM sodium acetate buffer pH 5 and stirred for 1 hr. Soluble protein was separated from un-extracted protein and pea seed debris by either centrifugation (8000 g, 20 minutes) or filtration through a 5 micron filter. Supernatant or filtrate, respectively, was collected. To this crude protein extract, solid ammonium sulfate was added to 50% wt/v saturation. The solution was stirred for 1 hour and then centrifuged. To the supernatant from this step, ammonium sulfate was added to bring to 90% wt/v saturation. The solution was stirred for 1 hour, and then centrifuged to collect the pea albumin proteins in the pellet. The pellet was stored at -20°C until further use. Protein was recovered from the pellet and prepared for use as described above, with the exception that final buffer can contain 0-500 mM sodium chloride.

In some embodiments, the flour was suspended in 10 volumes of 50 mM NaCl, pH 3.8 and stirred for 1 hour. Soluble protein was separated from un-extracted protein and pea seed debris by centrifugation (8000 g, 20 minutes). The supernatant was collected and filtered through a 0.2 micron membrane and concentrated using a 10 Kda cutoff PES membrane.

(ii) Pea-globulins: Dry green pea flour was used to extract pea globulin proteins. The flour was suspended in 10 volumes of 50 mM potassium phosphate buffer pH 8 and 0.4M sodium chloride and stirred for 1hr. Soluble protein was separated from pea seed debris by centrifugation. The supernatant was subjected to ammonium sulfate fractionation in two steps at 50% and 80% saturation. The 80% pellet containing globulins of interest was stored at -20°C until further use. Protein was recovered from the pellet and prepared for use as described above.

iii) Soybean 7S and 11S globulins: Globulins from soybean flour were isolated by first suspending lowfat/defatted soy flour in 4-15 volumes of 10 (or 20) mM potassium phosphate pH 7.4. The slurry was centrifuged at 8000 rcf for 20 mins or clarified by 5 micron filtration and the supernatant was collected. The crude protein extract contained both the 7S and 11S globulins. The solution then was 0.2 micron filtered and

concentrated using a 10 kDa molecular weight cutoff PES membrane on a Spectrum Labs KrosFlo hollow fiber tangential flow filtration system or by passing over anion-exchange resin prior to use in experiments. The 11S globulins were separated from the 7S proteins by isoelectric precipitation. The pH of the crude protein extract was adjusted to 6.4 with dilute HCl, stirred for 30 min-1 hr and then centrifuged to collect the 11S precipitate and 7S proteins in the supernatant. The 11S fraction was resuspended with 10mM Potassium phosphate pH 7.4 and the protein fractions were microfiltered and concentrated prior to use.

Soybean proteins also can be extracted by suspending the defatted soy flour in 4-15 volumes (e.g., 5 volumes) of 20 mM sodium carbonate, pH 9 (or water, pH adjusted to 9 after addition of the flour) or 20 mM potassium phosphate buffer pH 7.4 and 100 mM sodium chloride to decrease off-flavors in the purified protein. The slurry is stirred for one hour and centrifuged at 8000 xg for 20 minutes. The extracted proteins are ultrafiltered and then processed as above or alternatively, the supernatant was collected and filtered through a 0.2 micron membrane and concentrated using a 10 KDa cutoff PES membrane.

(iv) Moong bean 8S globulins: Moong bean flour was used to extract 8S globulins by first suspending the flour in 4 volumes of 50 mM KPhosphate buffer pH 7 (+ 0.5M NaCl for lab scale purifications). After centrifugation, proteins in the supernatant were fractionated by addition of ammonium sulfate in 2 steps at 50% and 90% saturation respectively. The precipitate from the 90% fraction contained the 8S globulins and was saved at -20˚C until further use. Protein was recovered from the pellet and prepared for use as described above.

Moong bean globulins also can be extracted by suspending the flour in 4 volumes of 20 mM sodium carbonate buffer, pH 9 (or water adjusted to pH 9 after addition of the moong flour) to reduce off-flavors in the purified protein fractions. The slurry is centrifuged (or filtered) to remove solids, ultrafiltered and then processed as described above.

(v) Late embryogenesis abundant proteins: Flour (including but not limited to moong bean and soy flour) was suspended in 20 mM Tris–HCl, pH 8.0, 10 mM NaCl,

and stirred at room temperature for 1 hour then centrifuged. Acid (HCl or acetic acid) was added to the supernatant to a 5% concentration (v/v), stirred at room temperature then centrifuged. The supernatant was heated to 95°C for 15 minutes, and then centrifuged. The supernatant was precipitated by adding Trichoroacetic acid to 25%, centrifuged, then washed with acetone. Heating and acid wash steps can be carried out in the reverse direction as well.

(vi) Pea-Prolamins: Dry green pea flour was suspended in 5x (w/v) 60% ethanol, stirred at room temperature for one hour, then centrifuged (7000g, 20min) and the supernatant collected. The ethanol in the supernatant was evaporated by heating the solution to 85°C and then cooling to room temperature. Ice-cold acetone was added (1: 4 v/v) to precipitate the proteins. The solution then was centrifuged (4000g, 20min), and protein recovered as the light-beige colored pellet.

(vii) Zein-Prolamins: Corn protein concentration or flour was suspended in 5x (w/v) 60% ethanol, stirred at room temperature for one hour, then centrifuged. Ethanol in supernatant was evaporated with heat, and then the solution is centrifuged, and the protein recovered as the pellet.

(viii) RuBisCO was fractionated from alfalfa greens by first grinding leaves with 4 volumes of cold 50 mM potassium phosphate buffer pH 7.4 buffer (0.5M NaCl + 2mM DTT + 1mM EDTA) in a blender. The resulting slurry was centrifuged to remove debris, and the supernatant (crude lysate) was used in further purification steps. Proteins in the crude lysate were fractionated by addition of ammonium sulfate to 30% (wt/v) saturation. The solution was stirred for 1hr and then centrifuged. The pellet from this step was discarded and additional ammonium sulfate was added to the supernatant to 50 % (wt/v) ammonium sulfate saturation. The solution was centrifuged again after stirring for 1hr. The pellet from this step contains RuBisCO, and was kept at -20°°C until used. Protein was recovered from the pellet and prepared for use as described above.

RuBisCO also can be purified by adjusting the crude lysate to 0.1M NaCl and applying to an anion exchange resin. The weakly bound protein contaminants are washed with 50 mM KPhosphate buffer pH 7.4 buffer + 0.1M NaCl. RuBisCO was then eluted with high ionic strength buffer (0.5M NaCl).

RuBisCO solutions were decolorized (pH 7-9) by passing over columns packed with activated carbon. The colorants bound to the column while Rubisco was isolated in the filtrate.

RuBisCO solutions were also alternatively decolorized by incubating the solution with FPX66 (Dow Chemicals) resin packed in a column (or batch mode). The slurry is incubated for 30mins and then the liquid is separated from the resin. The colorants bind to the resin and RuBisCO was collected in the column flow-through.

In some embodiments, RuBisCO was isolated from spinach leaves by first grinding the leaves with 4 volumes of 20mM potassium Phosphate buffer pH 7.4 buffer + 150 mM NaCl + 0.5 mM EDTA) in a blender. The resulting slurry was centrifuged to remove debris, and the supernatant (crude lysate) was filtered through a 0.2 micron membrane and concentrated using a 10 KDa cutoff PES membrane.

In some embodiments, RuBisCO was extracted from alfalfa or wheatgrass juice powder by mixing the powder with 4 volumes of 20mM potassium Phosphate buffer pH 7.4 buffer + 150 mM NaCl + 0.5 mM EDTA) in a blender. The resulting slurry was centrifuged to remove debris, and the supernatant (crude lysate) was filtered through a 0.2 micron membrane and concentrated using a 10 KDa cutoff PES membrane.

(ix) Leghemoglobin. Soy root nodules were suspended and lysed in 20 mM potassium phosphate pH 7.4, 100mM potassium chloride and 5mM EDTA using grinder-blender. During this process leghemoglobin is released into the buffer. Root-nodule lysate containing leghemoglobin was cleared from cell debris by filtration through 5 micron filter. In some embodiments, filtration was followed by centrifugation (7000g, 20min). Clarified lysate containing leghemoglobin was then filtered through 0.2 micron filter and applied onto anion-exchange chromatography column (High Prep Q; High Prep DEAE, GE Healthtcare) on fast protein liquid chromatography instrument (GE Healthcare). Leghemoglobin was collected in flowthrough fraction and concentrated over 3kDa molecular weight cutoff PES membrane on a Spectrum Labs KrosFlo hollow fiber tangential flow filtration system to a desired concentration. Purity (partial abundance) of purified leghemoglobin was analyzed by SDS-PAGE gel: in lysate leghemoglobin is present at 20-40%, while after anion-exchange purification it is present at 70-80%. In

another embodiment, soybean leghemoglobin flow through from anion-exchange chromatography was applied onto size-exclusion chromatography (Sephacryl S-100 HR, GE Healthcare). Soybean leghemoglobin eluted as two fractions corresponding to dimer and monomer species. Purity (partial abundance) of leghemoglobin was analyzed by SDS-PAGE and determined to be ~ 90-100%. Analysis of UV-VIS spectra (250-700nm) revealed spectral signature consistent with heme loaded leghemoglobin.

(x) Non-symbiotic hemoglobin from moong bean was cloned into pJexpress401 vector (DNA2.0) and transformed into *E. coli* BL21. Cells were grown in LB media containing soytone instead of tryptone, kanamycin, 0.1mM ferric chloride and 10 μg/ml 5-aminolevulinic acid. Expression was induced by 0.2mM IPTG and cells grown at 30°C for 20hr. *E.coli* cells expressing moong bean non-symbiotic hemoglobin were collected and resuspended in 20mM MES buffer pH 6.5, 50mM NaCL, 1mmM $MgCl_2$, 1mM $CaCl_2$. Add a bit of DNAaseI, and protease inhibitors. Cells were lysed by sonication. Lysate was cleared from cell debris by centrifugation at 16 000g for 20 min, followed by filtration over 200nm filter. Cell lysate was then loaded over FF-S resin on fast protein liquid chromatography instrument (GE Healthcare). Moong bean non-symbiotic hemoglobin bound to FF-S column and was eluted using sodium chloride gradient (50mM- 1000mM). Purity (partial abundance) of moong bean non-symbiotic hemoglobin was analyzed by SDS-PAGE and determined to be: *E.coli* lysate 13 %, after purification on FFQ 35%. UV-Vis analysis of purified protein showed spectra characteristic of heme bound protein.

(xi) Hemeproteins were synthesized with an N-terminal His6 epitope tag and a TEV cleavage site, cloned into pJexpress401 vector (DNA2.0), and transformed into *E. coli* BL21. Transformed cells were grown in LB media containing soytone instead of tryptone, kanamycin, 0.1 mM ferric chloride and 10 μg/ml 5-aminolevulinic acid. Expression was induced by 0.2mM IPTG and cells grown at 30°C for 20hr. *E.coli* cells expressing heme proteins were collected and resuspended in 50 mM potassium phosphate pH 8, 150 mM NaCl, 10 mM imidazole, 1 mM $MgCl_2$, 1 mM $CaCl_2$, DNAaseI, and protease inhibitors. Cells were lysed by sonication and clarified by centrifugation at 9000 x g. Lysate was incubated with NiNTA resin (MCLAB), washed with 5 column volumes

(CV) of 50 mM potassium phosphate pH 8, 150 mM NaCl, 10 mM imidazole, and eluted with 50 mM potassium phosphate pH 8, 150 mM NaCl, 500 mM imidazole. SDS-PAGE and UV-vis spectra confirmed expected molecular weights and complete heme-loading, respectively.

In some embodiments, transformed cells were grown in seed media comprised of 10g/L glucose monohydrate, 8g/L Monopotassium Phosphate, 2.5g/L Sensient Amberferm 6400, 2.5g/L Sensient Tastone 154, 2g/L Diammonium Phosphate, 1mL/L Trace Metals Mixture (Teknova 1000x Trace Metals Mixture Cat. No. T1001), 1g/L Magnesium Sulfate, 0.25mL 0.1M solution Ferric Chloride, 0.5mL/L Sigma Anti-foam 204, 1mL/L Kanamycin Sulfate 1000x solution. 250mL of media was used in a (4)-1L baffeled shakeflasks, innoculated with 0.25mL each from a single vial of glycerol stock culture. Shakeflasks were grown for 5.5 hours, with 250RPM agitation at 37°C. 40L of seed media was steam-sterlized in a 100L bioreactor, cooled to 37°C, pH-adjusted to 7.0 and innoculated with 800mL of shakeflask culture once a shakeflask OD of 2.5 was achieved. Aeration to the bioreactor was supplied at 40L/m and agitation was 250RPM. After 2.2 hours of growth, an OD of 2.20 was reached and 22L of culture was transferred to the final $4m^3$ bioreactor. The starting media for the final bioreactor comprised of the following components steamed-in-place: 1775L deionised water, 21.75kg Monopotassium Phosphate, 2.175kg Diammonium Phosphate, 4.35kg Ammonium Ferric Citrate, 8.7kg Ammonium Sulfate, 10.875kg Sensient Amberferm 6400, 10.875kg Sensient Tastone 154. After 30 minutes of steaming, the media components were cooled to 37°C and post-sterilzation additions were made: 2.145L of 0.1M Ferric Chloride solution, 59.32kg 55%w/w Glucose Monohydrate, 3.9L of Trace Metals Mixture (Teknova 1000x Trace Metals Mixture Cat. No. T1001), 10.88L of 200g/L Diammonium Phosphate, 36.14L 1M Magnesium Sulfate, and 2.175L Sigma Anti-foam 204, 2.175L Kanamycin Sulfate 1000x solution. pH was controlled at 7.0 via the addition of 30% Ammonium Hydroxide. Aeration was supplied at $2.175m^3$/min, dissolved oxygen was controlled at 25% by varying agitation between 60-150RPM. At two timepoints (EFT=4 and EFT8), bolus additions of additional nutrients were supplied. Each addition added 5.5kg of Sensient Amberferm 6400, 5.5kg of Sensient Tastone 154 and 4.4kg of

Diammonium Phosphate, in autoclaved solutions (100g/L solution for Amberferm and Tastetone, 200g/L for Diammonium Phosphate). A sterile glucose solution of 55%w/w Glucose Monohydrate was fed into the bioreactor to maintain a level of residudual glucose of 2-5g/L. Once an OD of 25 was reached, the temperature was reduced to 25°C and the culture was induced with 0.648L of 1M Isopropyl β-D-1-thiogalactopyranoside. The culture was allowed to grow for a total time of 25 hours, at which point the culture was diluted 1:1 with deionized water, then centrifuged, concentrating the centrate to 50%v/v solids content. Cell centrate was frozen at -20°C. Centrate was thawed to 4°C and diluted in 20 mM potassium phosphate pH 7.8, 100 mM NaCl, 10 mM imidazole, and homogenized at 15,000 PSI. Homogenized cells were 0.2 um filtered by tangential flow filtration (TFF) and filtered lysate was loaded directly onto a zinc-charged IMAC column (GE). Bound proteins were washed with 10 column volumes (CV) 20 mM potassium phosphate pH 7.4, 100 mM NaCl, 5 mM histidine and eluted with 10 CV 500 mM potassium phosphate monobasic, 100 mM NaCl. Eluted leghemoglobin was concentrated and diafiltered using a 3kDa molecular weight cutoff PES membrane and TFF. The concentrated sample was reduced with 20 mM sodium dithionite and desalted using G-20 resin (GE). Desalted leghemoglobin samples were frozen in liquid nitrogen and stored at -20 C. Leghemoglobin concentration and purity were determined by SDS-PAGE and UV-vis analysis.

(xi) Oleosin. Sunflower oil bodies were purified from sunflower seeds. Sunflower seeds were blended in 100 mM sodium phosphate buffer pH 7.4, 50mM sodium chloride, 1 mM EDTA at 1:3 wt/v. Oil-bodies were collected by centrifugation (5000g, 20min), and resuspended at 1:5 (wt/v) in 50 mM sodium chloride, 2M urea and stir for 30min, 4°C. 2M urea wash and centrifugation steps were repeated. Oil-bodies collected by centrifugation were resuspended in 100 mM sodium phosphate buffer pH 7.4, 50mM sodium chloride. Centrifugation and washing steps were repeated once more, and the final washed oil-bodies fraction was obtained from a last centrifugation step. Oil-bodies were resuspended at 10% wt/w in 100 mM sodium phosphate buffer pH 7.4, 50mM sodium chloride, 2% wt/v vegetable oil fatty acid salts, homogenized at 5000 psi and incubated at 4°C for 12 hr. Solution was centrifuged (8000g, 30min), top layer removed

and soluble fraction collected. SDS-PAGE analysis suggested that oleosins are a major protein present in the soluble fraction. Oleosin concentration was 2.8 mg/ml.

(xii) Pea total proteins: Dry green or yellow pea flour was used to extract total pea proteins. The flour was suspended in 10 volumes of 20mM potassium phosphate buffer pH 8 and 100 mM sodium chloride and stirred for 1hr. Soluble protein was separated from pea seed debris by centrifugation. The supernatant was collected and filtered through a 0.2 micron membrane and concentrated using a 10 Kda cutoff PES membrane.

(xiii) Pea vicilin and Pea legumin: Dry green or yellow pea flour was used to extract total pea proteins as described above. The crude pea mixture obtained thereof was fractionated into pea vicilin and pea legumin using ion-exchange chromatography. Material was loaded on Q Sepharose FastFlow resin and fractions were collected as salt concentration was varied from 100 mM to 500 mM NaCl. Pea vicilin was collected at 350 mM sodium chloride while pea legumin was collected at 460 mM sodium chloride. The collected fractions were concentrated using a 10 KDa cutoff PES membrane.

(xv) Lentil total proteins: Air classified lentil flour was used to extract crude mixture of lentil proteins. Flour was suspended in 5 volumes of 20 mM potassium phosphate buffer pH 7.4 and 0.5 M sodium chloride and stirred for 1 hr. Soluble protein was separated from un-extracted protein and lentil seed debris by centrifugation (8000 g, 20 minutes). The supernatant was collected and filtered through a 0.2 micron membrane and concentrated using a 10 KDa cutoff PES membrane.

(xvi) Lentil albumins: Air classified lentil flour was suspended in 5 volumes of 50 mM sodium chloride, pH 3.8 and stirred for 1 hr. Soluble protein was separated from un-extracted protein and lentil seed debris by centrifugation (8000 g, 20 minutes). The supernatant was collected and filtered through a 0.2 micron membrane and concentrated using a 10 KDa cutoff PES membrane.

(xvii) Chickpea / Garbanzo bean total proteins: Garbanzo bean flour was suspended in 5 volumes of 20 mM potassium phosphate buffer pH 7.4 and 0.5 M sodium chloride and stirred for 1 hr. Soluble protein was separated from un-extracted protein and chickpea seed debris by centrifugation (8000 g, 20 minutes). The supernatant was

collected and filtered through a 0.2 micron membrane and concentrated using a 10 KDa cutoff PES membrane.

(xviii) Chickpea/ Garbanzo bean albumins: Garbanzo bean flour was suspended in 5 volumes of 50 mM sodium chloride, pH 3.8 and stirred for 1 hr. Soluble protein was separated from un-extracted protein and lentil seed debris by centrifugation (8000 g, 20 minutes). The supernatant was collected and filtered through a 0.2 micron membrane and concentrated using a 10 KDa cutoff PES membrane.

(xix) Amaranth flour dehydrins: Amaranth flour was suspended in 5 volumes of 0.5 M sodium chloride, pH 4.0 and stirred for 1 hr. Soluble protein was separated from un-extracted protein and lentil seed debris by centrifugation (8000 g, 20 minutes). The supernatant was collected and filtered through a 0.2 micron membrane and concentrated using a 3 KDa cutoff PES membrane. Further enrichment of dehydrins from this fraction was obtained by boiling the concentrated protein material, spinning at 8000 g for 10 minutes and collecting the supernatant.

EXAMPLE 2: CONSTRUCTING A MUSCLE TISSUE ANALOG

To prepare a muscle tissue replica, 8 ml of moong bean protein solution (114 mg/ml in 20 mM phosphate buffer (pH 7.4) and 400 mM sodium chloride) were mixed with 16 ml of leghemoglobin solution (6 mg/ml leghemoglobin in 20 mM potassium phosphate, 400 mM NaCl, pH 7.3). The resulting mixture was concentrated using Amicon spin concentrators (10 kDa cut-off) to a final concentration of moong bean 8S globulin 61 mg/ml, and of leghemoglobin 6.5 mg/ml. Approximately 400 mg of transglutaminase powder were added to the solution, which were thoroughly mixed, and divided into two 50 ml Falcon tubes and incubated overnight at room temperature. Final total protein concentrations was 67.5 mg/ml total protein. The muscle tissue replica formed an opaque gel of reddish-brown color, with small amounts (< 1 ml) of inclusions of dark red, venous blood colored liquid.

EXAMPLE 3: INCREASED TENSILE STRENGTH ADIPOSE TISSUE REPLICA

A 40 ml aliquot of rice bran oil and a 40 ml aliquot of moong bean protein (114 mg/ml) were combined in a 250ml Pyrex beaker. The beaker was placed in a water bath and emulsified using a Branson Sonifer 450 sonicator with a 12mm tip for a 6 minute, 60% duty cycle at power level 5.

In an 18cm x 18cm x 2.5cm synthetic rubber Ikea plastic ice cube tray, 48 mg of electrospun fibers (from connective tissue Example 14) were laid longitudinally and as homogenously as possible across the bottom of one triangular 13.97cm x 1.27cm x 1.5875cm mold. Approximately 20 ml of the rice bran oil/moong bean protein emulsions then were poured on top of the fibers. An additional 20 ml of the emulsions were then poured into a similarly sized blank mold on the same tray to be used as a control.

The ice cube tray was floated in boiling water for 15 minutes, removed, and cooled to room temperature.

Using a razor blade, each of the resulting gels was cut into 3 segments, each 4.66cm long with a cross-sectional area of $1cm^2$. A Stable Micro Systems TA XTExpress Enhanced texture analyser with attached TA-96B probe was used to assess the tensile strength. The fiber containing the fat replica had a tensile strength of 23 kPa, whereas the fat replica with no fibers had a tensile strength of 20 kPa.

EXAMPLE 4 HIGH PERCENTAGE FAT ADIPOSE-REPLICA

An adipose tissue replica comprising a protein-oil emulsification formed with 3.3% wt/v pea globulin, 70% v/v oil that consisted of an equal mixture of coconut, cocoa, olive, and palm oils, and 0.5% wt/v lecitin, was cross-linked with a 2% transglutaminase (Ajinomoto Activa® TI). After draining and dehydrating, the resulting gel was medium soft and the fat content was confirmed to be 75% (wt/wt).

An adiose tissue matrix comprising a protein-oil emulsification was formed with 1.6 % wt/v Rubisco and 80% v/v cocoa butter. The resulting gel was soft.

EXAMPLE 5: METHOD OF PREPARING ADIPOSE TISSUE REPLICAS

Oils are melted if necessary by warming to room temperature or gently heated. If the oils are solid at room temperature, they are kept near the melting point during the rest

of the procedure. Proteins are obtained per specified protocols (see Example 1). Lecithin is weighed and resuspended in water, then sonicated to create a homogenous solution. Components are combined at specified ratios and brought to volume with buffer if necessary (20 mM sodium phosphate pH 7.4 with 50 mM sodium chloride) then are subjected to homogenization or sonication to control particle size within an emulsion. Afterwards, emulsions are gelled by either: (a) heating/cooling, (b) cross-linking with a transglutaminase enzyme or (c) heating/cooling followed by addition of a transglutaminase enzyme. Control samples (no heating/cooling nor transglutaminase cross-linking treatments) were prepared for comparisons. Emulsions stabilized by heating/cooling treatment are prepared by placing emulsion in 90-100°C water bath for five minutes, then letting samples slowly cool to room temperature. Emulsions stabilized by transglutaminase cross-linking are prepared by adding transglutaminase to 2% wt/v and incubating at 37°C for 12-18hr. Emulsions stabilized by heating/cooling followed by addition of transglutaminase enzyme were prepared by first undergoing the heat/cool protocol, then adding the enzyme once the samples cooled to room temperature. All emulsifications are incubated for 8-12 hours at 37°C.

EXAMPLE 6: METHOD TO ANALYZE ADIPOSE TISSUE REPLICAS

After different gelling treatments, gelled emulsions are moved to room temperature for evaluation. The total volume of gelled emulsions, and volumes of phase separated water and/or oil volumes (if gelled emulsions are not in a single phase) are recorded. Firmness of the adipose tissue replica is evaluated by gentle poking of gelled emulsions. Cooking experiments are performed by transferring the mass to a heated surface and measuring the temperature of the liquid immediately after cooking.

EXAMPLE 7: ADIPOSE REPLICA OF BEEF FAT

An adipose tissue replica was made by gelling a solution of purified moong bean 8S protein emulsified with equal amounts of cocoa butter, coconut butter, olive oil and palm oil. Moong bean 8S protein was purified as described in Example 1, and had a concentration of 140 mg/ml in 20mM K-phosphate pH 7.4, 400mM NaCl. A fat mixture

was prepared by melting individual fats from solid to liquid state at 45°C for 30 min. Individual fats (cocoa butter, coconut butter, olive oil and palm oil) in liquid states were then mixed at a 1:1:1:1 (v/v) ratio. A protein-fat emulsion was formed by mixing a 70% v/v liquid fat mixture with 4.2% wt/v moong bean 8S protein, 0.4% wt/v soybean lecithin and emulsified by vortexing for 30 sec followed by sonication for 1 min. After homogenization, the fat-protein emulsion was in a single liquid phase as judged by visual observation.

One adipose tissue replica emulsion was stabilized by cross-linking with 0.2% wt/v transglutaminase enzyme at 37°C for 12hr. Another fat tissue replica was stabilized by gelling of proteins by heating to 100°C in a water bath followed by cooling to ambient room temperature. The resulting adipose tissue replicas were in a single phase. The adipose tissue replica matrix formed by the transglutaminase was a softer solid than the adipose tissue replica matrix formed by heat/cool induced gelling.

EXAMPLE 8: AN ADIPOSE REPLICA OF WAGYU BEEF FAT

An adipose tissue replica was made by gelling an emulsion of purified pea globulin proteins and equal amounts of cocoa butter, coconut butter, olive oil and palm oil. Pea globulin proteins were purified as described in Example 1 and had a concentration of 100 mg/ml, in 20mM K-phosphate pH 8, 400mM NaCl. A fat mixture was prepared by melting individual fats from solid to a liquid state at 45°C for 30 min. Individual fats (cocoa butter, mango butter, olive oil) in liquid state where then mixed at 2:1:1 (olive oil: cocoa butter: mango butter) v/v ratio. A protein-fat emulsion was formed by mixing a liquid fat mixture with a 5% wt/v solution of pea globulins in a 1:1 ration, and emulsifying using a hand-held homogenizer at the maximum setting for 30sec. After homogenization, the fat-protein emulsion was in a single liquid phase as judged by visual observation. Emulsion was stabilized by cross-linking with 0.2% wt/v transglutaminase enzyme at 37°C for 12hr. The resulting adipose tissue replica was in a single phase, was a soft solid and was salty in flavor.

EXAMPLE 9: ADIPOSE TISSUE REPLICA WITH FATTY ACID DISTRIBUTION OF BEEF:

An adipose tissue replica was made by gelling an emulsion of purified pea globulin proteins and equal amounts of cocoa butter, mango butter, olive oil and rice bran oil. Pea globulin proteins were purified as described in Example 1, and had a concentration of 100mg/ml, in 20mM K-phosphate pH 8, 400mM NaCl. A fat mixture was prepared by melting individual fats from solid to liquid state at 45°C for 30min. Individual fats (cocoa butter, mango butter, olive oil and rice bran oil) in a liquid state where then mixed at 1:1:1:1 v/v ratio. A protein-fat emulsion was formed by mixing 50% v/v liquid fat mixture with 5% wt/v Pea globulins, and emulsifying using a hand-held homogenizer at the maximum setting for 30sec. After homogenization, the fat-protein emulsion was in a single liquid phase as judged by visual observation. The emulsion was stabilized by cross-linking with 0.2% wt/v transglutaminase enzyme at 37°C for 12hr. The resulting adipose tissue replica was in a single phase, was a soft solid and was salty in flavor.

EXAMPLE 10: ADIPOSE TISSUE REPLICA WHERE FIRMNESS OF FAT TISSUE AT REFRIGERATION AND AMBIENT TEMPERATURES IS CONTROLLED BY MELTING TEMPERATURE OF FAT IN ADIPOSE TISSUE REPLICA.

A adipose tissue replica made as a stable emulsion of RuBisCo with sunflower oil is softer than a adipose tissue replica made as a stable emulsion of RuBisCo and cocoa butter. Adipose tissue replicas were formed with 0.18%, 1.6%, and 2.4% wt/v Rubisco with 70%, 80%, and 90% v/v sunflower or cocoa butters. Each adipose tissue replica that contained cocoa butter was firmer than the corresponding replicas that were formed with sunflower oil. Adipose tissue replicas comprising 0.18%, 1.6%, and 2.4% wt/v Rubisco with 70%, 80%, and 90% v/v cocoa butter were solid at room temperature but melted at close to mouth temperature. In the adipose tissue replicas formed with varying concentrations of Rubisco (0.18, 1.6 1.9 % wt/v) and 70-80% v/v sunflower oil, the replicas were firmer as the amount of protein in the adipose tissue replica matrix

increased. Adipose tissue replicas with 0.18% wt/v Rubisco were very soft; adipose tissue replicas with 1.6% wt/v Rubisco were soft; and adipose tissue replicas with 1.9% wt/v RuBisco were of medium firmness.

Adipose tissue replicas made as a stable emulsion of Moong Bean 8S protein with sunflower oil were softer than adipose tissue replicas made as a stable emulsion of Moong Bean 8S protein and cocoa butter. Adipose tissue replicas were formed with 2%, 1%, and 0.5% wt/v Moong Bean 8S protein with 70%, 80%, and 90% v/v sunflower or cocoa butters. Each adipose tissue replica that contained cocoa butter was firmer than the corresponding replica that was formed with sunflower oil.

Adipose tissue replicas made as stable emulsions of Moong Bean 8S protein with canola oil were softer than the corresponding adipose tissue replicas made as stable emulsions of Moong Bean 8S protein with an equal mixture of coconut, cocoa, olive, and palm oils. Adipose tissue replicas were formed with 1.4% wt/v Moong Bean 8S protein with 50%, 70%, and 90% v/v sunflower or mixture of oils. Each adipose tissue replica that contained a mixture of oils was firmer than the corresponding replica that was formed with sunflower oil. A adipose tissue replica comprising1.4% wt/v Moong Bean 8S protein with 50%, 70%, and 90% v/v of an equal mixture of coconut, cocoa, olive, and palm oil was solid at room temperature but melted at close to mouth temperature.

Adipose tissue replica made as a stable emulsion of soy proteins with sunflower oil was softer than the adipose tissue replica made as stable emulsion of soy proteins and cocoa butter. Adipose tissue replicas were formed with 0.6%, 1.6%, and 2.6% wt/v Soy with 50%, 70%, 80%, and 90% v/v sunflower or mixture of oils. Each adipose tissue replica that contained mixture of oils was firmer than the corresponding replica that was formed with sunflower oil. Adipose tissue replicas comprising 0.6%, 1.6%, and 2.6% wt/v soy proteins with 50%, 70%, 80%, and 90% v/v cocoa butter were solid at room temperature but melted at close to mouth temperature.

EXAMPLE 11: ADIPOSE TISSUE REPLICA COOKING: STRUCTURE OF FAT TISSUE MATRIX CONTROLS MELTING POINT DURING COOKING.

An adipose tissue comprising a stabilized protein-oil emulsion constructed as described

in Example 5 and Example 6 above, formed with 2% w/v Rubisco and 50%, 70%, and 90% v/v cocoa butter, melted at a higher temperature when formed upon heat/cool denaturation and at a lower temperature than when formed by cross-linking with a transglutaminase.

EXAMPLE 12: COOKING ADIPOSE TISSUE: ARRANGEMENT AND STRUCTURAL ORGANIZATION OF PROTEINS AND FAT WITHIN A FAT TISSUE MATRIX CONTROLS AMOUNT OF FAT RELEASED AND FAT RETAINED BY THE ADIPOSE TISSUE REPLICA DURING COOKING.

During cooking of a adipose tissue replica matrix comprising a protein-oil emulsion formed with 2% w/v Rubisco and 50%, 70%, or 90% v/v cocoa butter, more adipose tissue replica mass was retained after cooking when the adipose tissue replica was formed upon heat/cool denaturation than when formed by cross-linking with a transglutaminase. Released mass was liquid and appeared oily.

During cooking of adipose tissue replica matrix comprising a protein-oil emulsion formed with 2.6 and 0.6 % w/v Soybean protein and 50%, 70%, or 90% v/v cocoa butter, more adipose tissue replica mass was retained when formed upon heat/cool denaturation than when formed by cross-linking with transglutaminase. Released mass was liquid and appeared oily.

EXAMPLE 13: COOKING AN ADIPOSE TISSUE REPLICA: CONCENTRATION OF PARTICULAR PROTEINS WITHIN FAT TISSUE MATRIX CONTROLS MASS OF ADIPOSE TISSUE REPLICA THAT REMAINS AFTER COOKING

A series of adipose tissue replicas constructed from 1.4% wt/v moong bean 8S protein with 90% v/v canola oil and 0.45% wt/v soybean lecithin, were homogenized and increasing concentration of sunflower oleosins were added at varying concentrations to the emulsion. The concentration of oleosin varied from 1:10 to $1:10^6$ oleosin to triglyceride molar ratio. An increase in mass retention after cooking was observed when the ratio of oleosins to oil in adipose tissue replica was greater.

A series of adipose tissue replicas formed with varying concentrations of Rubisco with 70% v/v sunflower oil, retained more mass upon cooking as the concentration of Rubisco increased. Adipose tissue replicas containing RuBisCo at 0% wt/v completely melted, while 1.9% wt/v Rubisco retained 10% mass, and adipose tissue replica containing 2.4% wt/v Rubisco retained 20% mass upon cooking

EXAMPLE 14: CONNECTIVE TISSUE ANALOG

Connective tissue fiber replicas were manufactured by electrospinning a solution of moong bean globulin (22.5 mg/ml) containing 400 mM sodium chloride, 6.75% w/v of poly(vinyl alcohol) and trace amounts of sodium azide (0.007% w/v). The resulting solution was pumped at 3 µl/min using a syringe pump, from a 5 ml syringe through a Teflon tube and a blunted 21gauge needle. The needle was connected to a positive terminal of a Spellman CZE 30 kV high voltage supply set at 17kV and fixed 12 cm from a an aluminum drum (ca. 12 cm long, 5 cm in diameter) that was wrapped in aluminum foil. The drum was attached to a spindle that is rotated by an IKA RW20 motor at about 220 rpm. The spindle was connected to a ground terminal of the high voltage supply. The protein/polymer fibers that accumulated on the foil were scraped off and used as the connective tissue replicas.

EXAMPLE 15: EXTENDING THE LIFETIME OF REDUCED (HEME-FE2+) LEGHEMOGLOBIN

Equine myoglobin was purchased from Sigma. Myoglobin was resuspended at 10 mg/ml in 20 mM potassium phosphate, pH 8.0, 100 mM NaCl. SDS-PAGE analysis suggested that protein purity was ~ 90%.

Soy leghemoglobin was purified from *Glycine max* root nodules via ammonium sulfate precipitation (60%/90% fractionation) as detailed in Example 1. Resuspended 90% ammonium sulfate leghemoglobin was further purified by anion-exchange chromatography (HiTrap Q FF 5mL FPLC column) in 20 mM potassium phosphate, pH 8.0, 100 mM NaCl. The leghemoglobin eluted in flow through fractions. SDS-PAGE analysis suggested that protein purity was ~ 70%. Leghemoglobin was buffer exchanged

into 20 mM potassium phosphate, pH 7.4, 100 mM NaCl and concentrated to 10 mg/ml on 3.5kDa membrane concentrators.

Carbon monoxide treatment: Myoglobin at 10 mg/ml in 20 mM potassium phosphate, pH 8.0, 100 mM NaCl and leghemoglobin at 10 mg/ml in 20 mM potassium phosphate, pH 7.4, 100 mM NaCl were first degassed under vacuum for 1 hour at 4°C then perfused with carbon monoxide gas for 2 minutes. Globins were then reduced from heme-Fe^{3+} to heme-Fe^{2+} state by adding 10 mM sodium dithionite, 0.1mM sodium hydroxide for 2 minutes. Sodium dithionite and sodium hydroxide were removed from the protein solution by using size-exclusion chromatography (PD-10 desalting column) in 20 mM potassium phosphate, pH 8.0, 100 mM NaCl and 20 mM potassium phosphate, pH 7.4, 100 mM NaCl respectively. Globins fractions were collected as a peak red colored fractions as evaluated by visual estimation. UV-VIS spectra confirmed the presence of heme-Fe^{2+} state for both proteins. After desalting, the solution was again perfused with gas for another 2 minutes. The color of the solutions was evaluated by taking UV-Vis spectra (250nm- 700nm) every 20 minutes using the nanodrop spectrophotometer. Control samples were not treated with carbon monoxide.

Sodium nitrite treatment: Myoglobin at 10 mg/ml in 20 mM potassium phosphate, pH 8.0, 100 mM NaCl and leghemoglobin at 10 mg/ml in 20 mM potassium phosphate, pH 7.4, 100mM NaCl were reduced from heme-Fe^{3+} to heme –Fe^{2+} by adding 10 mM sodium dithionite, 0.1mM sodium hydroxide for 2 minutes. Sodium dithionite and sodium hydroxide were removed from the protein solution by using size-exclusion chromatography (PD-10 desalting column) in 20 mM potassium phosphate, pH 8.0, 100 mM NaCl and 20 mM potassium phosphate, pH 7.4, 100 mM NaCl, respectively. Globins fractions were collected as peak red colored fractions as evaluated by visual estimation. UV-VIS spectra confirmed the presence of heme-Fe^{2+} state for both proteins. Sodium nitrite was then added to a final concentration of 1 mM from 100 mM nitrite in phosphate buffer pH 7.4. The lifetime of heme-Fe^{2+} state was followed by recording UV-VIS spectra (250-700nm) using spectrophotometer as a function of time. Control samples were not treated with sodium nitrite.

Data analysis of heme-Fe^{2+} life-times for myoglobin and leghemoglobin samples treated with carbon monoxide and sodium nitrite was performed in Microsoft Excel by plotting the amplitude of the absorbance peak at the 540nm wavelength. The "baseline" of the 540 nm absorbance was determined by the state of the UV-vis spectrum of the globin solutions prior to the addition of any additives, dithionite reduction, or desalting. The built-in curve fit function was used to produce an exponential line-of-best-fit, the exponent of which directly relates to the half life of the peak amplitude.

The life-time of the heme-Fe^{2+} state and accompanying red color of myoglobin and leghemoglobin solutions in the absence of carbon monoxide and sodium nitrite were ~ 6hr and ~ 4hr respectively. Addition of sodium nitrite extended the life-time of the heme-Fe^{2+} state and accompanying red color to more than seven days. Addition of carbon monoxide extended the life-time of heme-$Fe2^{+}$ state and accompanying red color to more than two weeks.

EXAMPLE 16: PREPARATION OF MEAT REPLICAS IN WHICH THE PARTICLE SIZE OF INDIVIDUAL TISSUE REPLICA UNITS IS VARIED TO CONTROL AROMA GENERATION DURING COOKING.

Muscle tissue replica and adipose tissue replica were prepared separately and then combined into a meat tissue replica such that the size of individual tissue replica units was varied to control aroma generation during cooking. Individual fat, muscle and connective tissue replicas were constructed in the following manner.

A muscle tissue replica was prepared as in Example 2. The muscle tissue replica formed an opaque gel of reddish-brown color, with small amounts (< 1 ml) of inclusions of dark red, venous blood colored liquid. A connective tissue replica was prepared as in Example 14. An adipose tissue replica was prepared as in Example 7.

Meat replicas with a lean-to-fat ratio 85/15 were prepared by combining individual muscle, connective and adipose tissues such that particle size of individual tissues replicas varied. (a) 2.1 g of muscle replica with 0.9 g of chunks of fat replica 5-10 mm in size ("coarse mix"); (b) 2.1 g of muscle replica with 0.9 g of fat replica chopped to 2-3 mm in size ("fine mix"); and (c) 2.1 g of muscle replica with 0.9 g of fat replica

thoroughly blended to <1 mm in size ("blend"). "Muscle only" control sample contained 3 g of muscle replica alone. "Fat only" control sample contained 3 g of fat replica alone as 5-10 mm sized particle. Meat, muscle and fat tissue samples were cooked in sealed glass vials at 150°C for 10 min. Aroma profiles of the samples were analyzed by a panel of testers, and by GC-MS.

Sensory olfactory analysis of meat replica samples performed by a panel of testers, suggested that the size of individual tissue units and the extent of their mixing within meat tissue replicas correlated with generation of different aromas. Muscle tissue replica cooked by itself generated aromas associated with store-bought gravy, faint citrus and star anise. Adipose tissue replica cooked by itself generated aromas associated with musty, rancid and sweet aromas. Cooked meat tissue replica (coarse particle size) generated aromas of store-bought gravy, sweet, slightly musty and star-anise. Cooked meat tissue replica (fine particle size) generated aromas associated with soy sauce, musty, slightly rancid and beef bouillon. Cooked meat tissue replica (very fine particle size) generated aromas associated with sweet, musty and soy-sauce. All samples with the exception of adipose tissue replica generated aromas associated with burnt meat smell, however to varying intensities.

Analysis of GCMS data indicated that the size of individual tissue units and the extent of their mixing within meat tissue replicas had profound effects on the generation of aromatic compounds upon cooking. In particular, multiple aromatic compounds associated with fruity/grean bean/metallic (2-pentyl-furan); nutty/green (4-methylthiazole); peanut butter/musty (pyrazine, ethyl); raw potato/roasted/earthy (Pyrazine, 2,3-dimethyl); vinegary (acetic acid); spicy/caramel/almond (5-methyl-2-furancarboxaldehyde); creamy (butyrolactone); sweet (2,5-dimethyl-3-(3-methyl butyl) pyrazine); fruity/stale beer (2-cyclopentene-1-one, 2-hydroxy-3-methyl); musty/nutty/coumarin/licorice/walnut/bread (3-acetyl-1H-pyrolline); coconut/woody/sweet (pantolactone); penetrating (1-H-pyrrole-2-2carboxaldehyde, 1-methyl); minty (caprolactam); toasty caramel (4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl) aromas appeared only in mixed meat replicas, but not in individual tissue replicas. Some other aromatic compounds, for example associated with gasoline-

like (nonane, 2,6-dimethyl), petroleum-like (3-hexene, 3-methyl); sour/putrid/fish-like (pyridine); bland/woody/yogurt (acetoin); fatty/honey/citrus (octanal); pungent/sweet/caramelic (2-propanone, 1-hydroxy) and nutty/burnt green (ethenyl pyrazine) aromas appeared only in individual tissue replicas, but did not accumulate in mixed meat replicas. Furthermore, the levels to which all of the above compounds accumulated during cooking depended on the sizes of tissue units and how they are mixed (coarse particle size, fine particle size, or very fine particle size (blended)).

Similar to meat tissue replica, it was found that structural organization and particle size of beef tissues modify response of beef tissues to cooking. For example, the flavor of meat is modified by the size of the particles. Beef samples were prepared as following: samples of beef muscle and beef fat were cut separately with a knife and: (a) "ground", where knife-cut tissue cubes were passed through standard meat grinder. 80/20 (wt/wt) lean/fat ground beef sample was prepared by mixing muscle and fat tissue cubes at appropriate ratio prior to grinding. This sample preparation is referred to as a "fine size particle mix". (b) Ground tissue particle size was further reduced by freezing ground tissue in liquid nitrogen and crushing it using mortar and pestle to a very fine powder (particle size <1mm). This sample preparation is referred to as a "very fine size particle mix". All samples were cooked in sealed glass vials at 150 °C for 10 min. Aroma profiles of the samples were analyzed by a panel of testers, and by GC-MS, as described in Example 1. "Muscle only" control sample contained 3 g of muscle tissue alone. "Fat only" control sample contained 3 g of fat tissue alone. Ground beef sample contained 3 g of a 80/20 (wt/wt) muscle/fat mixture.

Sensory olfactory analysis of beef samples performed by a panel of testers, suggested that the size of individual tissue units and the extent of their mixing within the samples correlated with generation of different aromas. Beef muscle cooked by itself generated typical aromas associated with cooked ground beef. Fat tissue replica cooked by itself generated slightly sweet aromas, and aromas associated with burnt mushrooms. Cooked ground beef with "fine size particle mix" generated typical aromas associated with cooked ground beef, with presence of slightly sweet aromas characteristic of cooked fat. Cooked ground beef with "very fine size particle mix" generated aromas associated

with cooked ground beef, but no slightly sweet aroma characteristic of cooked fat was detected.

Analysis of GCMS data indicated that the particle size of the individual tissue units has effect on generation of aromatic compounds upon cooking. In particular generation and/or amount of multiple aromatic compounds by individual tissue samples or ground beef sample varied in correlation with particle size of the tissue. Some of the aromatic compounds that differed between fine and very fine particle size of muscle tissue: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, 3-Acetyl-1H-pyrolline, 1-(6-methyl-2-pyrazinyl)-1-ethanone, 2,5-dimethyl-3-(3-methyl butyl) pyrazine, 2-furancarboxyaldehyde, 5-methyl, Acetic acid, Ethenyl pyrazine, Pyrazine, 2,3-dimethyl , 2-Propanone, 1-hydroxy, Octanal, Acetoin, 4-Methylthiazole, Pseudo-2-pentyl-furan, 2-pentyl-furan. Some of the aromatic compounds that differed between fine and very fine particle size of fat tissue: triethylene glycol: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, Caprolactam, 1-(6-methyl-2-pyrazinyl)-1-ethanone, 2-Cyclopentene-1-one, 2-hydroxy-3-methyl, Butyrolactone, 2-furancarboxyaldehyde, 5-methyl, Ethanone, 1(2 furanyl), Acetic acid, 2-ethyl-5-methyl pyrazine, Pyrazine, 2,3-dimethyl, Pyrazine, ethyl, Octanal, Acetoin, 4-Methylthiazole, Pseudo-2-pentyl-furan, Pyridine, Nonane, 2,6-dimethyl. Some of the aromatic compounds that differed between fine and very fine particle size of 80/20 muscle/fat sample: 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl, Caprolactam, 1H-1Pyridine, 3-carbonitrile, 4-ethyl-2-oxo-2,5, 1-H-Pyrrole-2-2carboxaldehyde, 1-methyl, 2-Cyclopentene-1-one, 2-hydroxy-3-methyl , 2,5-dimethyl-3-(3-methyl butyl) pyrazine, Butyrolactone, 2-furancarboxyaldehyde, 5-methyl, Ethanone, 1(2 furanyl), Acetic acid, Ethenyl pyrazine, 2-ethyl-5-methyl pyrazine, Pyrazine, 2,3-dimethyl; 2-Propanone, 1-hydroxy, Octanal, Acetoin, 2-pentyl-furan.

EXAMPLE 17: LEGHEMOGLOBIN CONTRIBUTION TO FLAVOR

Beef flavors and aromas can be created in non beef consumables by addition of heme proteins. Ground chicken (90% lean, 10% fat) was strained with cheesecloth and mixed with recombinant soy leghemoglobin or recombinant bovine myoglobin to a final

concentration of 0.5-1.0% wt/wt. The recombinant heme proteins were expressed in *E. coli* and purified by nickel affinity purification as described in Example 1. Prior to being mixed with chicken, the heme proteins were reduced with 20 mM Na Dithionite. Na dithionite was removed from the sample with a Zeba desalting column (Thermo Scientific). Leghemoglobin was desalted into 20 mM potassium phosphate pH 7.4, 100 mM NaCl. Myoglobin was desalted into either 20 mM potassium phosphate pH 7.4, 100 mM NaCl or 20 mM Na citrate pH 6.0, 100 mM NaCl. The reduced heme protein samples were divided in two, and half the sample was bubbled with carbon monoxide (CO) for 2 minutes. After mixing the heme protein samples with ground chicken, the mixture was poured into nugget-shaped molds and incubated overnight at 4°C. The nuggets were oven baked or pan fried at 165°C until each nugget reached an internal temperature of 165°C. A panel of judges tasted nuggets containing chicken alone, chicken mixed with buffer, chicken mixed with either leghemoglobin or myoglobin +/- CO, or beef (90% lean, 10% fat). Judges filled out a survey to evaluate the aroma and flavor of each nugget. Judges rated the aroma and flavor of each nugget as follows: 1 = chicken, 2 = chicken + faint beef, 3 = 50/50 chicken + beef, 4 = beef + faint chicken, 5 = beef. Shown in Table 2 are the average scores received for each nugget. Percentages indicate the final concentration of heme protein wt/wt (abbreviations: KP = 20 mM potassium phosphate pH 7.4, 100 mM NaCl buffer. NC = 20 mM Na citrate pH 6.0, 100 mM NaCl buffer. n/d = not determined). Adding recombinant leghemoglobin or myoglobin to chicken resulted in an increased beef aroma and flavor. The perceived levels of beef flavor and aroma increased with the myoglobin and leghemoglobin content. Leghemoglobin and myoglobin provide the same benefit to the flavor and aroma.

TABLE 2

	Oven Baked		Pan Fried	
	Aroma	Flavor	Aroma	Flavor
Chicken	1	1	1	1
Chicken KP	1	1	2.5	1.2
Chicken NC	1.5	1.5	1.5	1
Chicken 0.5% legH KP	1.5	2.5	3.67	3.2
Chicken 0.5% legH+CO KP	2.5	2.5	2.67	2.2

Chicken 0.5% Myo NC	2	2	1.5	2.4
Chicken 0.5% Myo+CO NC	2	2	2.5	3
Chicken 0.5% Myo+CO KP	2.5	2.5	2.33	2
Chicken 0.8% Myo+CO NC	2	3	4	2.6
Chicken 1% Myo NC	4.5	4	n/d	n/d
Chicken 1% legH NC	4	4	n/d	n/d
Beef	5	5	5	5

EXAMPLE 18: PREPARATION OF A NON-DAIRY CREAM LIQUOR

A cream liquor was made from a sunflower cream fraction, RuBsiCo and whiskey (Jameson). A sunflower cream fraction was made by blending sunflower seeds in 40 mM sodium phosphate pH 8.0, 400 mM sodium chloride buffer. Seed debris was pelleted by centrifugation at 5000g for 20 min, and the cream fraction collected. The cream fraction was resuspended in 10 mM potassium phosphate pH 7.4 buffer and collected by centrifugation at 5000g, 20 min. Rubsico was purified as described in Example 1 and used as 25mg/ml stock solution in 20mM K-phosphate pH 7.0, 150mM NaCl.

In one example, cream liquor was made as follows: 11.4% wt/v of sunflower cream fraction, 40% v/v Jameson whiskey, 0.4-1.6% wt/v Rubsico, 0.5% wt/v vanilla extract, 0.5% v/v espresso coffee, and 1.5% wt/v chocolate powder. The resulting mixture was homogenized at 5000 psi.

In another example, cream liquor was made from sunflower cream fraction and whiskey (Jameson) and sugar: 11.4% wt/v of sunflower cream fraction, 40% v/v Jameson whiskey, 0.5% v/v vanilla extract, 0.5% v/v espresso coffee, 1.5% wt/v chocolate powder and 8% wt/v sugar.

Beverages were served either at ambient room temperature or chilled. Resulting beverages were beige to light chocolate in color. The tasting results suggested that the beverage had a very creamy alcoholic flavor similar to dairy cream liquor. Chilled product was preferred. The emulsion was stable at room temperature for at least 1 week (maximum time tested).

EXAMPLE 19 –CHOCOLATE SPREAD

A chocolate spread was made from 34% (wt/wt) cane sugar, 22 % (wt/wt) cocoa powder, 19 % (wt/v) pistachio cream fraction, and 12 % (v/v) almond milk. Cane sugar and cocoa powder (Ghirardelli) were commercially purchased. Almond skim milk was made in a following manner: Almonds were blanched by immersion into 100°C water for 30 sec. Blanched nuts were recovered and cooled by immersion into ice-cold water. Almonds were air dried. Almonds were then rehydrated by immersion in 2°C water for 16hr. Rehydrated almonds were drained, mixed with water at 1:2 wt/v ratio and blended in a Vitamix blender for 5 min. The blended slurry was collected in chilled container and stirred with a frozen cooling stick to cool. Once the slurry cooled to 10°C, the slurry was placed at 2°C for up to 12 hours. Almond skim and cream were separated by centrifugation at 7480g for 30min at 4°C. Almond milk separated into 3 layers, a dense pellet of insoluble solids, a clear to translucent aqueous layer (which is referred to as the "almond skim milk"), and a lighter, creamy, opaque layer (which is referred to as the "almond cream"). Almond milk was then was pasteurized at 75°C for 16 seconds, chilled and stored at 2°C.

A pistachio cream fraction was prepared by blending pistachios in 100 mM sodium carbonate pH 9.5 buffer with 400 mM sodium chloride and 1 mM EDTA, then centrifuging 5000xg for 20 min. The cream fraction was collected and washed into the same buffer once more. After centrifugation at 5000g for 20 min, the cream fraction was collected and washed into 20 mM sodium phosphate pH 7.4 buffer with 50 mM sodium chloride and 1 mM EDTA. After centrifugation at 5000g for 20 min, the cream fraction was collected an washed once more in the neutral (pH 7.4) buffer, centrifuged at 5000g for 20 min. Pistachio cream fraction was collected and stored at 4°C.

Chocolate spread was made in the following way. Cane sugar was melted in almond milk, cocoa powder was added to sugar-milk mixture with stirring and melted. Sugar, milk and cocoa were then added to the pistachio cream fraction and whisked together. Resulting mixture was then poured into molds and let sit for 24 hours at refrigeration and freezer temperatures.

In another example, chocolate spread was made from 42% (wt/wt) cane sugar, 27 % (wt/wt) cocoa powder, 31 % (wt/v) sunflower cream fraction, and 23% (v/v) almond skim milk. All ingredients and procedures apart from sunflower cream fraction were as described above.

The sunflower cream fraction was created from blending sunflower seeds in 5 times the weight to volume of a solution of 40 mM potassium phosphate pH 8, with 400 mM NaCl, 1 mM EDTA, then cooled to 20°C, and the slurry was then centrifuged. The top cream layer was removed and is blended in the same buffer, followed by heating for 1 hour at 40°C. The slurry is cooled down to 20°C then centrifuged; the cream layer is removed and mixed with 5 times the weight to volume of 100 mM sodium carbonate pH 10 with 400 mM NaCl, then centrifuge. The top layer is then mixed with 5 times the weight to volume of water and centrifuged again, the resulting cream fraction is very creamy, white, and neutral tasting.

In another example, a chocolate spread was made from 37% (wt/wt) cane sugar, 23 % (wt/wt) cocoa powder, 13 % (wt/v) sunflower cream fraction, and 7 % (wt/wt) cocoa butter, and 20 % v/v almond skim milk.

In another example, a chocolate spread was made from 37% (wt/wt) cane sugar, 23 % (wt/wt) cocoa powder, 13 % (wt/v) sunflower cream fraction, and 7 % (wt/wt) coconut oil 20 % v/v almond skim milk.

In another example a chocolate spread was made from 37% (wt/wt) cane sugar, 23 % (wt/wt) cocoa powder, 13 % (wt/v) sunflower cream fraction, and 7 % (wt/wt) palm oil, and 20 % v/v almond skim milk.

In another example a chocolate spread was made from 1.8% (wt/wt) cane sugar, 1.13% (wt/wt) cocoa powder, 88 % (wt/v) pistachio cream fraction, and 9 % almond skim milk, by whisking equal amounts of spread described above and pistachio oil bodies.

In another example, a chocolate spread was made from 8.5 % (wt/wt) cane sugar, 5.4 % (wt/wt) cocoa powder, 81 % (wt/v) sunflower cream fraction, and 4.6 % (v/v) almond skim milk, by mixing chocolate spread described above with sunflower cream at ratio 2:1.

Visual and textural inspection of all products suggested that they formed stable, solid, creamy spreads at room temperatures. All products were firm solids at refrigeration and freezer temperatures. Tasting results of all products suggested very pleasant, rich creamy texture with product melting in the mouth reviewed positively by tasters. Individual tasters preferences varied with respect to like or dislike of pistachio flavor, coconut flavor, preference for more or less sweet product and more or less cocoa flavor. One particular sample was described as being similar to a milk chocolate spread, as the sunflower cream fraction contributed a neutral flavor.

EXAMPLE 20 - Generation of Adipose Tissue Replica

Adipose tissue replicas were generated using the ingredients recited in Table 3.

TABLE 3

Adipose Tissue Replica	
Ingredient	**%**
Coconut oil	65
Pea vicillin protein in buffer	21.3
Cocoa butter	10
Buffer	2.7
Lecithin slurry, 50 mg/ml	1
Total	100

Lecithin (SOLEC^{TM} F Deoiled Soy Lecithin, The Solae Company, St. Louis, MO) was prepared at a concentration of 50 mg/ml in 20 mM potassium phosphate, 100 mM NaCl, pH 8.0 buffer and sonicated (Sonifier Analog Cell Disruptor model 102C, BRANSON Ultrasonics Corporation, Danbury, Connecticut) for 30 seconds.

The pea vicilin protein was supplied as a liquid containing approximately 140 mg/g pea vicilin in 20 mM potassium phosphate, 100 mM NaCl, pH 8.0 buffer.

Coconut oil (Shay and Company, Milwaukie, OR) and cocoa butter (Cocoa Family, Duarte, CA) were melted by heating to 50 - 70°C and then combined and held warm until needed.

The buffered protein solution, additional buffer, and lecithin slurry were mixed in a 32 ounce sized metal beaker and equilibrated to room temperature. An emulsion was formed using a hand held homogenizer (OMNI model GLH fitted with G20-195ST

20mm generator probe, OMNI International, Kennesaw, GA). The homogenizer probe was put into the protein lecithin mixture and turned on to speed 4. The warmed oil was then added slowly over the course of about 2 minutes while continuously moving the probe around in the mixture.

The emulsion was then heat set by placing the metal beaker into a 95°C waterbath. Using a clean spatula, the emulsion was stirred every 20 seconds for 3 minutes total. The beaker was then removed from the waterbath and stored at 4°C for several hours until thoroughly cooled.

EXAMPLE 21 - Generation of Raw Tissue Replica

A raw tissue replicawas generated using the ingredients recited in Table 4.

TABLE 4:

Raw Tissue Replica	
Ingredient	**%**
Buffer	41.6
Heme protein in buffer	26.7
Pea legumin in buffer, dried	12.1
Pea vicillin in buffer, dried	9.4
Flavor precursor mix, 17x	6.2
Transglutaminase preparation	4
Total	100

The buffer was 20 mM potassium phosphate, 100 mM NaCl, pH 7.4. The heme protein was prepared at a concentration of 55 mg/g in 20 mM potassium phosphate, 100 mM NaCl, pH 7.4 buffer. The 17x flavor precursor mix precursor is described in Example 27. The pea legumin was prepared in 20 mM potassium phosphate, 500 mM NaCl, pH 8 buffer and then freeze dried prior to use. The final protein concentration of the dried material was 746 mg/g. The pea vicilin was prepared in 20 mM potassium phosphate, 200mM NaCl, pH 8 buffer and then freeze dried prior to use. The final protein concentration of the dried material was 497 mg/g.

The liquid ingredients (buffer, heme, and flavor precursor mix) were mixed in a plastic beaker. The dry pea legumin and pea vicilin were then added and allowed to fully rehydrate while gently stirring for 1 hour at room temperature. The dry transglutaminase preparation (ACTIVA® TI, Ajinomoto, Fort Lee, NJ) was then added and stirred for about 5 minutes until dissolved. The stirring was then turned off and the mixture was allowed to gel at room temperature until firm. After the gel had formed the raw tissue replicawas stored refrigerated until used.

EXAMPLE 22 - Hard Connective tissue replica

A hard connective tissue replica was made as following using soy protein isolate (Supro Ex38, Solae), wheat gluten (Cargill), and bamboo fiber (Alpha-Fiber B-200, The Ingredient House). The purified proteins were freeze-dried and milled using a standard coffee grinder. Commercially available powders of soy protein isolate and wheat gluten were used as received.

The connective tissue replica contained 49% soy protein isolate, 49% wheat gluten and 2% bamboo fiber. The ingredients were thoroughly mixed and loaded into the loading tube of the extruder's batch feeder. A twin-screw extruder (Nano 16, Leistritz Extrusion Corp.), with a high-pressure water injection pump (Eldex) and custom-made die nozzles (stainless steel tube, 3 mm ID, 15 cm length, pressure rating 3000+ PSI) attached using a Hy-Lok two-ferule tube fitting and a custom-made die with a threaded nozzle and 10 mm ID, 20 mm long flow channel was used.

The dry mixture was fed into the extruder at the rate 1 g/min. Water was fed by the pump into the second zone of the extruder's barrel. The rate of water feeding is adjusted to the rate of dry mixture feeding such as to provide 55% moisture level in the final extrudiate. A temperature gradient was maintained along the extruder barrel as follows: feed zone - 25°C, zone 1 – 30°C, zone 2 – 60°C, zone 3 – 130°C, zone 4 – 130°C. The die plate was neither actively heated, nor cooled. The die nozzle was actively cooled (by applying moist tissue replica) to maintain extrudate temperature below 100°C.

Hard connective tissue replica obtained by this process was dark off-white ("cappuccino") colored material shaped into 3 mm thick filaments that had tensile strength similar to animal connective tissue (3 MPa).

Example 23. Soft Connective tissue replica

A soft connective tissue replica was made as in Example 22, except that the rate of water feeding was adjusted to the rate of dry mixture feeding to provide 60% moisture level in the final extrudate. A temperature gradient was maintained along the extruder barrel as follows: feed zone - 25°C, zone 1 – 30°C, zone 2 – 60°C, zone 3 – 115°C, zone 4 – 115°C. The die plate was neither actively heated, nor cooled. The die nozzle was actively cooled (by applying moist tissue) to maintain extrudate temperature below 100°C.

Soft connective tissue replica obtained by this process was light off-white colored material shaped into 3 mm thick filaments that had low tensile strength (< 0.1 MPa) and had a significant propensity to split longitudinally into bands and thin fibers

EXAMPLE 24. Thin connective tissue replica (zein fibers) process:

A thin connective tissue replica was prepared using zein protein powder, glycerol (FCC grade), polyethylene glycol (PEG 400 or PEG3350), ethanol, sodium hydroxide (FCC grade), and water. The zein powder and PEG3350 at a 35% w/w ratio to zein were dissolved in 85% aqueous ethanol to reach a final zein concentration of 57% w/w. The pH of the solution was adjusted to 7.0 with 1M solution of sodium hydroxide in ethanol. A syringe pump with A 1-12 ml syringe, spinning nozzle (hypodermic needle, 18-27 gauge, or a plastic nozzle, 18-24 gauge), heating silicon tape, and a heating fan was used, with a collector assembled using a computer-controlled motor rotating a Delrin rod, which serves as a collector.

This solution was loaded into the syringe, which was mounted onto the syringe pump, with an 18 gauge plastic tip attached. A silicon heating tape was looped around the tip to maintain it at elevated temperature. After solution was extruded out of the tip and forms a drop, it was picked up with a spatula and carefully transferred towards the

collector rod to form a filament between the tip and the collector. The extrusion rate was optimized to produce even flow of material from the tip (5 ml/h for a 12 ml syringe and 18 gauge tip). The collector rotation speed was 3 RPM. The heating fan was positioned to blow hot air onto the spooling fiber. After spooling, the fibers were cured in a 120°C oven for 24 hours.

Thin connective tissue replica obtained by this process was semi-clear yellow colored material shaped into 300-micrometer thick fibers that were semi-flexible in air, and became very flexible and elastic in presence of water, maintaining high tensile strength similar to animal connective tissue (6 MPa)

EXAMPLE 25. Noodles

Noodles were prepared using purified pea vicilin (freeze-dried), and soy protein isolate (Supro EX38 by Solae, Solbar Q842 (CHS)) or soy protein concentrate (Hisolate, Harvest Innovations). To prepare the noodles, 67% soy protein concentrate or isolate, and 33% ground pea vicilin powder were thoroughly mixed and loaded into the loading tube of the extruder's batch feeder. The dry mixture was fed into the extruder at the rate in the range of 1-2 g/min. Water was fed by the pump into the second zone of the extruder's barrel at the rate 3.6-5.3 ml/min such as to maintain the final moisture content in the extrudate at 72.5%. A temperature gradient was maintained along the extruder barrel as follows: feed zone - 25 °C, zone 1 – 30 °C, zone 2 – 60 °C, zone 3 – 100 °C, zone 4 – 100 °C. Zone 1 temperature can be varied in the range of 25 – 45 °C. Zone 2 temperature can be varied in the range of 45 – 65°C. Zone 3 and 4 temperatures can be varied in the range of 95 – 100°C. The die plate was neither actively heated, nor cooled. The die nozzle was passively cooled by ambient air ensuring extrudate temperature below 100°C.

Noodles obtained by this process were light yellow colored material shaped into 1.5 mm thick filaments that had low tensile strength (< 0.1 MPa) and a moderately sticky texture.

Example 26. Sticky Tissue Replica Preparation

Sticky tissue replica was prepared using purified pea vicilin (freeze-dried) and purified pea legumin (freeze-dried). To prepare the sticky tissue replica, 50% ground pea vicilin powder and 50% ground pea legumin powder were thoroughly mixed and loaded into the loading tube of the extruder's batch feeder. The dry mixture was fed into the extruder at the rate in the range 0.4 – 0.8 g/min. Water was fed by the pump into the second zone of the extruder's barrel at the rate in the range 1.6 – 3.2 ml/min, such that the final moisture content of the extrudate was maintained at 80%. As the total throughput increased from 2 g/min to 4 g/min, the screw speed was increased from 100 to 200 RPM. Larger die diameters (4 mm and above) are also helpful for preventing backflow at higher throughputs. A temperature gradient was maintained along the extruder barrel as follows: feed zone - 25 °C, zone 1 – 30 °C, zone 2 – 60 °C, zone 3 – 90 °C, zone 4 – 90 °C. The die plate was neither actively heated, nor cooled. The die nozzle was passively cooled by ambient air. The die nozzle is kept free of obstructions by solidifying gel material.

Sticky tissue replica obtained by this process was semi-transparent water-white colored material shaped into irregular 1-5 cm sized bulbs that had sticky paste-like texture.

Example 27. Preparation of the flavor precursor mix

A flavor precursor mix was prepared by mixing concentrated stock solutions of each additive to make a 17X solution. Table 5 contains the chemical composition of the mix and the mM concentration of each component in the final burger. The concentrated flavor precursor mix was sterile filtered and adjusted to pH 5.5 – 6.0 using NaOH, and used at a 1X concentration in burgers.

TABLE 5.

Chemical composition of the flavor precursor mix.

Chemical entity	mM
Alanine	5.6
Arginine	0.6
Asparagine	0.8

Aspartate	0.8
Cysteine	0.8
Glutamic acid	3.4
Glutamine	0.7
Glycine	1.3
Histidine	0.6
Isoleucine	0.8
Leucine	0.8
Lysine	0.7
Methionine	0.7
Phenylalanine	0.6
Proline	0.9
Threonine	0.8
Tryptophan	0.5
Tyrosine	0.6
Valine	0.9
glucose	5.6
Ribose	6.7
Maltodextrin	5.0
Thiamine	0.5
GMP	0.2
IMP	0.6
Lactic acid	1.0
creatine	1.0
NaCl	10
KCl	10
Kphos pH 6.0	10

Example 28 - Freeze-alignment to produce texturized protein for use as muscle replicas

This example describes a non-extrusion based method to produce texturized protein materials that can be used in meat replicas.

A muscle tissue-replica was prepared by first preparing a gel of lentil proteins by mixing a 4.5% (w/v) solution of lentil proteins in 20mM potassium phosphate buffer pH 7.4 + 100mM sodium chloride with 20% (v/v) canola oil (from Jedwards International). The mixture was gelled by heating at 95°C for 15 minutes and slow cooled to room

temperature (at the rate of 1°C/minute). The gel was then poured into a vessel and frozen at -40°C by placing above a liquid nitrogen bath until completely frozen. The frozen material was then dried in a freeze-dryer. When the material was completely dried, the material was stabilized by autoclaving (121°C, 15 minutes). The resulting material is a texturized muscle tissue replica formed with plant proteins.

The aligned muscle replica then was presoaked in water for 5 minutes, cut into pieces of length 3-4mm and then combined with 10g adipose-replica, 10g connective tissue replica and 5g cold-set gels to form 50g beef patty replicas. An in-house sensory panel attributed inclusion of freeze-aligned tissue to impart improved fibrous texture to the patty.

A muscle-tissue replica also was prepared by first forming freeze-aligned material as described above. After the tissue replica was steam cooked at 121°C for 10 minutes, the material was allowed to soak in a solution of heat-denatured pea vicilins (at 6% w/v in 20mM potassium phosphate buffer pH 7.4 + 100mM sodium chloride, heat denatured by heating at 95°C for 30 minutes), 1% equine myoglobin (w/v) (Sigma), and 40% (v/v) canola oil (from Jedwards International). Gelation of the medium was induced by addition of calcium chloride at 20 mM. The sample was allowed to sit for 5 minutes at room temperature to allow for gel formation. The resulting muscle-replica contained aligned material in a cold-set gel reminiscent of beef muscle in steak.

EXAMPLE 29 - Cold gelation of proteins for meat applications

In one example, a cold-set gel comprising myoglobin was prepared by first heat denaturing a 6% (w/v) solution of pea-vicilins in 20mM potassium phosphate buffer, pH 7.4 with 100mM sodium chloride at 100°C for 30 minutes. The solution was allowed to cool back to room temperature. Canola oil (from Jedwards International) and equine myoglobin (Sigma) were added to a final concentration of 20% (v/v) and 1% (w/v), respectively. Gel formation was induced by adding calcium chloride at 20mM. A 50g beef patty replica was formed by combining 5g of the cold gel with 10g of adipose tissue-replica, 10g of connective tissue-replica, and 25g of muscle tissue-replica. Five mls of a

7% (w/w) solution of crude lentil protein was added to the mixture and patties were formed.

Example 30 - Binding materials in meat replicas

In one example, a beef replica was made by first preparing a coacervate from a 3% (w/v) solution of pea vicilins and legumins (vicilin:legumin ratio of 3:1) in 20 mM potassium phosphate pH 7.4 + 100 mM sodium chloride. Melted palm oil (from Jedwards International) was added to the solution to a final concentration of 5% and mixed by vortexing. The emulsion then was acidified by adding hydrochloric acid while stirring to a pH of 5. The slurry was then centrifuged at 5000 x g for 10 minutes and the liquid top layer was decanted from the coacervate.

A 50g beef patty replica was formed by combining the coacervate at 10% with adipose tissue-replica (20%), connective tissue-replica (20%) and muscle tissue-replica (50%). Five mls of a 7% solution of crude lentil protein was added to the mixture and patties were formed. Patties that included coacervate as binding material were observed to be more cohesive than patties that did not.

EXAMPLE 31 - Assembly of a sticky and noodle type ground tissue replica and burger replica

Ground tissue replica and burger replicas were prepared using the ingredients in Table 6. During all pre-processing steps, the temperature of the materials was maintained cold (4 – 15°C).

TABLE 6

Composition of sticky and noodle type formulation	
Ingredient	**%**
Adipose	26
Soft connective	20.6
Sticky	12
Raw	10
Noodles	10

Hard connective	10
Flavor and heme solution	10
k-carrageenan	1.4
Total	100

Adipose tissue replica from Example 6 was chilled following the final heat-cool step into a solid block. Optionally 0.2% by weight of heme protein can be added as a 20 mg/ml liquid solution and manually worked into the adipose. The adipose was then crumbled manually into small bits 3 – 7 mm in diameter while cold.

Soft connective tissue replica from Example 23 was produced by an extrusion process as long string-like pieces. The soft connective was chopped with a mini chopper (Mini-Prep® Plus Processor model DLC-2L Cuisinart, Stamford, CT) in a single step process. Approximate 200g of soft connective was placed in the mini chopper and processed on the chop setting for 60 seconds to yield pieces of 1 – 3 mm in length with ragged edges.

Sticky tissue replica and noodles tissue replica (see Examples 25 and 26) were produced by an extrusion process as long noodle-like pieces or amorphous pieces, respectively. Raw tissue replica of Example 21 was provided from the enzymatic crosslinking process as a solid block. All three of these tissue replicas were manually broken down into pieces 1 - 3 cm in diameter.

Hard connective tissue replica from Example 22 was produced from an extrusion process as long string-like pieces. The hard connective tissue replica was chopped to three levels, named coarse, intermediate, and fine, in a mini chopper (Mini-Prep® Plus Processor model DLC-2L Cuisinart, Stamford, CT). 160 – 200 g of hard connective tissue replica was placed in the mini chopper and processed on the chop setting for 90 seconds. One third of the material was removed as the coarse chopped fraction. The material remaining in the chopper was then processed for an additional 60 seconds and one third of the original weight was removed as the intermediate chopped fraction. The material remaining in the chopper was then processed for an additional 30 seconds to produce the fine fraction.

Leghemoglobin was freeze-dried and then reconstituted in a 17x flavor precursor mix (see Example 27) adjusted to pH 6.0 with 10 N NaOH to make the flavor and heme protein solution.

Following pre-processing described above, the soft connective, sticky, raw, noodles, hard connective, and 2/3 of the adipose were mixed by hand in a bowl. A typical batch size was 100 g to 2000 g. The flavor and heme solution was then dribbled onto the mixed tissue replicas and mixed gently by hand, then k-carrageenan powder was tsprinkled over the mixture and mixed in by hand. During the assembly, grinding, and forming all materials were kept cold (4 – 15°C). The mixture was ground using a stand mixer fitted with a food grinder attachment (KitchenAid® Professional 600 Series 6 Quart Bowl-Lift Stand Mixer model KP26M1XER and KitchenAid® Food Grinder model FGA, St. Joseph, MI) on speed setting 1. The food grinder material was fed by a screw conveyor past a rotating knife installed in front of a fixed hole plate.

The ground tissue replica mix was collected in a bowl and the remaining 1/3 of the crumbled adipose was then added to the ground tissue replica mix and mixed in by hand. Approximately 30 g or 90 g portions of ground tissue replica were then formed by hand into round patty shapes. Typical dimensions for 30 g patties were 50 mm x 12 mm. Typical dimensions for 90 g patties were 70 mm x 18 mm. Patties were refrigerated until cooked. Cooked patties had appearance, texture, and flavor similar to ground beef as judged by a trained sensory panel. In addition to cooking in patty format, the ground tissue replica can also be used in a variety of dishes such as taco filling, casseroles, sauces, toppings, soups, stews, or loaves.

EXAMPLE 32 - Assembly of a Wheat Gluten containing ground tissue replica and burger replicas

Ground tissue replica and burgers were prepared using the ingredients in Table 7.

TABLE 7:

Composition of Wheat Gluten Type formulation

Ingredient	%
Adipose	25
Soft connective	30
Raw tissue replica with heme and flavor	35
Wheat gluten	5
Hard connective	5
Total	100

Adipose, soft connective, and hard connective were preprocessed as described in Example 31. During all pre-processing steps, the temperature of the materials was maintained cold (4 – 15°C).

Raw muscle with heme protein and flavor was then prepared as follows. Freeze-dried pea vicilin and pea legumin were dissolved in water and 16x flavor stock. The freeze-dried heme protein was dissolved in this mixture and the pH was adjusted with citric acid to 5.8. A dry transglutaminase preparation (ACTIVA® TI Ajinomoto Fort Lee, NJ) was then added and mixed for about 5 min to fully dissolve. The mixture was then allowed to stir for an additional 10 minutes until some increase in viscosity was observed. The soft connective and hard connective were then added and the mixture was allowed to sit for 1 hour at room temperature to cure and form a solid mass.

Wheat gluten powder (vital wheat gluten, Great Northern, item 131100, Giusto's Vita-Grain, South San Francisco, CA) then was added to the gelled raw muscle and mixed to distribute. This mixture was then immediately ground using a stand mixer fitted with a food grinder attachment as described in the previous example. Ground tissue replica was then chilled for 5 min at -20°C. Finally, the chopped adipose, pre-chilled to 4°C, was added to the chilled ground tissue replica.

Ground tissue replica mix with adipose tissue replica added was then formed by hand into two 90 g round patties. 90 g patties are typically 70 mm x 18 mm. A typical batch size was 180 - 200 g and produced two patties. The patties were then allowed to rest at room temperature for 30 minutes. After resting the patties can be cooked or refrigerated until ready to cook.

EXAMPLE 33: Generation of beef flavor in replica burgers by the addition of Heme and flavor precursors.

Characteristic flavor and fragrance components in meat are mostly produced during the cooking process by chemical reactions molecules (precursors) including amino acids, fats and sugars that are found in plants as well as meat. Flavor precursors along with 1% Leghemoglobin were added to the muscle component of the burger replicas as indicated in Table 8. Three replicas, one with no precursors, and two different mixtures of precursors, along with 80:20 beef were cooked then served to a trained sensory panel to describe the flavor attributes shown in Table 9. The addition of precursors increased the beefy flavor, the bloody notes, overall flavor quanity, and decreased the off notes in the replica. The replicas and beef sample also were analyzed by GCMS by adding 3 grams of uncooked replica or beef into a GCMS vial. All samples were cooked at 150°C for 3 mins, cooled to 50°C to extract for 12 minutes using GCMS (SPME fiber sampling of headspace). A search algorithm analyzed the retention time and mass fingerprint information to assign chemical names to peaks. In the replica burger with 1% Leg hemeoglobin, and precursor mixture 2, 136 beef compounds were created. In Table 10, all the compounds created in the replica burger that were also identified by GCMS in the beef samples are indicated.

TABLE 8

Flavor precursors added to the beef replicas before cooking.

Samples	767	804	929
Additive (mm)	Precursor Mix 1	No Precursors	Precursor Mix 2
Alanine	5.61		5.61
Cysteine	0.83		0.83
Glutamic acid	3.40		3.40
Leucine	0.76		0.76
Lysine	0.68		0.68
Methionine	0.67		0.67
Tryptophan	0.49		0.49
Tyrosine	0.55		0.55
Valine	0.85		0.85

Glucose	5.55		5.55
Ribose	6.66		6.66
Lactic acid	1.00		1.00
creatine	1.00		1.00
Thiamine	0.50		0.50
IMP + GMP	0.40		0.40
Sucrose			2.00
Fructose			2.00
Xylose			2.00
Maltodextrin	0.50%		0.50%

TABLE 9

The sensory score determined by the sensory panel for the replica burgers and 80:20 beef sample.

Sample #		**Beef**	**767**	**804**	**929**
Flavor Quality	mean	7.0	3.8	3.2	4.3
	STDEV	0.0	1.3	1.0	1.2
Flavor Intensity	mean	4.3	4.2	4.3	4.3
	STDEV	0.8	1.2	1.4	1.2
Flavor: Beefy	mean	5.8	3.3	2.3	4.2
	STDEV	1.0	1.2	0.8	1.2
Flavor: bloody/Metallic	mean	4.5	2.0	2.2	3.2
	STDEV	1.2	1.0	0.9	1.4
Flavor: Savory	mean	3.3	3.8	3.7	4.2
	STDEV	1.2	1.2	1.2	1.8
Off Flavors: chemical/oxidized/beany	mean	1.5	2.3	3.5	2.7
	STDEV	0.8	1.2	1.8	1.0

TABLE 10

Beef flavor compounds created in replica burger with 1% LegH and precursor mix 2 as detected by GCMS.

3-octen-2-one	octanoic acid	(Z)-2-decenal,
1-penten-3-ol	octane	carbon disulfide

n-caproic acid vinyl ester	octanal	butyrolactone
2-acetylthiazole	nonanal	butanoic acid
thiophene	4,7-dimethyl-undecane,	3-methyl-butanal,
methyl-thiirane,	methyl ethanoate	2-methyl-butanal,
thiazole	methional	butanal
styrene	methacrolein	3,6,6-trimethyl-bicyclo[3.1.1]hept-2-ene,
	isovaleric acid	benzyl alcohol
pyrrole	isopropyl alcohol	1,3-dimethyl-benzene
pyridine	hexanoic acid	benzene
trimethyl-pyrazine	2,2,4,6,6-pentamethyl-heptane,	benzaldehyde
tetramethyl-pyrazine,	2-methyl-heptane,	acetophenone
methyl-pyrazine,	heptane	acetonitrile
ethyl-pyrazine	heptanal	acetone
3-ethyl-2,5-dimethyl-pyrazine	furfural	acetoin
2,5-dimethyl-pyrazine	furaneol	acetic acid ethenyl ester
2,3-dimethyl-pyrazine	3-methyl-furan	acetic acid
2-ethyl-5-methyl-pyrazine	2-propyl-furan	acetamide
2-ethenyl-6-methyl-pyrazine,	2-pentyl-furan	acetaldehyde
pyrazine	2-methyl-furan	4-methyl-5-thiazoleethanol
2-methyl-propanal,	2-ethyl-furan	6-methyl-5-hepten-2-one
propanal	furan	trans-2-(2-pentenyl)furan
phenylacetaldehyde	formamide	(E)-4-octene,
phenol	ethyl acetate	4-cyclopentene-1,3-dione
pentanoic acid	1-(2-furanyl)-ethanone	4-cyanocyclohexene
3-ethyl-2,2-dimethyl-pentane	1-(1H-pyrrol-2-yl)-ethanone	dihydro-2-methyl-3(2H)-furanone,
pentanal	dimethyl trisulfide	(E,E)-3,5-octadien-2-one
p-cresol	dimethyl sulfide	3,5-octadien-2-one
oxalic acid, butyl propyl ester	d-limonene	2,2-dimethyl-undecane
1-heptene	1-octen-3-ol	toluene
1-ethyl-5-methylcyclopentene	1-octanol	1-pentanol
1-butanol	1-hexanol	1-octen-3-one
1H-pyrrole-2-carboxaldehyde	2-methyl-1H-pyrrole	2-butanone
2-nonanone	3-methyl-2-butenal	2-thiophenecarboxaldehyde
2-n-butylacrolein	3-ethylcyclopentanone	2-pyrrolidinone
2-methyl-2-heptene	2(5H)-furanone	2-propenal
(E)-2-hexenal,	dihydro-5-pentyl-2(3H)-furanone	1-hydroxy-2-propanone

(E)-2-heptenal,	5-ethyldihydro-2(3H)-furanone	1-(acetyloxy)-2-propanone
6-methyl-2-heptanone	5-acetyldihydro-2(3H)-furanone	2-pentanone
2-heptanone	2,6-dimethylpyrazine	(E)-2-octenal
2-furanmethanol	(E,E)-2,4-nonadienal	2-octanone
3-ethyl-2-1,4-dioxin	(E,E)-2,4-heptadienal	(E)-2-nonenal
3-ethyl-2-methyl-1,3-hexadiene	(E,E)-2,4-decadienal	8-methyl-1-undecene
2-butenal	2,3-dimethyl-5-ethylpyrazine	1-propanol
1-penten-3-one		

EXAMPLE 34 - Removal of off-flavors from plant protein solutions

i) Synthesis of ligand modified LOX removal resin

A 1 mL settled volume of CM Sepharose resin (Sigma Aldrich Catalog # CCF100) was loaded into a BioRad minicolumn. 3 mL of 50 mM MES (2-morpholinoethanesulfonic acid) buffer preset to a pH range of 5.5 to 6 was allowed to pass through the resin bed. Separately, to 1 mL of the same buffer was added in succession, 0.044 mL of 4,7,10- trioxa-1,13-tridecanediamine, 0.030 mL of 12 N HCl, 23 mg of NHS (N-hydroxy-succinimide), and 38 mg of EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) with dissolution after each addition. The resulting solution was added to the top of the column bed and allowed to gravity flow through and the effluent was collected. The resulting effluent was returned to the top of the column bed. The cycle of addition of solution, elution and return was done four times. When the last elution was finished, 3 mL of 50 mM MES buffer preset to a pH of 5.5 to 6 was allowed to gravity flow through the column. Linoleic acid (0.03 mL) was dissolved in 0.5 mL DMF (N,N-dimethylformamide), followed in sequence by 12 mg NHS, 19 mg EDC, and 0.5 mL of 50 mM MES buffer preset to a pH of 5.5 to 6. The NHS/EDC mixture was shaken to mix, resulting in a two-phase liquid which was applied to the top of the column and eluted through the resin bed, allowed to gravity flow through the column and effluent collected. The effluent was returned to the top of the column bed. The cycle of addition of solution, elution and return was done four times. Once the last collection was finished a solution of 70% ethanol in water (5 mL) was added to the

top of the column, followed by 3 mL of 0.1 M sodium hydroxide. This was followed by 0.1 M buffer of potassium phosphate preadjusted to pH 7 to 8.

ii) Removal of off-flavors from pea proteins using the ligand modified LOX removal resin.

A solution of pea proteins (30 mls at 20 mg/ml protein concentration in 20 mM potassium phosphate buffer pH 7.4, 100mM sodium chloride) was passed through a 100 ml resin bed described above. All unbound material was collected and the resin further washed with 200 mls of buffer. Both fractions were combined and resin was stripped of bound protein by washing with 2 bed volumes of 20 mM potassium phosphate, 1M sodium chloride. Resin bed was regenerated by washing with 2 bed volumes of 0.1M NaOH followed by 3 bed volumes of water and re-equilibration with buffer.

Depletion of LOX activity in the pooled unbound fraction was confirmed by assaying for enzyme activity. Sodium linoleate was used as substrate for LOX and formation of hydroperoxide intermediates was monitored by absorbance at 234nm. Assays were performed at pH9 in 50mM sodium borate buffer. Assays confirmed depletion of LOX activity in the pooled unbound fraction. LOX was removed from resin in washes with 0.5M and 1M sodium chloride.

Improvement in flavor in the LOX depleted protein solutions (both as-is and incubated with canola oil at 10%) was confirmed by a panel of 4 tasters. Samples of pea proteins at same concentration, but not depleted of LOX was used at control. Tasters described LOX -depleted samples as mild tasting and control samples as having beany, planty taste. In addition, GC-MS analysis of samples showed 5X reduction in overall volatiles in the LOX-depleted samples.

Reduction of off-flavors using dialysis and activated carbon

A solution of pea albumins (30 mls at 40 mg/ml in 20mM potassium phosphate buffer pH 7.4, 100 mM sodium chloride) was dialyzed against 100 volumes of buffer overnight at 4°C. The solution was then poured over a bed of activated carbon (100-mesh, Sigma-Aldrich) prewetted with buffer. The slurry was centrifuged at 5000 x g for

10 minutes and the supernatant containing protein was decanted off. This solution was tested for improvement in flavor by both taste and GC-MS. Improved flavor (less bitter, less soapy flavor) was noted by tasters and GC-MS confirmed a >2X reduction in volatile compounds; in particular, samples treated with activated carbon showed a decrease in C6- and C7 compounds (e.g., 1-heptanal, 2-heptenal, or 2-heptanone) that are associated with green/grassy/planty flavors.

Reduction of off-flavors using antioxidants and/or LOX inhibitors

A 7% solution of pea-vicilins was heated with coconut oil (20%) to 95°C for 15 min in the presence of antioxidants or LOX inhibitors and compared for off-flavors sample against a control without any antioxidants or LOX inhibitors. In a similar experiment, soymilk was heated in the presence of antioxidants or LOX inhibitors and tasted in-house for off-flavors. Table 11 summarizes off-flavors noted by tasters. Both epigallocetechin gallate and propyl gallate were effective at minimizing off-flavors in pea samples. However, in case of soymilk, epigallocatechin gallate did not appear to reduce beaniness; propyl gallate and α-tocopherol were found to slightly improve flavor in soymilk.

Table 11

Compound	soymilk	Pea
α-tocopherol	Slightly improved flavor	Oxidized oil
caffeic acid (0.02%)	Beany	Oxidized oil
epigallocatechin gallate	Beany	Improved flavor
propyl gallate (0.02%)	Slightly improved flavor	Improved flavor
β-carotene	Beany	Oxidized oil

EXAMPLE 35 - ADIPOSE REPLICA WITH LECITHIN GRADIENT

Lecithin (SOLEC™ F Deoiled Soy Lecithin, The Solae Company, St. Louis, MO) was prepared at a concentration of 50 mg/mL in 20 mM potassium phosphate, 100 mM NaCl, pH 8.0 buffer and sonicated (Sonifier Analog Cell Disruptor model 102C,

BRANSON Ultrasonics Corporation, Danbury, Connecticut) for 30 seconds. Moong protein was supplied as a liquid in 20 mM potassium phosphate, 100 mM NaCl, pH 8.0 buffer. Coconut oil was melted by heating to 50 - 70°C and held warm until needed. The coconut oil, buffered protein solution, additional buffer, and lecithin slurry were mixed at 70°C, and an emulsion was formed using a hand held homogenizer. The emulsion was then heat set by placing the tube into a 95°C waterbath for a total of 5 mins. The tube was then removed from the waterbath and stored at 4°C for twelve hours or longer prior to analysis.

To observe the effect of lecithin on the adipose tissue replica replica, a formulation was prepared that contained 1% w/v moong protein and 75% v/v coconut oil, with the lecithin amount increasing from 0%, 0.05%, 0.25%, 0.5%, and 1.0% w/v.

Properties of the adipose replica were measured by weighing small portions of the material and forming uniform round balls, which were then cooked on a non-stick pan with temperature slowly ramping up to 150°C. The temperature of the pan at which fat visibly released from the balls was measured as the fat release temperature. After cooking to completion, the point at which fat no longer released, the total fat released was measured.

An increase in the amount of lecithin in the adipose replica correlated with an increase in the percent fat released, and with a decrease in the fat release temperature (see FIGs. 2A and 2B). With 0% lecithin, there was an average of 40% fat released, and when lecithin was increased to 0.05%, there was an average of 82% fat released, which further increased to 88% with 0.25% lecithin, then leveled off to an average of 60% with further increases in lecithin. With 0% lecithin, a high temperature of 217°C was required to begin the fat release. The fat release temperature decreased to 122°C with 0.25% lecithin, then leveled off to an average of 62°C with further increases of lecithin.

Firmness of the adipose replica was measured by texture analyzer (TA XT plus). A probe penetrated a flat surface of the adipose replica, and the force at 2 mm penetration was recorded. A small amount of lecithin (0.05%) increased the firmness, and at 0.25% and above, the firmness of the adipose replica decreased. See FIG. 2C.

EXAMPLE 36 - ADIPOSE REPLICA WITH VARYING VEGETABLE OIL TYPE

To observe the effect of vegetable oil type on the adipose replica, a formulation was prepared that contained a 1.5% w/v moong protein and 0.05% w/v lecithin, and 75% v/v of different vegetable oils (canola oil, cocoa butter, coconut oil, and olive oil) using the same methodology as Example 35. Properties of the adipose replica were measured by weighing small portions of the material and forming uniform round balls, which were then cooked on a non-stick pan with temperature slowly ramping up to 250°C. The temperature of the pan at which fat visibly released from the balls was measured as the fat release temperature. After cooking to completion, the point at which fat no longer released, the total fat released was measured.

Varying the vegetable oil type had a large effect on the adipose replica. Oils with a higher amount of unsaturated fats, including canola and rice bran oils, released very little fat (1 and 2% fat released, see FIG. 3), while oils with a higher amount of saturated fats, including cocoa butter and coconut oil released significantly more fat (30% and 50% fat released, see FIG. 4). The adipose replicas with canola and rice bran oils required a temperature greater than 250°C to melt (not measured), while the adipose replicas with cocoa butter and coconut oil released fat at a lower temperature (82°C and 137°C).

EXAMPLE 37 - ADIPOSE REPLICA MADE WITH COACERVATE

Lecithin (SOLECTM F Deoiled Soy Lecithin, The Solae Company, St. Louis, MO) was prepared at a concentration of 50 mg/ml in 20 mM potassium phosphate, 100 mM NaCl, pH 8.0 buffer and sonicated (Sonifier Analog Cell Disruptor model 102C, BRANSON Ultrasonics Corporation, Danbury, Connecticut) for 30 seconds. Pea legumin and pea vicilin proteins in 20 mM potassium phosphate, 100 mM NaCl, pH 8.0 buffer, were mixed to a 1:1 ratio. Cocoa butter was melted by heating to 70°C and held warm until needed. The cocoa butter was added to 2% and 10% w/v to the protein mixtures, and was added at 60°C to maintain cocoa butter in the liquid state. While the solutions were still warm, the mixtures were sonicated 1-3 minutes, until cocoa butter particles were visibly emulsified. The pH of the samples was adjusted to 5.5 with HCl,

and the mixtures turned into a milky white color, then centrifuged at 5,000xg for ten minutes. After centrifugation, a pellet was collected, comprising a coacervate of protein and cocoa butter. The 2% fat coacervates were sticky and stretchy, while the 10% fat coacervates were fatty and pliable. The coacervates were sealed in plastic and subjected to high pressure processing (HPP). The sample was sealed in a heat-sealable food-saver plastic bag and then subject to high pressure processing (85k psi for 5 minutes in an Avure 2L Isostatic Food Press). After HPP, the 2% coacervate samples formed a semi-firm, cohesive material. The 10% coacervate samples were crumbly, soft, and oily.

Properties of the processed coacervate samples were measured by breaking off small portions of the material and cooking on a non-stick pan with temperature slowly ramping up to 250°C. The coacervate samples did not release any fat upon cooking to this temperature.

OTHER EMBODIMENTS

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.

WHAT IS CLAIMED IS:

1. A consumable product comprising an isolated and purified plant protein, wherein the isolated and purified plant protein has (i) a solubility in a solution of at least 25 g/L at a temperature between about 2°C and about 32°C, wherein the solution has a pH between 3 and 8, and has a sodium chloride content of 0 to 300 mM or (ii) a solubility in a solution of at least 1 mg/ml at a temperature of between 90°C and 110°C, wherein the solution has a pH between 5 and 8 and has a sodium chloride content of 0 to 300 mM.

2. The consumable product of claim 1, wherein said product is a beverage, a protein supplement, a baked good, a condiment, a meat product, or a meat substitute product.

3. The consumable product of claim 2, wherein said beverage is an alcoholic beverage or a protein drink.

4. The consumable product of claim 3, wherein said alcoholic beverage is a cream liquor.

5. The consumable product of claim 4, said cream liquor further comprising a non-dairy lipid emulsion, wherein said cream liquor is free of animal products.

6. The consumable product of claim 3, wherein said protein drink is a meal replacement beverage, a beer supplemented with said protein, or distilled alcoholic beverage supplemented with said protein.

7. The consumable product of claim 2, wherein said condiment is a mayonnaise replica.

8. The consumable product of claim 2, wherein said meat product is a pate, a sausage replica, or a meat substitute.

9. The consumable product of any one of claims 1-8, wherein said isolated and purified plant protein is at least 10 kDa in size.

10. The consumable product of any one of claims 1-9, wherein said isolated and purified plant protein is not fully denatured.

11. The consumable product of any one of claims 1-10, wherein said isolated and purified plant protein is not derived from soy.

12. The consumable product of any one of claims 1-11, wherein said isolated and purified plant protein comprises one or more of RuBisCo, Moong 8S globulin, a pea globulin, a pea albumin, a lentil protein, zein, or an oleosin.

13. The consumable product of any one of claims 1-12, wherein said isolated and purified plant protein comprises a dehydrin, a hydrophilin, an intrinsically disordered protein, or a protein identified based on its ability to stay soluble after boiling at a pH and a salt concentration comparable to a food.

14. The consumable product of any one of claims 1-13, said consumable product further comprising a plant derived lipid or a microbial-derived lipid.

15. The consumable product of any one of claims 1-14, further comprising a second isolated and purified protein.

16. The consumable product of any one of claims 1-15, further comprising a seasoning agent, a flavoring agent, an emulsifier, a gelling agent, a sugar, or a fiber.

17. A consumable product comprising a coacervate comprising one or more isolated and purified proteins.

18. The consumable product of claim 17, wherein said consumable product is a meat replica.

19. The consumable product of claim 17 or claim 18, said product further comprising a plant derived lipid or a microbial-derived lipid.

20. The consumable product of claim 19, wherein said plant derived lipid or microbial-derived lipid comprises lecithin and/or an oil.

21. The consumable product of claim 20, said product comprising up to about 1% lecithin by weight.

22. The consumable product of claims 20 or claim 21, wherein said product comprises lecithin and said oil.

23. The consumable product of any one of claims 20-22, wherein said oil is canola oil, palm oil, or cocoa butter.

24. The consumable product of any one of claims 20-23, said product comprising about 1% to about 9% of said oil.

25. The consumable product of any one of claims 17-24, wherein said one or more isolated and purified proteins comprise plant proteins.

26. The consumable product of claim 25, wherein said one or more plant proteins comprise one or more pea proteins, chickpea proteins, lentil proteins, lupine proteins, other legume proteins, or mixtures thereof.

27. The consumable product of claim 26, wherein said one or more pea proteins are legumins, vicilin, a convicilin, or a mixture thereof.

28. A meat replica comprising a muscle replica, a connective tissue replica; an adipose tissue replica, and a coacervate comprising one or more isolated and purified proteins.

29. The meat replica of claim 28, wherein said coacervate further comprises a plant-derived lipid or microbial-derived lipid.

30. The meat replica of claim 29, wherein said plant derived lipid or microbial-derived lipid is lecithin and/or an oil.

31. The meat replica of any one of claims 28-30, wherein said meat replica is a ground beef replica.

32. A consumable product comprising a comprising a cold set gel comprising one or more isolated and purified proteins from a non-animal source and a salt.

33. The consumable product of claim 32, wherein said isolated and purified plant protein comprises one or more of RuBisCo, moong 8S globulin, a pea globulin, a pea albumin, a lentil protein, zein, or an oleosin.

34. The consumable product claim 32, wherein said isolated and purified plant protein comprises a dehydrin, a hydrophilin, or an intrinsically disordered protein.

35. The consumable product of any one of claims 32-35, wherein said cold set gel further comprises a plant derived lipid or microbial derived lipid.

36. The consumable product of claim 35, wherein said plant derived lipid or microbial derived lipid is lecithin and/or oil.

37. An adipose tissue replica comprising one or more isolated plant proteins, one or more plant or algal derived oils, and optionally a phospholipid.

38. The adipose tissue replica of claim 37, wherein said phospholipid is lecithin.

39. The adipose tissue replica of claim 37 or claim 38, wherein said plant based oils are selected from the group consisting of corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil, sunflower oil, flax seed oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, rice bran oil, and combinations thereof.

40. The adipose tissue replica of any one of claims 37-39, wherein the fat release temperature of said adipose tissue replica is between 23°C to 33°C, 34°C to 44°C, 45°C to 55°C, 56°C to 66°C, 67°C to 77°C, 78°C to 88°C, 89°C to 99°C, 100°C to 110°C, 111°C to 121°C, 122°C to 132°C, 133°C to 143°C, 144°C to 154°C, 155°C to 165°C, 166°C to 167°C, 168°C to 169°C, 170°C to 180°C, 181°C to 191°C, 192°C to 202°C, 203°C to 213°C, 214°C to 224°C, 225°C to 235°C, 236°C to 246°C, 247°C to 257°C, 258°C to 268°C, 269°C to 279°C, 280°C to 290°C, or 291°C to 301°C.

41. The adipose tissue replica of any one of claim 37-40, wherein the percent fat release of said adipose tissue replica is 0 to 10%, 10% to 20%, 20% to 30%, 30% to 40%, 40% to 50%, 50% to 60%, 60% to 70%, 70% to 80%, 80% to 90%, or 90% to 100% upon cooking.

42. The adipose tissue replica of any one of claims 37-41, wherein said isolated and purified plant protein comprises one or more of RuBisCo, moong 8S globulin, a pea globulin, a pea albumin, a lentil protein, zein, or an oleosin.

43. The adipose tissue replica of any one of claims 37-42, wherein said adipose tissue replica comprises from about 40% to about 90% of said oil.

44. The adipose tissue replica of any one of claims 37-43, wherein said adipose tissue replica comprises from about 1% to about 6% of said isolated and purified plant protein.

45. The adipose tissue replica of any one of claims 37-44, wherein said adipose tissue replica comprises from about 0.05 to about 2% of said phospholipid.

46. The adipose tissue replica of any one of claims 37-45, wherein the firmness of said adipose tissue replica is similar to that of beef adipose tissue.

47. A consumable product comprising a heme-containing protein and (i) carbon monoxide and/or (ii) a nitrite, wherein said consumable product does not comprise meat.

48. The consumable product of claim 47, wherein said heme-containing protein accounts for at least 0.01% of the composition.

49. The consumable product of claim 47 or claim 48, further comprising one or more ammonium, sodium, potassium, or calcium salts.

50. The consumable product or meat replica of any one of claims 17-49, wherein said isolated and purified proteins are crosslinked.

51. A consumable product comprising a gelled emulsion, said gelled emulsion comprising:

a) an isolated and purified protein;

b) a first lipid that when not in said consumable product is solid at a selected temperature range; and

c) a second lipid that when not in said consumable product is liquid at said selected temperature range; wherein the melting temperature of the mixture of said first and second lipids is similar to the melting temperature of lipids found in meat, and wherein said first and second lipids are plant derived lipids or microbial derived lipids.

52. A method for making a consumable product comprising:

a) preparing a solution comprising an isolated and purified plant protein, wherein said isolated and purified plant protein has (i) a solubility in said solution of at least 25 at a temperature between about 2°C and 32°C, wherein said solution has a pH between 3 and 8, and has a sodium chloride content of 0 to 300 mM or (ii) a solubility in said solution of at least 1 mg/ml at a temperature of between 90°C and 110°C, wherein said solution has a pH between 5 and 8 and has a sodium chloride content of 0 to 300 mM; and

b) adding said solution to a beverage.

53. The method of claim 52 wherein said solution comprises two or more isolated and purified plant proteins.

54. The method of claim 52 or claim 53, wherein said beverage is clear.

55. The method of any one of claim 52-54, wherein said isolated and purified plant protein is at a concentration of at least 1% by weight in said solution.

56. The method of any one of claims 52-55, wherein said isolated and purified plant protein is selected from the group consisting of RuBisCo, a moong globulin, a soy globulin, a pea globulin, a pea albumin, a prolamin, a lentil protein, a dehydrin, a hydrophilin, and an intrinsically disordered protein.

57. The method of any one of claims 52-56, wherein said isolated and purified plant protein is lyophilized prior to making said solution.

58. The method of any one of claims 52-57, wherein said beverage has an improved mouthfeel compared to a corresponding beverage without said isolated and purified protein.

59. A method for extending the shelf-life of a meat-free consumable product, said method comprising adding a heme-containing protein to said consumable product, wherein said heme containing protein oxidizes more slowly than myoglobin under equivalent storage conditions.

60. The method of claim 59, wherein said heme-containing protein comprises an amino acid sequence with at least 70% homology to an amino acid sequence set forth in any one of SEQ ID NOs: 1-27.

61. A method for making a meat replica comprising a cold set gel comprising:

a) denaturing a solution comprising at least one isolated and purified protein from a non-animal source under conditions wherein said isolated and purified protein does not precipitate out of said solution;

b) optionally adding any heat-labile components to said solution of denatured protein;

c) gelling said solution of denatured protein at about 4°C to about 25°C by increasing the ionic strength of said solution to form a cold set gel, and

d) incorporating said cold set gel into a meat replica.

62. The method of claim 61, wherein gelling is induced using 5 to 100 mM sodium or calcium chloride.

63. The method of claim 61 or claim 62, wherein said heat-labile components are proteins or lipids, or mixtures thereof.

64. The method of claim 63, wherein said protein is a heme-containing protein.

65. The method of claim 63, wherein said cold set gel is formed in a matrix comprising a freeze-aligned plant protein.

66. The method of any one of claims 61-65, wherein said isolated and purified protein from a non-animal source is a plant protein.

67. The method of claim 66, wherein said plant protein is selected from the group consisting of RuBisCo, a moong globulin, a soy globulin, a pea globulin, a pea albumin, a prolamin, a lentil protein, a dehydrin, a hydrophilin, and an intrinsically disordered protein.

68. An adipose tissue replica, comprising

a) an isolated and purified non-animal protein;

b) a non-animal lipid; and

c. a three-dimensional matrix comprising fibers derived from non-animal sources, wherein said lipid and said protein are dispersed in said three-dimensional matrix, and wherein said three-dimensional matrix stabilizes the structure of the adipose tissue replica.

69. A connective tissue replica comprising one or more isolated and purified proteins assembled into fibrous structures by a solution spinning process.

70. The connective tissue replica of claim 69 wherein the fibrous structures are stabilized by a cross-linking agent.

71. A method for imparting a beef like flavor to a consumable product, comprising adding to said consumable composition a heme-containing protein, wherein after cooking, a beef-like flavor is imparted to said consumable.

72. A method for making a poultry or a fish composition taste like beef, said method comprising adding a heme protein to said poultry or fish composition, respectively.

73. The method of claim 71 or claim 72, wherein said heme-containing protein has an amino acid sequence with at least 70% homology to any one of the amino acid sequences set forth in SEQ ID NOs: 1-27.

74. A method of making a coacervate, said method comprising

a) acidifying a solution of one or more plant proteins to a pH between 3.5 and 5.5, wherein said solution comprises 100 mM or less of sodium chloride; and

b) isolating said coacervate from said solution.

75. The method of claim 74, wherein said pH is between 4 and 5.

76. The method of claim 74 or claim 75, wherein said plant proteins comprise one or more pea proteins, chickpea proteins, lentil proteins, lupine proteins, other legume proteins, or mixtures thereof.

77. The method of claim 76, wherein said pea proteins comprise isolated and purified legumins, isolated and purified vicilins, isolated and purified convicilins, or combinations thereof.

78. The method of claim 77, wherein said isolated and purified pea proteins comprise isolated and purified vicilins and isolated and purified convicilins.

79. The method of any one of claims 74-78, wherein said acidifying step is done in the presence of a plant derived lipid or microbial derived lipid.

80. The method of claim 79, wherein said plant derived lipid or microbial derived lipid comprises oils and/or phospholipids.

81. A method of making an adipose tissue replica, said method comprising forming an emulsion comprising one or more isolated plant proteins, one or more plant or algal derived oils, and optionally a phospholipid.

82. The method of claim 81, wherein said phosoholipid is included, and is lecithin.

83. The method of claim 81 or claim 82, wherein said plant based oils are selected from the group consisting of corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil,

sunflower oil, flax seed oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, rice bran oil, and combinations thereof.

84. The method of any one of claims 81-83, wherein the fat release temperature of said adipose tissue replica is between 23°C to 33°C, 34°C to 44°C, 45°C to 55°C, 56°C to 66°C, 67°C to 77°C, 78°C to 88°C, 89°C to 99°C, 100°C to 110°C, 111°C to 121°C, 122°C to 132°C, 133°C to 143°C, 144°C to 154°C, 155°C to 165°C, 166°C to 167°C, 168°C to 169°C, 170°C to 180°C, 181°C to 191°C, 192°C to 202°C, 203°C to 213°C, 214°C to 224°C, 225°C to 235°C, 236°C to 246°C, 247°C to 257°C, 258°C to 268°C, 269°C to 279°C, 280°C to 290°C, or 291°C to 301°C.

85. The method of any one of claim 81-84, wherein the percent fat release of said adipose tissue replica is 0 to 10%, 10% to 20%, 20% to 30%, 30% to 40%, 40% to 50%, 50% to 60%, 60% to 70%, 70% to 80%, 80% to 90%, or 90% to 100% upon cooking.

86. The method of any one of claims 81-85, wherein said isolated and purified plant protein comprises one or more of RuBisCo, moong 8S globulin, a pea globulin, a pea albumin, a lentil protein, zein, or an oleosin.

87. The method of any one of claims 81-86, wherein said emulsion comprises from about 40% to about 90% of said oil.

88. The method of any one of claims 81-87, wherein said emulsion comprises from about 1% to about 4% of said isolated and purified plant protein.

89. The method of any one of claims 81-88, wherein said adipose tissue replica comprises from about 0.05 to about 1% of said phospholipid.

90. The method of any one of claims 81-89, wherein the emulsion is formed by high-pressure homogenization, sonication, or hand homogenization.

91. A method of minimizing undesirable odors in a composition comprising plant proteins, said method comprising contacting said composition with a ligand having affinity for one or more lipoxygenases.

92. A method of minimizing undesirable odors in a composition comprising plant proteins, said method comprising contacting said composition with activated carbon then removing the activated carbon from said composition.

93. A method of minimizing undesirable odors in a composition comprising plant proteins, said method comprising adding a lipoxygenase inhibitor and/or an antioxidant to said composition.

94. A chocolate flavored spread comprising:

a) sugar

b) a chocolate flavoring, and

c) a cream fraction from a plant based milk.

95. A method for altering the texture of a consumable during or after cooking comprising incorporating within the consumable one or more plant proteins with a low denaturation temperature.

96. The method of claim 95, wherein at least one of the one or more plant proteins is isolated and purified.

97. The method of claim 95, wherein the one or more plant proteins are selected from the group consisting of rubisco, pea proteins, lentil proteins, or other legume proteins.

98. The method of claim 97, wherein the pea proteins comprise pea albumin proteins.

99. The method of claim 95, wherein the consumable becomes firmer during or after cooking.

100. A tissue replica comprising a freeze-aligned non-animal protein.

101. The tissue replica of claim 100, wherein the non-animal protein is a plant protein.

102. The tissue replica of claim 101, wherein the non-animal protein is isolated and purified.

103. The tissue replica of claim 100, which is a muscle tissue replica.

104. A meat replica comprising a tissue replica comprising a freeze-aligned non-animal protein.

FIGURE 1

SEQ ID NO:1 *Vigna radiata*

MTTTLERGFTEEQEALVVKSWNVMKKNSGELGLKFFLKIFEIAPSAQKLFSFLRDSTVP
LEQNPKLKPHAVSVFVMTCDSAVQLRKAGKVTVRESNLKKLGATHFRTGVANEHFEVTK
FALLETIKEAVPEMWSPAMKNAWGEAYDQLVDAIKYEMKPPSS

SEQ ID NO:2 *Methylacidiphilum infernorum*

MIDQKEKELIKESWKRIEPNKNEIGLLFYANLFKEEPTVSVLFQNPISSQSRKLMQVLG
ILVQGIDNLEGLIPTLQDLGRRHKQYGVVDSHYPLVGDCLLKSIQEYLGQGFTEEAKAA
WTKVYGIAAQVMTAE

SEQ ID NO:3 *Aquifex aeolicus*

MLSEETIRVIKSTVPLLKEHGTEITARMYELLFSKYPKTKELFAGASEEQPKKLANAII
AYATYIDRLEELDNAISTIARSHVRRNVKPEHYPLVKECLLQAIEEVLNPGEEVLKAWE
EAYDFLAKTLITLEKKLYSQP

SEQ ID NO:4 *Glycine max*

MGAFTEKQEALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNPKL
TGHAEKLFGLVRDSAGQLKANGTVVADAALGSIHAQKAITDPQFVVVKEALLKTIKEAV
GDKWSDELSSAWEVAYDELAAAIKKAF

SEQ ID NO:5 *Hordeum vulgare*

MSAAEGAVVFSEEKEALVLKSWAIMKKDSANLGLRFFLKIFEIAPSARQMFPFLRDSDV
PLETNPKLKTHAVSVFVMTCEAAAQLRKAGKITVRETTLKRLGGTHLKYGVADGHFEVT
RFALLETIKEALPADMWGPEMRNAWGEAYDQLVAAIKQEMKPAE

Magnaporthe oryzae, (SEQ ID NO:6)

```
   1 mdgavrldwt gldltgheih dgvpiasrvq vmvsfplfkd qhiimsskes psrksstigq
  61 strngscqad tqkgqlppvg ekpkpvkenp mkklkemsqr plptqhgdgt yptekkltgi
 121 gedlkhirgy dvktllamvk sklkgeklkd dktmlmervm qlvarlptes kkraeltdsl
 181 inelwesldh pplnylgpeh syrtpdgsyn hpfnpqlgaa gsryarsvip tvtppgalpd
 241 pglifdsimg rtpnsyrkhp nnvssilwyw atiiihdifw tdprdintnk sssyldlapl
 301 ygnsqemqds irtfkdgrmk pdcyadkrla gmppgvsvll imfnrfhnhv aenlalineg
 361 grfnkpsdll egeareaawk kydndlfqva rlvtsglyin itlvdyvrni vnlnrvdttw
 421 tldprqdaga hvgtadgaer gtgnavsaef nlcyrwhsci sekdskfvea qfqnifgkpa
 481 sevrpdemwk gfakmeqntp adpgqrtfgg fkrgpdgkfd dddlvrcise avedvagafg
 541 arnvpqamkv vetmgiiqgr kwnvaglnef rkhfhlkpys tfedinsdpg vaealrrlyd
 601 hpdnvelypg lvaeedkqpm vpgvgiapty tisrvvlsda vclvrgdrfy ttdftprnlt
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```
 661 nwgykevdyd lsvnhgcvfy klfirafpnh fkqnsvyahy pmvvpsenkr ilealgradl
 721 fdfeapkyip prvnitsygg aeyiletqek ykvtwheglg flmgegglkf mlsgddplha
 781 qqrkcmaaql ykdgwteavk afyagmmeel lvsksyflgn nkhrhvdiir dvgnmvhvhf
 841 asqvfglplk taknptgvft eqemygilaa ifttiffdld psksfplrtk trevcqklak
 901 lveanvklin kipwsrgmfv gkpakdepls iygktmikgl kahglsdydi awshvvptsg
 961 amvpnqaqvf aqavdyylsp agmhyipeih mvalqpstpe tdalllgyam egirlagtfg
1021 syreaavddv vkedngrqvp vkagdrvfvs fvdaardpkh fpdpevvnpr rpakkyihyg
1081 vgphaclgrd asqiaitemf rclfrrrnvr rvpgpqgelk kvprpggfyv ymredwgglf
1141 pfpvtmrvmw dde
```

Fusarium oxysporum (SEQ ID NO:7)

```
   1 mkgsatlafa lvqfsaasql vwpskwdeve dllymqggfn krgfadalrt cefgsnvpgt
  61 qntaewlrta fhdaithdak agtggldasi ywessrpenp gkafnntfgf fsgfhnprat
 121 asdltalgtv lavgacngpr ipfragrida ykagpagvpe pstnlkdtfa aftkagftke
 181 emtamvacgh aiggvhsvdf peivgikadp nndtnvpfqk dvssfhngiv teylagtskn
 241 plvasknatf hsdkrifdnd katmkklstk agfnsmcadi ltrmidtvpk svqltpvlea
 301 ydvrpyitel slnnknkihf tgsvrvritn nirdnndlai nliyvgrdgk kvtvptqqvt
 361 fqggtsfgag evfanfefdt tmdakngitk ffiqevkpst katvthdnqk tggykvddtv
 421 lyqlqqscav leklpnaplv vtamvrdara kdaltlrvah kkpvkgsivp rfqtaitnfk
 481 atgkkssgyt gfqaktmfee qstyfdivlg gspasgvqfl tsqampsqcs
```

Fusarium graminearum (SEQ ID NO:8)

```
   1 masatrqfar aatratrngf aiaprqvirq qgrryyssep aqksssawiw ltgaavagga
  61 gyyfygnsas satakvfnps kedyqkvyne iaarleekdd yddgsygpvl vrlawhasgt
 121 ydketgtggs ngatmrfape sdhganagla aardflqpvk ekfpwitysd lwilagvcai
 181 qemlgpaipy rpgrsdrdvs gctpdgrlpd askrqdhlrg ifgrmgfndq eivalsgaha
 241 lgrchtdrsg ysgpwtfspt vltndyfrll veekwqwkkw ngpaqyedks tkslmmlpsd
 301 ialiedkkfk pwvekyakdn daffkdfsnv vlrlfelgvp faqgtenqrw tfkpthqe
```

SEQ ID NO: 9 *Chlamydomonas eugametos*

MSLFAKLGGREAVEAAVDKFYNKIVADPTVSTYFSNTDMKVQRSKQFAFLAYALGGASE
WKGKDMRTAHKDLVPHLSDVHFQAVARHLSDTLTELGVPPEDITDAMAVVASTRTEVLN
MPQQ

SEQ ID NO:10 *Tetrahymena pyriformis*

MNKPQTIYEKLGGENAMKAAVPLFYKKVLADERVKHFFKNTDMDHQTKQQTDFLTMLLG
GPNHYKGKNMTEAHKGMNLQNLHFDAIIENLAATLKELGVTDAVINEAAKVIEHTRKDM
LGK

SEQ ID NO:11 *Paramecium caudatum*

MSLFEQLGGQAAVQAVTAQFYANIQADATVATFFNGIDMPNQTNKTAAFLCAALGGPNA
WTGRNLKEVHANMGVSNAQFTTVIGHLRSALTGAGVAAALVEQTVAVAETVRGDVVTV

SEQ ID NO:12 *Aspergillus niger*

MPLTPEQIKIIKATVPVLQEYGTKITTAFYMNMSTVHPELNAVFNTANQVKGHQARALA
GALFAYASHIDDLGALGPAVELICNKHASLYIQADEYKIVGKYLLEAMKEVLGDACTDD
ILDAWGAAYWALADIMINREAALYKQSQG

SEQ ID NO:13 *Zea mays*

MALAEADDGAVVFGEEQEALVLKSWAVMKKDAANLGLRFFLKVFEIAPSAEQMFSFLRD
SDVPLEKNPKLKTHAMSVFVMTCEAAAQLRKAGKVTVRETTLKRLGATHLRYGVADGHF
EVTGFALLETIKEALPADMWSLEMKKAWAEAYSQLVAAIKREMKPDA

SEQ ID NO:14 *Oryza sativa subsp. japonica*

MALVEGNNGVSGGAVSFSEEQEALVLKSWAIMKKDSANIGLRFFLKIFEVAPSASQMFS
FLRNSDVPLEKNPKLKTHAMSVFVMTCEAAAQLRKAGKVTVRDTTLKRLGATHFKYGVG
DAHFEVTRFALLETIKEAVPVDMWSPAMKSAWSEAYNQLVAAIKQEMKPAE

SEQ ID NO:15 *Arabidopsis thaliana*

MESEGKIVFTEEQEALVVKSWSVMKKNSAELGLKLFIKIFEIAPTTKKMFSFLRDSPIP
AEQNPKLKPHAMSVFVMCCESAVQLRKTGKVTVRETTLKRLGASHSKYGVVDEHFEVAK
YALLETIKEAVPEMWSPEMKVAWGQAYDHLVAAIKAEMNLSN

SEQ ID NO:16 *Pisum sativum*

MGFTDKQEALVNSSWESFKQNLSGNSILFYTIILEKAPAAKGLFSFLKDTAGVEDSPKL
QAHAEQVFGLVRDSAAQLRTKGEVVLGNATLGAIHVQRGVTDPHFVVVKEALLQTIKKA
SGNNWSEELNTAWEVAYDGLATAIKKAMT

SEQ ID NO:17 *Vigna unguiculata*

MVAFSDKQEALVNGAYEAFKANIPKYSVVFYTTILEKAPAAKNLFSFLANGVDATNPKL
TGHAEKLFGLVRDSAAQLRASGGVVADAALGAVHSQKAVNDAQFVVVKEALVKTLKEAV
GDKWSDELGTAVELAYDELAAAIKKAY

SEQ ID NO:18 Bos taurus

MGLSDGEWQLVLNAWGKVEADVAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKAS
EDLKKHGNTVLTALGGILKKKGHHEAEVKHLAESHANKHKIPVKYLEFISDAIIHVLHA
KHPSDFGADAQAAMSKALELFRNDMAAQYKVLGFHG

SEQ ID NO:19 *Sus scrofa*
MGLSDGEWQLVLNVWGKVEADVAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKAS
EDLKKHGNTVLTALGGILKKKGHHEAELTPLAQSHATKHKIPVKYLEFISEAIIQVLQS
KH PGDFGADAQGAMSKALELFRNDMAAKYKELGFQG

SEQ ID NO: 20 *Equus caballus*

MGLSDGEWQQVLNVWGKVEADIAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKAS
EDLKKHGTVVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHS
KH PGDFGADAQGAMTKALELFRNDIAAKYKELGFQG

SEQ ID NO: 21 Nicotiana benthamiana
MSSFTEEQEALVVKSWDSMKKNAGEWGLKLFLKIFEIAPSAKKLFSFLKDSNVPL
EQNAKLKPHSKSVFVMTCEAAVQLRKAGKVVVRDSTLKKLGATHFKYGVADE
HFEVTKFALLETIKEAVPEMWSVDMKNAWGEAFDQLVNAIKTEMK

SEQ ID NO: 22 Bacillus subtilis
MGQSFNAPYEAIGEELLSQLVDTFYERVASHPLLKPIFPSDLTETARKQKQFLTQY
LGGPPLYTEEHGHPMLRARHLPFPITNERADAWLSCMKDAMDHVGLEGEIREFL
FGRLELTARHMVNQTEAEDRSS

SEQ ID NO: 23 Corynebacterium glutamicum

MTTSENFYDSVGGEETFSLIVHRFYEQVPNDDILGPMYPPDDFEGAEQRLKMFLS
QYWGGPKDYQEQRGHPRLRMRHVNYPIGVTAAERWLQLMSNALDGVDLTAEQ
REAIWEHMVRAADMLINSNPDPHA

SEQ ID NO: 24 Synechocystis PCC6803
MSTLYEKLGGTTAVDLAVDKFYERVLQDDRIKHFFADVDMAKQRAHQKAFLTY
AFGGTDKYDGRYMREAHKELVENHGLNGEHFDAVAEDLLATLKEMGVPEDLIA
EVAAVAGAPAHKRDVLNQ

SEQ ID NO: 25 Synechococcus sp. PCC 7335
MDVALLEKSFEQISPRAIEFSASFYQNLFHHHPELKPLFAETSQTIQEKKLIFSLAAI
IENLRNPDILQPALKSLGARHAEVGTIKSHYPLVGQALIETFAEYLAADWTEQLA
TAWVEAYDVIASTMIEGADNPAAYLEPELTFYEWLDLYGEESPKVRNAIATLTH
FHYGEDPQDVQRDSRG

SEQ ID NO: 26 Nostoc commune
MSTLYDNIGGQPAIEQVVDELHKRIATDSLLAPVFAGTDMVKQRNHLVAFLAQIF
EGPKQYGGRPMDKTHAGLNLQQPHFDAIAKHLGERMAVRGVSAENTKAALDR
VTNMKGAILNK

SEQ ID NO: 27 Bacillus megaterium
MREKIHSPYELLGGEHTISKLVDAFYTRVGQHPELAPIFPDNLTETARKQKQFLT
QYLGGPSLYTEEHGHPMLRARHLPFEITPSRAKAWLTCMHEAMDEINLEGPER
DELYHRLILTAQHMINSPEQTDEKGFSH

FIGURE 2A

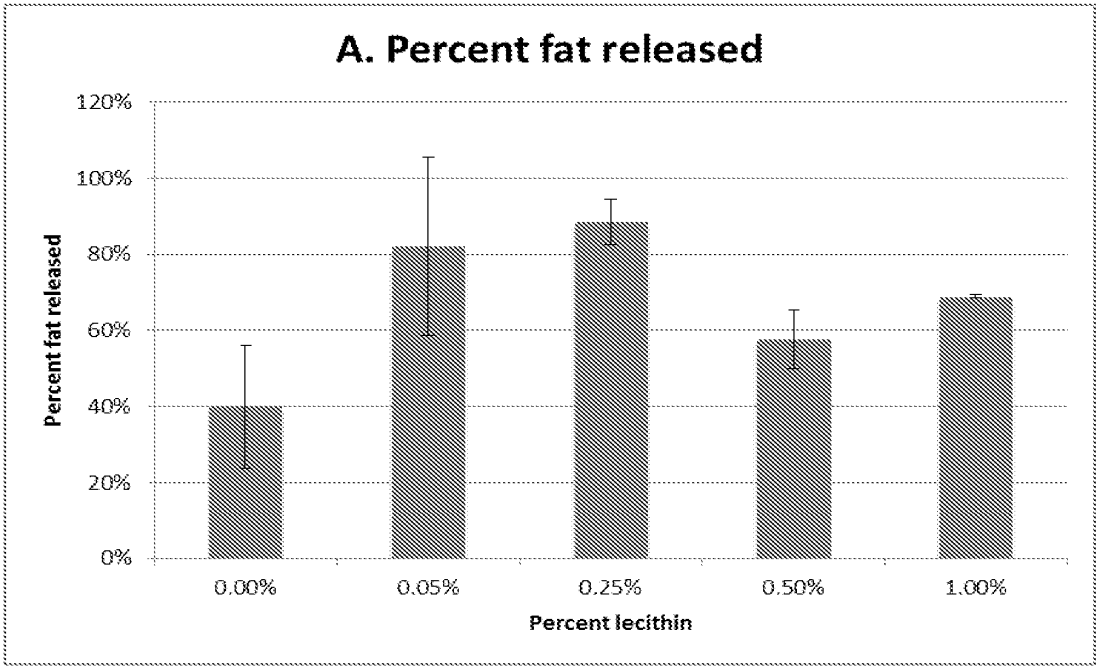


FIGURE 2B

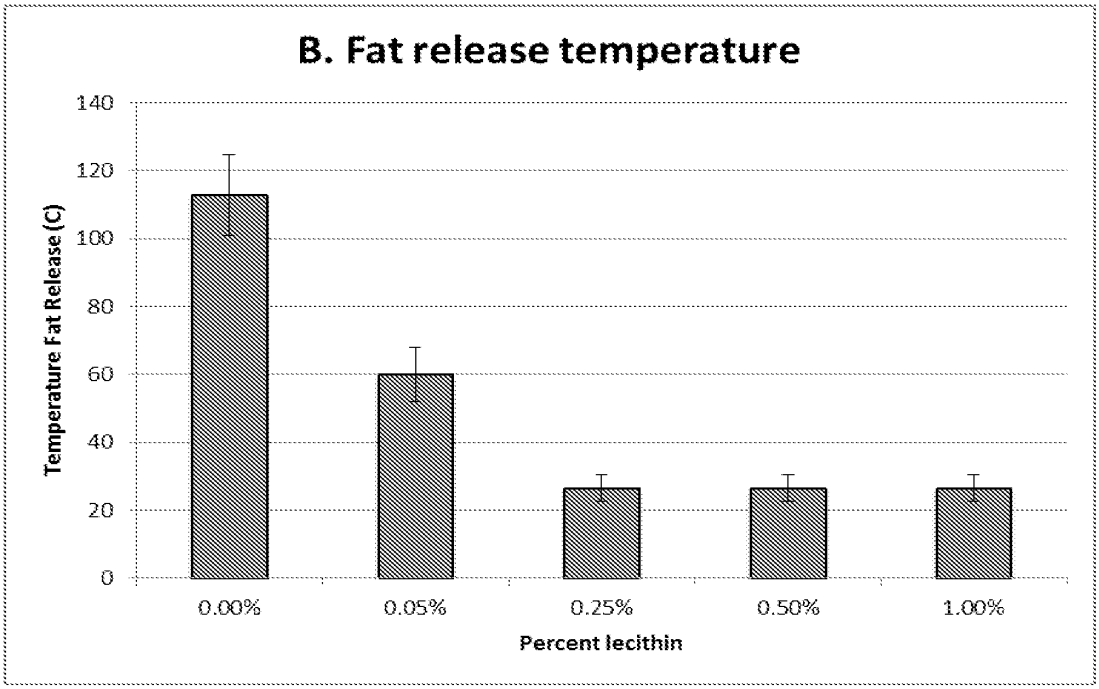


FIGURE 2C

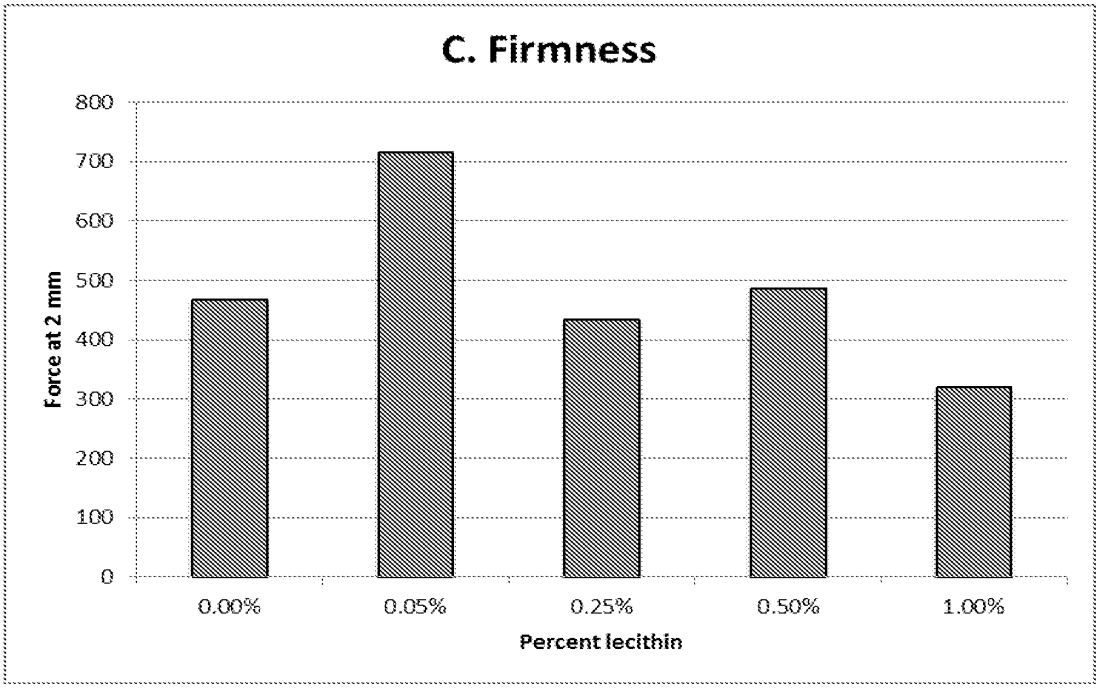

C. Firmness
800
700
600
500
400
300
200
100
0
Force at 2 mm
0.00%
0.05%
0.25%
0.50%
1.00%
Percent lecithin

FIGURE 3

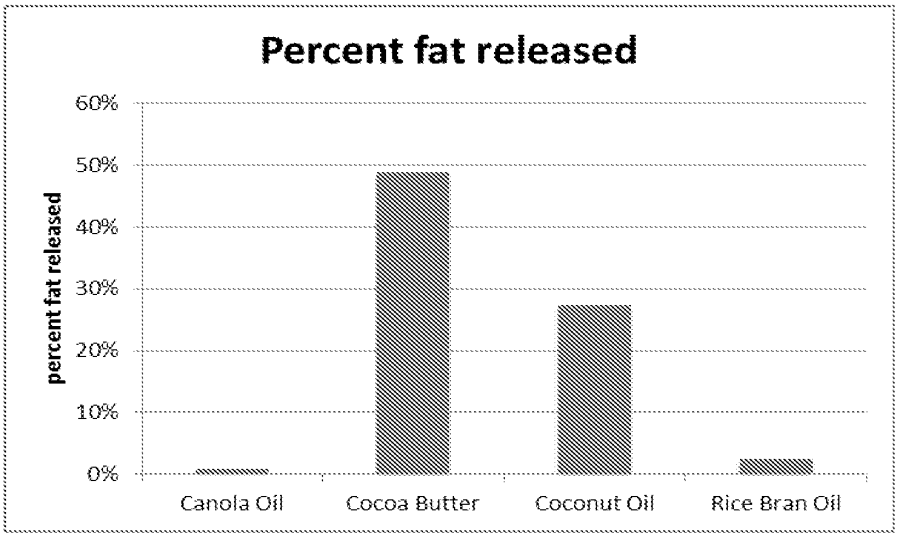
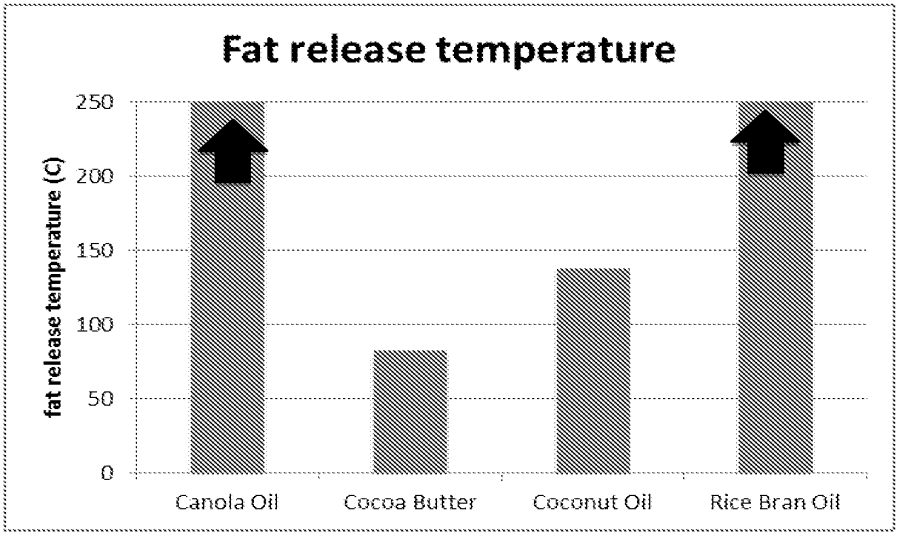

Percent fat released
percent fat released
60%
50%
40%
30%
20%
10%
0%
Canola Oil
Cocoa Butter
Coconut Oil
Rice Bran Oil

FIGURE 4



60902413.doc

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/11361

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A23L 1/212 (2014.01)
USPC - 424/478, 479
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)
IPC(8): A23L 1/212, 1/10, 1/216; A23K 1/100 (2014.01)
USPC: 424/615, 481, 479, 478, 574, 531

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; Google Patents; Science Direct; ProQuest; consumable, plant, 'plant protein,' peptide, coacervate, 'food product,' 'meat substitute,' 'sodium chloride,' soluble, mayo, beverage

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 3,870,801 (TOMBS, MP) March 11, 1975; abstract; column 1, lines 64-65; column 3, lines 12-13; column 6, lines 20-21; column 8, lines 56-57; column 14, lines 3-5; column 19, lines 32-34	1, 2, 8, 9/1, 9/2, 9/8 ----- 3-7, 9/3-9/7, 18, 52, 53, 54/52, 54/53
Y	EP 1361264 B1 (TSUBAKI, K et al.) April 18, 2007; paragraphs [0001], [0029], [0038], [0039], [0044], [0045]	3-7, 9/3-9/7, 19-21, 22/20, 22/21, 52, 53, 54/52, 54/53
X --- Y	US 2013/0004617 A1 (ZHANG, N et al.) January 03, 2013; paragraphs [0004], [0006]	17 ----- 18-21, 22/20, 22/21, 53, 54/53

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
28 May 2014 (28.05.2014)	16 JUN 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/11361

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 10-16, 23-27, 40-46, 50, 55-58, 66, 67, 79, 80, 84-90
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Group I: Claims 1-9, 17-22, 52-54

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/11361

-***-Continued from Box No. III: Lack of Unity of Invention:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-9, 17-22 and 52-54 are directed toward a consumable product comprising an isolated and purified plant protein, wherein the isolated and purified plant protein has (i) a solubility in a solution of at least 25 g/L at a temperature between about 2 degrees C and about 32 degress C, wherein the solution has a pH between 3 and 8, and has a sodium chloride content of 0 to 300 mM or (ii) a solubility in a solution of at least 1 mg/ml at a temperature of between 90 degrees C and 110 degrees C, wherein the solution has a pH between 5 and 8 and has a sodium chloride content of 0 to 300 mM, and a method of making said consumable product.

Group II: Claims 28-31, 37-39, 68-70 and 81-83 are directed toward a meat replica comprising a muscle replica, a connective tissue replica; an adipose tissue replica, and a coacervate comprising one or more isolated and purified proteins, said adipose tissue replica including one or more isolated plant proteins, one or more plant or algal derived oils, optionally a phospholipid, and a three-dimensional matrix comprising fibers derived from non-animal sources, wherein said lipid and said protein are dispersed in said three-dimensional matrix, and wherein said three-dimensional matrix stabilizes the structure of the adipose tissue replica; and wherein the connective tissue replica comprises proteins assembled into fibrous structures by a solution spinning process.

Group III: Claims 32-36 and 61-65 are directed toward a consumable product comprising a cold set gel comprising one or more isolated and purified proteins from a non-animal source and a salt, and a method of making said consumable product.

Group IV: Claims 47-49 are directed toward a consumable product comprising a heme-containing protein and (i) carbon monoxide and/or (ii) a nitrite, wherein said consumable product does not comprise meat.

Group V: Claim 51 is directed toward a consumable product comprising a gelled emulsion, said gelled emulsion comprising: a) an isolated and purified protein; b) a first lipid that when not in said consumable product is solid at a selected temperature range; and c) a second lipid that when not in said consumable product is liquid at said selected temperature range; wherein the melting temperature of the mixture of said first and second lipids is similar to the melting temperature of lipids found in meat, and wherein said first and second lipids are plant derived lipids or microbial derived lipids.

Groups VI+: Claims 59, 60 and SEQ ID NO: 1 are directed toward a method for extending the shelf-life of a meat-free consumable product, said method comprising adding a heme-containing protein to said consumable product, wherein said heme containing protein oxidizes more slowly than myoglobin under equivalent storage conditions. (SEQ ID NO: 1-27). + type Markush group.

The method for extending the shelf-life of a meat-free consumable product comprising adding a heme-containing protein to said consumable product, wherein said heme containing protein oxidizes more slowly than myoglobin under equivalent storage conditions can be searched to the extent that it encompasses a sequence at least 70% identical to SEQ ID NO: 1 (Vigna radiata amino acid sequence). Additional sequences will searched upon the payment of additional fees. It is believed that Claims 59 and 60 (in-part) encompass this first named invention and thus these claims can be searched, upon appropriate payment, to the extent that they encompass SEQ ID NO: 1 (Vigna radiata amino acid sequence). Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) may result in only the first claimed invention to be searched/examined. An exemplary election would be SEQ ID NO: 2 (Methylacidiphilum infernorum amino acid sequence).

Groups VII+: Claims 71-73 and SEQ ID NO: 1 are directed toward a method for imparting a beef like flavor to a consumable product, including a poultry or fish composition, comprising adding to said consumable composition a heme-containing protein, wherein after cooking, a beef-like flavor is imparted to said consumable.

Group VIII: Claims 74-78 are directed toward a method of making a coacervate, said method comprising: a) acidifying a solution of one or more plant proteins to a pH between 3.5 and 5.5, wherein said solution comprises 100 mM or less of sodium chloride; and b) isolating said coacervate from said solution.

Group IX: Claims 91-93 are directed toward methods of minimizing undesirable odors in a composition comprising plant proteins.

Group X: Claim 94 is directed toward a chocolate flavored spread comprising: a) sugar; b) a chocolate flavoring; and c) a cream fraction from a plant based milk.

Group XI: Claims 95-99 are directed toward a method for altering the texture of a consumable during or after cooking comprising incorporating within the consumable one or more plant proteins with a low denaturation temperature.

Group XII: Claims 100-104 are directed toward tissue replicas comprising a freeze-aligned non-animal protein.

-***-Continued Within the Next Supplemental Box-***-

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/11361

-***-Continued from Previous Supplemental Page:

The inventions listed as Groups I-XII do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Group I include isolated and purified plant protein has (i) a solubility in a solution of at least 25 g/L at a temperature between about 2 degrees C and about 32 degrees C, wherein the solution has a pH between 3 and 8, and has a sodium chloride content of 0 to 300 mM or (ii) a solubility in a solution of at least 1 mg/ml at a temperature of between 90 degrees C and 110 degrees C, wherein the solution has a pH between 5 and 8 and has a sodium chloride content of 0 to 300 mM, not present in any other groups; the special technical features of Group II including a meat replica comprising a muscle replica, a connective tissue replica; an adipose tissue replica, or a coacervate, which are not present in any other group; the special technical features of Group III including a cold set gel comprising one or more isolated and purified proteins from a non-animal source and a salt, which are not present in any other Group; the special technical features of Group IV including a consumable product comprising a heme-containing protein and (i) carbon monoxide and/or (ii) a nitrite, not present in any other group; the special technical features of Group V including a consumable product comprising a gelled emulsion, said gelled emulsion comprising: a) an isolated and purified protein; b) a first lipid that when not in said consumable product is solid at a selected temperature range; and c) a second lipid that when not in said consumable product is liquid at said selected temperature range; wherein the melting temperature of the mixture of said first and second lipids is similar to the melting temperature of lipids found in meat, which are not not present in any other group; the special technical features of Groups VI+ including a method for extending the shelf-life of a meat-free consumable product, which are not present in any other group; the special technical features of Groups VII+ including a method for imparting a beef like flavor to a consumable product, which are not present in any other group; the special technical features of Group VIII including a method of making a coacervate comprising: a) acidifying a solution of one or more plant proteins to a pH between 3.5 and 5.5, which are not present in any other group; the special technical features of Group IX including methods of minimizing undesirable odors in a composition comprising plant proteins, which are present in any other group; the special technical features of Group X including a chocolate flavored spread, which are not present in any other Group; the special technical features of Group XI including a method for altering the texture of a consumable, which not present in any other group; the special technical features of Group XII including a freeze-aligned non-animal protein.

Groups I-XII share the technical features including consumables. Groups I, II, V, VIII, IX, X and XI share the technical features including plants. Groups I, II, VIII, IX and XI share the technical features including plant proteins. Groups I-V, VI+, VII+, VIII, IX, XI and XII share the technical features including proteins. Groups I, III, V, VII+, XI and XII share the technical features including temperature. Groups I and VIII share the technical features including pH. Groups I, III and VIII share the technical features including sodium chloride. Groups I-III, VI+, VII+, VIII and XI share the technical features including a method of making a consumable product. Groups II, III and XII share the technical features including a meat replica. Groups II and VIII share the technical features including a coacervate. Groups II and V share the technical features including plant or algal derived oils or lipids. Groups II and V share the technical features comprising an emulsion. Groups III and V share the technical features including a gel. Groups IV, VI+ and VII+ share the technical features including a heme-containing protein. Groups IV and VI+ share the technical features including meat-free products. Groups VI+ and VII+ share the technical features including a heme-containing protein comprising a sequence at least 70% identical to any of SEQ ID NOs: 1-27.

Groups VI+ share the technical features including a method for extending the shelf-life of a meat-free consumable product, said method comprising adding a heme-containing protein to said consumable product, wherein said heme containing protein oxidizes more slowly than myoglobin under equivalent storage conditions.

Groups VII+ share the technical features including a method for imparting a beef like flavor to a consumable product, including a poultry or fish composition, comprising adding to said consumable composition a heme-containing protein, wherein after cooking, a beef-like flavor is imparted to said consumable.

However, these shared technical features are previously disclosed by WO 2001/015714 A1 (ZLOTKIN) in view of US 2010/0074998 A1 to Espeleta-Vega et al. (hereinafter 'Espeleta-Vega'), US 2011/0083681 A1 to Sengupta, et al. (hereinafter 'Sengupta'), the publication entitled 'Comparative Analysis Of Autoxidation Of Haemoglobin' by Jensen (hereinafter 'Jensen'), US 4,986,998 A to Yoo, et al. (hereinafter 'Yoo') and GenBank Accession AFK42304 by Krishnakumar, et al. (hereinafter 'Krishnakumar').

Zlotkin discloses consumables (compositions including micronutrients (consumables); abstract) including plants (chicory fructooligosaccharide (plants); page 11, lines 15-19); proteins (proteins; page 15, lines 20-28); temperature (temperature; page 17, lines 10-25); a method of making a consumable product (a process for producing a composition comprising micronutrients (a method of making a consumable product); page 8, lines 22-27); plant or algal derived oils or lipids (soybean oil (plant or algal derived oils or lipids); page 15, lines 1-4); meat-free products (cereals; purees of fruit, vegetables, milk, yogurt, cottage cheese (meat-free products); page 18, lines 1-11), and a method for preventing or reducing the discoloration and taste alteration of a food due to the addition of an iron compound thereto (a method for preventing or reducing the discoloration and taste alteration of a food due to the addition of an iron compound thereto; page 14, lines 21-28).

Zlotkin does not disclose plant proteins; pH, sodium chloride; a meat replica; a coacervate; an emulsion; a gel; a heme-containing protein; a heme-containing protein comprising a sequence at least 70% identical to any of SEQ ID NOs: 1-27; a method for extending the shelf-life of a meat-free consumable product, said method comprising adding a heme-containing protein to said consumable product, wherein said heme containing protein oxidizes more slowly than myoglobin under equivalent storage conditions; and a method for imparting a beef like flavor to a consumable product, including a poultry or fish composition, comprising adding to said consumable composition a heme-containing protein, wherein after cooking, a beef-like flavor is imparted to said consumable.

-***-Continued Within the Next Supplemental Box-***-

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/11361

-***-Continued from Previous Supplemental Page:

Espeleta-Vega discloses plant proteins (vegetable protein (plant proteins); abstract), pH (a pH modifier; paragraph [0010]), sodium chloride (salt (sodium chloride); paragraph [0038]), a meat replica (substitute meat food product (a meat replica); abstract); an emulsion (an emulsion; paragraph [0011]); a gel (a gel; paragraph [0011]).

Sengupta discloses a process for preparing coacervate gel particles comprising plant materials including proteins (a process for preparing coacervate gel particles comprising plant materials including proteins; paragraph [0015]).

Jensen discloses a heme-containing protein (hemoglobin; abstract) wherein the oxidation of hemoglobin and myoglobin is significantly slower than free heme (oxidation of hemoglobin and myoglobin is significantly slower than free heme; page 2029, column 1, paragraph 2), and wherein the oxidation of tetrameric hemoglobin is significantly slower than either monomeric hemoglobin or myoglobin (wherein the oxidation of tetrameric hemoglobin is significantly slower than either monomeric hemoglobin or myoglobin; page 2030, column 1, paragraph 1).

Yoo discloses a method for imparting (flavoring (imparting); abstract) a beef (cow (beef); column 4, lines 26-32) like flavor to a consumable product (flavor to a consumable product; abstract, column 2, lines 49-50), comprising adding to said consumable composition a heme-containing protein (comprising adding to said consumable a powdered blood product (comprising adding to said consumable composition a heme-containing protein); abstract), wherein after cooking, a beef-like flavor is imparted to said consumable (providing a meat-like flavor (beef-like flavor is imparted to said consumable); column 3, lines 1-6).

Krishnakumar discloses a heme-containing protein comprising a sequence at least 70% identical to SEQ ID NO: 1 (a globin (a heme-containing protein comprising a sequence at least 70% identical to SEQ ID NO: 1); page 1).

It would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the disclosure of Zlotkin by combining the consumable disclosed by Zlotkin with a meat replica, such as that disclosed by Espeleta-Vega, for providing the micronutrients in the consumable in a form acceptable to a consumer, such as in a meat replica, for enhancing the nutrient content of a meat replica, particularly the iron content of said replica, providing the previous disclosure of Zlotkin. Also, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the disclosure of Zlotkin regarding micronutrient-comprising consumables with the coacervate preparation process of Sengupta, for enabling the preparation of particulates comprising the micronutrients disclosed by Zlotkin using a plant polysaccharide, such as pectin, as disclosed by Sengupta, for providing micronutrients in a consumable and easily digestible form to enable a consumer to absorb the nutrients. Furthermore, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the disclosure of Zlotkin, for including the use of hemoglobin in preference to free iron or other forms of iron, in order to provide the iron to the consumer in a biologically useful form and oxidation state. Moreover, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have therefore modified the disclosure of Zlotkin, for providing a method for extending the shelf-life of a meat-free consumable product, such as the consumables disclosed by Zlotkin, said method comprising adding a heme-containing protein to said consumable product, wherein said heme containing protein oxidizes more slowly than myoglobin under equivalent storage conditions, such as adding hemoglobin, based on the disclosure of Jensen that tetrameric hemoglobin oxidizes more slowly than other forms of heme-containing proteins. Also, it would have been obvious to a person of ordinary skill in the art to have modified the disclosure of Zlotkin, for including a meat-like flavoring including an iron-containing protein based on the disclosure of Yoo, in order to provide a beef-like flavor to a consumable food, including a poultry or fish composition, to provide a beef-flavored product not containing beef, but with lower fat, such as a consumable comprising a plant protein, fish, or poultry protein. Finally, it would have been obvious to a person of ordinary skill in the art, at the time of the invention, to have modified the disclosure of Zlotkin regarding micronutrient-comprising consumables comprising iron, for including a plant-based iron-comprising proteins, such as the globin disclosed by Krishnakumar, in order to provide iron from non-animal sources having high absorbability and utilization by a consumer.

Since none of the special technical features of the Groups I-XII inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Zlotkin, Espeleta-Vega, Sengupta, Jensen, Yoo and Krishnakumar references, unity of invention is lacking.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: MONICA MCCORMICK GRAHAM
FISH & RICHARDSON P.C.
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing *(day/month/year)* 20 MAR 2015
Applicant's or agent's file reference 387670009WO1	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 14/55227	International filing date *(day/month/year)* 11 September 2014 (11.09.2014)
Applicant IMPOSSIBLE FOODS INC.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46).

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

   For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
   - ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.
   - ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

   Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

   Within **19 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 Telephone No. PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2014)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 3678709309WO1	**FOR FURTHER ACTION**	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US 14/55227	International filing date *(day/month/year)* 11 September 2014 (11.09.2014)	(Earliest) Priority Date *(day/month/year)* 11 September 2013 (11.09.2013)
Applicant IMPOSSIBLE FOODS INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 7 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (see Box No. II).

3. ☒ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. _____

☐ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☒ none of the figures is to be published with the abstract.

Form PCT/ISA/210 (first sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/55227

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)
   - ☐ on paper
   - ☒ in electronic form

   b. (time)
   - ☒ in the international application as filed
   - ☐ together with the international application in electronic form
   - ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore ver 6.4.1 SEQ ID NOs: 1, 4, 33, 35, 55

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/55227

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-9, 13-18, 25, 26, 31, 32, 37-39, 43-47, 54
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-----please go to Extra Sheet for Box NO. III-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-4, 33-36 and 49-53

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/55227

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 21/04; C12P 21/06; C12N 15/00; C12N 1/20 (2015.01)
CPC - C07K 2319/02; C12N 15/75
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)
CPC: C07K 2319/02; C12N 15/75

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C07K 2319/02; C12N 15/75 (text search)
USPC: 435/69.1, 69.7, 320.1, 252.3, 252.31; 536/23.4 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Scholar; Google Patents; GenCore sequence search (AA)
Search terms: heme-containing polypeptide, vector, signal or leader sequence, His-tag or affinity tag, secretion, periplasmic space or cell medium, B. subtilis, globin, cytochrome

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	LONDER et al. Production and preliminary characterization of a recombinant triheme cytochrome c(7) from Geobacter sulfurreducens in Escherichia coli. Biochem Biophys Acta 1 July 2002 Vol 1554 No 3 Pages 202-211. Especially pg 204 fig 1 and fig 2, pg 205 col 1 para 1, pg 206 col 1 para 1, pg 206 col 2 para 3-4.	49-52 ----- 1-4, 33-36, 53
Y	WU et al. Efficient production of a functional single-chain antidigoxin antibody via an engineered Bacillus subtilis expression-secretion system. Bio/Technology (NY) January 1993 Vol 11 No1 Pages 71-76. Especially abstract, pg 76 fig 1B.	1-4
Y	US 2008/0166757 A1 (BRON et al.) 1 June 2008 (01.06.2008). Especially SEQ ID NO: 58	3, 4, 33-36
Y	WO 2012/083424 A1 (LI et al.) 28 June 2012 (28.06.2012). Especially sheet 3 fig 3B.	53

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
02 March 2015 (02.03.2015)	20 MAR 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/55227

Continuation of Box III (Lack of Unity of Invention)

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-4, 33-36 and 49-53, drawn to a recombinant bacterium cell capable of secreting a heme-containing polypeptide, said cell comprising at least one exogenous nucleic acid, said exogenous nucleic acid comprising first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide, wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide. Compositions of Group I may specifically contain a vector encoding said exogenous nucleic acids (e.g. claims 33, 49).

Group II: Claims 10-12 and 55-57, drawn to a method for producing a heme-containing polypeptide, said method comprising culturing a recombinant bacterium cell in a culture medium under conditions that allow said heme-containing polypeptide to be secreted into said culture medium, said recombinant bacterium cell comprising at least one exogenous nucleic acid, said exogenous nucleic acid comprising first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide, wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide, and wherein upon secretion of said fusion polypeptide from said cell into said culture medium, said signal peptide is removed from said heme-containing polypeptide.

Group III: Claims 19-24, drawn to a recombinant plant or plant cell producing a heme-containing polypeptide, said plant comprising at least one exogenous nucleic acid encoding a heme-containing polypeptide, wherein said plant or plant cell is from a species other than Nicotiana.

Group IV: Claims 27-30, drawn to a method of producing a heme-containing polypeptide, said method comprising growing a recombinant plant, said recombinant plant comprising at least one exogenous nucleic acid encoding said heme-containing polypeptide, wherein said
plant is from a species other than Nicotiana, and purifying said heme-containing polypeptide from a tissue of said plant.

Group V: Claims 40-42, 48 and 58-60, drawn to a composition comprising:
(a) a purified heme-containing polypeptide; and
(b) a non-naturally occurring component of a recombinant Bacillus cell or a recombinant plant cell other than a Nicotiana plant cell. In certain Group V compositions (e.g., claim 58), the composition is a purified fusion polypeptide comprising:
(a) a heme-containing polypeptide; and
(b) a tag.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Groups I, III and V have the special technical feature of being compositions, not required by Groups II or IV.

Groups II and IV have the special technical feature of being methods, not required by Groups I, III, V.

Group I has the special technical feature of being a recombinant bacterium cell capable of of secreting a heme-containing polypeptide, not required by Groups III or V.

Group III has the special technical feature of being a recombinant plant or plant cell producing a heme-containing polypeptide, not required by Groups I or V.

Group V has the special technical feature of being a composition comprising an isolated and purified heme-containing polypeptide and isolated and purified a non-naturally occurring component [e.g. signal peptide or tag] of a recombinant Bacillus cell or a recombinant plant cell other than a Nicotiana plant cell, not required by Groups I or III.

Group II has the special technical feature of being a method of producing a heme-containing polypeptide in a bacterium, where the heme-containing polypeptide is part of a fusion protein that is secreted from the bacterium, not required by Group IV.

Group IV has the special technical feature of being a method of producing a heme-containing polypeptide in a recombinant plant, where the heme-containing polypeptide is not secreted and is purified from a plant tissue, not required by Group II.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/55227

----continued from previous sheet----

Common Technical Features:

1. Groups I-V share the common technical feature of a heme-containing polypeptide or a nucleic acid encoding said heme-containing polypeptide.

2. Groups I and II share the common technical feature of a recombinant bacterium for producing a heme-containing polypeptide, where said bacterium comprises first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide, wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide, and wherein the heme-containing polypeptide is secreted.

3. Groups III and IV share the common technical feature of a transgenic plant comprising at least one exogenous nucleic acid encoding a heme-containing polypeptide, wherein said plant or plant cell is from a species other than Nicotiana.

However said common technical features do not represent a contribution over the prior art, and are obvious over the publication titled "Production and preliminary characterization of a recombinant triheme cytochrome c(7) from Geobacter sulfurreducens in Escherichia coli" by LONDER et al. (hereinafter "Londer") [Biochem Biophys Acta 1 July 2002 Vol 1554 No 3 Pages 202-211], in view of the publication titled "Efficient production of a functional single-chain antidigoxin antibody via an engineered Bacillus subtilis expression-secretion system" by WU et al. (hereinafter "Wu") [Bio/Technology (NY) January 1993 Vol 11 No1 Pages 71-76] in further view of US 2006/0053515 A1 to GEIGENBERGER et al. (hereinafter "Geigenberger").

Concerning common technical features #1, Londer teaches a heme-containing polypeptide or a nucleic acid encoding said heme-containing polypeptide (abstract; We cloned the gene for cytochrome c7 and expressed it in Escherichia coli together with the cytochrome c maturation gene cluster, ccmABCDEFGH, on a separate plasmid. We designed two constructs, with and without an N-terminal His-tag. The untagged version provided a good yield (up to 6 mg/l of aerobic culture) of the fully matured protein, with all three hemes attached, while the N-terminal His-tag appeared to be detrimental for proper heme incorporation. The recombinant protein (untagged) is properly folded, it has the same molecular weight and displays the same absorption spectra, both in reduced and in oxidized forms, as the protein isolated from G. sulfurreducens")

Concerning common technical feature #2, Londer teaches a recombinant bacterium for producing a heme-containing polypeptide (abstract), where said bacterium comprises first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide (pg 206 col 2 para 3-4; "Another construct was designed based on vector pASK40, a pUC derivative [38]. This vector features the lac promoter and OmpA leader sequences and has been used in our lab for expression of antibody light chain fragments [...] This construct named pCK32 provided expression of a polypeptide consisting of the OmpA leader sequence and the mature cytochrome sequence. The protein produced in strain HM125 was purified in two major steps: first, the periplasmic fraction was isolated by osmotic shock"; pg 205 fig 2-vector construct indicated). Londer further teaches that the heme-containing polypeptide is secreted into the extracellular periplasmic space (pg 206 col 2 para 4; The protein produced in strain HM125 was purified in two major steps: first, the periplasmic fraction was isolated by osmotic shock. The protein is basic, therefore, for purification it was applied to High S cation exchange resin at pH 8.5 and eluted with a NaCl gradient. The elution profile is shown in Fig. 5. [...] The protein concentration for each fraction determined by the Bradford method, is compared with the heme concentration measured by absorbance of the reduced protein at 552 nm in Table 1"; pg 208 Table 1). Londer does not specifically teach secretion into the culture medium, which, in fact, is known in the art NOT to occur in gram negative host bacteria such as E. coli, as was used by Londer. Wu teaches secretion of a recombinant protein into the culture medium using gram positive host Bacillus subtilis: "We have applied a Bacillus subtilis expression-secretion system to produce a functional antidigoxin SCA (single-chain antibody consisting of VL-linker-VH) and the individual variable domains of light (VL) and heavy (VH) chains. The secreted antidigoxin SCA can be affinity purified in one step by applying the culture supernatant directly to a ouabain-Sepharose column. N-terminal sequence determination indicated that the protein has the expected N-terminus with the signal peptide properly processed. Affinity and ligand specificity studies demonstrated that the engineered antidigoxin SCA has almost identical properties as those of the parental monoclonal antibody. The use of B. subtilis WB600, an engineered, six-extracellular protease-deficient strain, is vital for the production of antidigoxin SCA in high quality and quantity (5 mg/liter in a shake flask culture). All the secreted SCAs are biologically active"). An artisan of ordinary skill in the art would have recognized that it would have been obvious to have tried to use to the Bacillus subtilis recombinant expression system of Wu to express the heme-containing protein taught by Londer in order to enable secretion into the culture medium, with the expectation of a reasonable probability of success and without undue effort.

Concerning common technical feature #3, Geigenberger teaches a transgenic plant comprising at least one exogenous nucleic acid encoding a heme-containing polypeptide (para [0017-0018]; " hemoglobin- or leghemoglobin- and hemoglobin expressing transformed plants are employed in the method according to the invention for modifying the storage reserve content in plants. Accordingly, the present invention also relates to leghemoglobin- and/or hemoglobin-expressing transformed plants and to their use. [0018] In accordance with the invention, hemoglobin is understood as meaning iron(II) complexes of protoporphyrin"; para [0056]-" The invention furthermore relates to a nucleotide sequence as shown in sequence No. 1 coding for leghemoglobin for use in a plant and a corresponding gene structure or vector and their use for transforming a plant with the invention"), wherein said plant or plant cell is from a species other than Nicotiana (para [0054]- Especially preferred are tuber-producing plants, in particular potato plants, or seed-producing plants, in particular Arabidopsis thaliana, oilseed rape or soybean").

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/55227

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As the common technical feature was known in the art at the time of the invention, they cannot be considered as common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I-V lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note: Claims 5-9, 13-18, 25, 26, 31, 32, 37-39, 43-47, 54 are multiple dependent claims and are held unsearchable because they are not drafted according to the second and third sentences of PCT Rule 6.4(a).

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: MONICA MCCORMICK GRAHAM
FISH & RICHARDSON P.C.
P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)*	20 MAR 2015
Applicant's or agent's file reference: 387670009WO1	FOR FURTHER ACTION: See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 14/55227	11 September 2014 (11.09.2014)	11 September 2013 (11.09.2013)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12P 21/04; C12P 21/06; C12N 15/00; C12N 1/20 (2015.01)
CPC - C07K 2319/02; C12N 15/75

Applicant: IMPOSSIBLE FOODS INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1 *bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer:
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	02 March 2015 (02.03.2015)	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 14/55227

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:
   - ☒ the international application in the language in which it was filed.
   - ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a))

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing filed or furnished:
   a. (means)
      - ☐ on paper
      - ☒ in electronic form
   b. (time)
      - ☒ in the international application as filed
      - ☐ together with the international application in electronic form
      - ☐ subsequently to this Authority for the purposes of search

4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

5. Additional comments:
GenCore ver 6.4.1 SEQ ID NOs: 1, 4, 33, 35, 55

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 14/55227

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 5-9, 13-18, 25, 26, 31, 32, 37-39, 43-47, 54

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. ---see below--- are so unclear that no meaningful opinion could be formed *(specify)*:

Claims 5-9, 13-18, 25, 26, 31, 32, 37-39, 43-47, 54 are multiple dependent claims and are held unsearchable because they are not drafted according to the second and third sentences of PCT Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 5-9, 13-18, 25, 26, 31, 32, 37-39, 43-47, 54

☐ a meaningful opinion could not be formed without the sequence listing: the applicant did not, within the prescribed time limit

- ☐ furnish a sequence listing on paper complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ furnish a sequence listing in electronic form complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in a form and manner acceptable to it.
- ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

Form PCT/ISA/237 (Box No. III) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 14/55227

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:
   - ☐ paid additional fees.
   - ☐ paid additional fees under protest and, where applicable, the protest fee.
   - ☐ paid additional fees under protest but the applicable protest fee was not paid.
   - ☒ not paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is
   - ☐ complied with.
   - ☒ not complied with for the following reasons:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-4, 33-36 and 49-53, drawn to a recombinant bacterium cell capable of secreting a heme-containing polypeptide, said cell comprising at least one exogenous nucleic acid, said exogenous nucleic acid comprising first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide, wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide. Compositions of Group I may specifically contain a vector encoding said exogenous nucleic acids (e.g. claims 33, 49).

Group II: Claims 10-12 and 55-57, drawn to a method for producing a heme-containing polypeptide, said method comprising culturing a recombinant bacterium cell in a culture medium under conditions that allow said heme-containing polypeptide to be secreted into said culture medium, said recombinant bacterium cell comprising at least one exogenous nucleic acid, said exogenous nucleic acid comprising first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide, wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide, and wherein upon secretion of said fusion polypeptide from said cell into said culture medium, said signal peptide is removed from said heme-containing polypeptide.

Group III: Claims 19-24, drawn to a recombinant plant or plant cell producing a heme-containing polypeptide, said plant comprising at least one exogenous nucleic acid encoding a heme-containing polypeptide, wherein said plant or plant cell is from a species other than Nicotiana.

Group IV: Claims 27-30, drawn to a method of producing a heme-containing polypeptide, said method comprising growing a recombinant plant, said recombinant plant comprising at least one exogenous nucleic acid encoding said heme-containing polypeptide, wherein said plant is from a species other than Nicotiana, and purifying said heme-containing polypeptide from a tissue of said plant.

Group V: Claims 40-42, 48 and 58-60, drawn to a composition comprising:
(a) a purified heme-containing polypeptide; and
(b) a non-naturally occurring component of a recombinant Bacillus cell or a recombinant plant cell other than a Nicotiana plant cell. In certain Group V compositions (e.g., claim 56), the composition is a purified fusion polypeptide comprising:
(a) a heme-containing polypeptide; and
(b) a tag.

The inventions listed as Groups I-V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

-----continued in Supplemental Box-----

4. Consequently, this opinion has been established in respect of the following parts of the international application:
   - ☐ all parts.
   - ☒ the parts relating to claims Nos. 1-4, 33-36, 49-53

Form PCT/ISA/237 (Box No. IV) (July 2011)

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 14/55227

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-4, 33-36, 53	YES
	Claims	49-52	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	1-4, 33-36, 49-53	NO
Industrial applicability (IA)	Claims	1-4, 33-36, 49-53	YES
	Claims	NONE	NO

2. Citations and explanations:

Claims 49-52 lack novelty under PCT Article 33(2) as being anticipated by the publication titled "Production and preliminary characterization of a recombinant triheme cytochrome c(7) from Geobacter sulfurreducens in Escherichia coli" by LONDER et al. (hereinafter "Londer").

As to claim 49, Londer teaches a vector (pg 204 fig 1; vector pCH33) comprising a polynucleotide sequence encoding a heme-containing polypeptide (pg 204 fig 1; nucleic acid encoding the gene for mature triheme cytochrome c7 in vector pCH33) a signal peptide (pg 204 fig 1; pelA signal peptide in vector pCH33) and a tag (pg 204 fig 1 ; 6-His tag), wherein expression of the polynucleotide sequence in a host cell produces a fusion protein containing said heme-containing polypeptide, said signal peptide, and said tag (pg 205 col 1 para 1: " The cells were harvested, resuspended in Extraction/Wash buffer (Clontech; 50 mM sodium phosphate, pH 7.0, 300 mM NaCl) with 50 mM imidazole and then disrupted by sonication. The supernatant after centrifugation (20-25 min, 25000 x g, 4C) was loaded onto TALON resin (Clontech) [i.e. column to purify via his tag]. Electrophoretically pure protein was eluted with the same buffer containing 100 mM imidazole")

As to claim 50, Londer further teaches that said tag is an affinity tag (pg 204 fig 1; His tag; pg 206 col 1 para 1:" The His-affinity tag was intended to facilitate the purification procedure)

As to claim 51, Londer further tag comprises a 6-histidine tag (pg 204 fig 1; pg 206 col 1 para 1).

As to claim 52, Londer further teaches that the tag is detectable (pg 205 col 1 para 1).

Claims 1-2 lack an inventive step under PCT Article 33(3) and being obvious over Londer, in view of the publication titled "Efficient production of a functional single-chain antidigoxin antibody via an engineered Bacillus subtilis expression-secretion system" by WU et al. (hereinafter "Wu")

As to claim 1, Londer teaches a recombinant bacterium cell capable of [secreting into the periplasmic space] a heme-containing polypeptide (abstract; "We cloned the gene for cytochrome c7 and expressed it in Escherichia coli together with the cytochrome c maturation gene cluster, ccmABCDEFGH, on a separate plasmid. We designed two constructs, with and without an N-terminal His-tag. The untagged version provided a good yield (up to 6 mg/l of aerobic culture) of the fully matured protein, with all three hemes attached [...] The recombinant protein (untagged) is properly folded, it has the same molecular weight and displays the same absorption spectra, both in reduced and in oxidized forms, as the protein isolated from G. sulfurreducens"), said cell comprising at least one exogenous nucleic acid, said exogenous nucleic acid comprising first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide, wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide (pg 206 col 2 para 3-4; "Another construct was designed based on vector pASK40, a pUC derivative [38]. This vector features the lac promoter and OmpA leader sequences and has been used in our lab for expression of antibody light chain fragments [...] This construct named pCK32 provided expression of a polypeptide consisting of the OmpA leader sequence and the mature cytochrome sequence. The protein produced in strain HM125 was purified in two major steps: first, the periplasmic fraction was isolated by osmotic shock"; pg 205 fig 2-vector construct indicated). Londer further teaches that the heme-containing polypeptide is secreted into the extracellular periplasmic space (pg 206 col 2 para 4, The protein produced in strain HM125 was purified in two major steps: first, the periplasmic fraction was isolated by osmotic shock. The protein is basic, therefore, for purification it was applied to High S cation exchange resin at pH 6.5 and eluted with a NaCl gradient. The elution profile is shown in Fig. 5. [...] The protein concentration for each fraction determined by the Bradford method, is compared with the heme concentration measured by absorbance of the reduced protein at 552 nm in Table 1"; pg 206 Table 1). Londer does not specifically teach secretion into the culture medium, which, in fact, is known in the art NOT to occur in gram negative host bacteria such as E. coli, es was used by Londer. [claim 1 continued]

-----continued in Supplemental Box---------

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 14/55227

Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**
Continuation of:
Box IV-3 (Lack of Unity of Invention)

Special Technical Features:

Groups I, III and V have the special technical feature of being compositions, not required by Groups II or IV.

Groups II and IV have the special technical feature of being methods, not required by Groups I, III, V.

Group I has the special technical feature of being a recombinant bacterium cell capable of of secreting a heme-containing polypeptide, not required by Groups III or V.

Group III has the special technical feature of being a recombinant plant or plant cell producing a heme-containing polypeptide, not required by Groups I or V.

Group V has the special technical feature of being a composition comprising an isolated and purified heme-containing polypeptide and isolated and purified a non-naturally occurring component [e.g. signal peptide or tag] of a recombinant Bacillus cell or a recombinant plant cell other than a Nicotiana plant cell, not required by Groups I or III.

Group II has the special technical feature of being a method of producing a heme-containing polypeptide in a bacterium, where the heme-containing polypeptide is part of a fusion protein that is secreted from the bacterium, not required by Group IV.

Group IV has the special technical feature of being a method of producing a heme-containing polypeptide in a recombinant plant, where the heme-containing polypeptide is not secreted and is purified from a plant tissue, not required by Group II.

Common Technical Features:

1. Groups I-V share the common technical feature of a heme-containing polypeptide or a nucleic acid encoding said heme-containing polypeptide.

2. Groups I and II share the common technical feature of a recombinant bacterium for producing a heme-containing polypeptide, where said bacterium comprises first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide, wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide, and wherein the heme-containing polypeptide is secreted.

3. Groups III and IV share the common technical feature of a transgenic plant comprising at least one exogenous nucleic acid encoding a heme-containing polypeptide, wherein said plant or plant cell is from a species other than Nicotiana.

However said common technical features do not represent a contribution over the prior art, and are obvious over the publication titled "Production and preliminary characterization of a recombinant triheme cytochrome c(7) from Geobacter sulfurreducens in Escherichia coli" by LONDER et al. (hereinafter "Londer") [Biochem Biophys Acta 1 July 2002 Vol 1554 No 3 Pages 202-211], in view of the publication titled "Efficient production of a functional single-chain antidigoxin antibody via an engineered Bacillus subtilis expression-secretion system" by WU et al. (hereinafter "Wu") [Bio/Technology (NY) January 1993 Vol 11 No1 Pages 71-76] in further view of US 2006/0053515 A1 to GEIGENBERGER et al. (hereinafter "Geigenberger").

Concerning common technical features #1, Londer teaches a heme-containing polypeptide or a nucleic acid encoding said heme-containing polypeptide (abstract; We cloned the gene for cytochrome c7 and expressed it in Escherichia coli together with the cytochrome c maturation gene cluster, ccmABCDEFGH, on a separate plasmid. We designed two constructs, with and without an N-terminal His-tag. The untagged version provided a good yield (up to 6 mg/l of aerobic culture) of the fully matured protein, with all three hemes attached, while the N-terminal His-tag appeared to be detrimental for proper heme incorporation. The recombinant protein [untagged] is properly folded, it has the same molecular weight and displays the same absorption spectra, both in reduced and in oxidized forms, as the protein isolated from G. sulfurreducens")

-----continued in next Supplemental Box-----

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

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Supplemental Box

In case **the space in any of the preceding boxes is not sufficient.**

Continuation of:
Box IV-3 (Lack of Unity of Invention) and previous Supplemental Box

Concerning common technical feature #2, Londer teaches a recombinant bacterium for producing a heme-containing polypeptide (abstract), where said bacterium comprises first and second nucleic acid sequences, wherein said first nucleic acid sequence encodes a signal peptide and said second nucleic acid sequence encodes a heme-containing polypeptide wherein said first and second nucleic acid sequences are operably linked to produce a fusion polypeptide comprising said signal peptide and said heme-containing polypeptide (pg 206 col 2 para 3-4; "Another construct was designed based on vector pASK40, a pUC derivative [38]. This vector features the lac promoter and OmpA leader sequences and has been used in our lab for expression of antibody light chain fragments [...] This construct named pCK32 provided expression of a polypeptide consisting of the OmpA leader sequence and the mature cytochrome sequence. The protein produced in strain HM125 was purified in two major steps: first, the periplasmic fraction was isolated by osmotic shock"; pg 205 fig 2-vector construct indicated). Londer further teaches that the heme-containing polypeptide is secreted into the extracellular periplasmic space (pg 206 col 2 para 4; The protein produced in strain HM125 was purified in two major steps: first, the periplasmic fraction was isolated by osmotic shock. The protein is basic, therefore, for purification it was applied to High S cation exchange resin at pH 8.5 and eluted with a NaCl gradient. The elution profile is shown in Fig. 5. [...] The protein concentration for each fraction determined by the Bradford method, is compared with the heme concentration measured by absorbance of the reduced protein at 552 nm in Table 1"; pg 208 Table 1). Londer does not specifically teach secretion into the culture medium, which, in fact, is known in the art NOT to occur in gram negative host bacteria such as E. coli, as was used by Londer. Wu teaches secretion of a recombinant protein into the culture medium using gram positive host Bacillus subtilis: "We have applied a Bacillus subtilis expression-secretion system to produce a functional antidigoxin SCA (single-chain antibody consisting of VL-linker-VH) and the individual variable domains of light (VL) and heavy (VH) cheins. The secreted antidigoxin SCA can be affinity purified in one step by applying the culture supernatant directly to a ouabain-Sepharose column. N-terminal sequence determination indicated that the protein has the expected N-terminus with the signal peptide properly processed. Affinity and ligand specificity studies demonstrated that the engineered antidigoxin SCA has almost identical properties as those of the parental monoclonal antibody. The use of B. subtilis WB600, an engineered, six-extracellular protease-deficient strain, is vital for the production of antidigoxin SCA in high quality and quantity (5 mg/liter in a shake flask culture). All the secreted SCAs are biologically active"). An artisan of ordinary skill in the art would have recognized that it would have been obvious to have tried to use to the Bacillus subtilis recombinant expression system of Wu to express the heme-containing protein taught by Londer in order to enable secretion into the culture medium, with the expectation of a reasonable probability of success and without undue effort.

Concerning common technical feature #3, Geigenberger teaches a transgenic plant comprising at least one exogenous nucleic acid encoding a heme-containing polypeptide (para [0017-0018]; " hemoglobin- or leghemoglobin- and hemoglobin expressing transformed plants are employed in the method according to the invention for modifying the storage reserve content in plants. Accordingly, the present invention also relates to leghemoglobin- and/or hemoglobin-expressing transformed plants and to their use. [0018] In accordance with the invention, hemoglobin is understood as meaning iron(II) complexes of protoporphyrin"; para [0056]-" The invention furthermore relates to a nucleotide sequence as shown in sequence No. 1 coding for leghemoglobin for use in a plant and a corresponding gene structure or vector and their use for transforming a plant with the invention") wherein said plant or plant cell is from a species other than Nicotiana (para [0054]- Especially preferred are tuber-producing plants, in particular potato plants, or seed-producing plants, in particular Arabidopsis thaliana, oilseed rape or soybean").

As the common technical feature was known in the art at the time of the invention, they cannot be considered as common special technical features that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I-V lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Note: Claims 5-9, 13-18, 25, 26, 31, 32, 37-39, 43-47, 54 are multiple dependent claims and are held unsearchable because they are not drafted according to the second and third sentences of PCT Rule 6.4(a).

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 14/55227

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of
Box V-2 and previous Supplemental Box

[continuation of claim 1]: Wu teaches secretion of a recombinant protein into the culture medium using gram positive host Bacillus subtilis: (abstract; "We have applied a Bacillus subtilis expression-secretion system to produce a functional antidigoxin SCA (single-chain antibody consisting of VL-linker-VH) and the individual variable domains of light (VL) and heavy (VH) chains. The secreted antidigoxin SCA can be affinity purified in one step by applying the culture supernatant directly to a ouabain-Sepharose column. N-terminal sequence determination indicated that the protein has the expected N-terminus with the signal peptide properly processed. Affinity and ligand specificity studies demonstrated that the engineered antidigoxin SCA has almost identical properties as those of the parental monoclonal antibody. The use of B. subtilis WB600, an engineered, six-extracellular protease-deficient strain, is vital for the production of antidigoxin SCA in high quality and quantity (5 mg/liter in a shake flask culture). All the secreted SCAs are biologically active"). An artisan of ordinary skill in the art would have recognized that it would have been obvious to have tried to use to the Bacillus subtilis recombinant expression system of Wu to express the heme-containing protein taught by Londer in order to enable secretion into the culture medium, with the expectation of a reasonable probability of success and without undue effort. Consequently, it would have been obvious for one of ordinary skill to combine all claim elements, as taught by Londer, with bacterium cell capable of secreting a polypeptide, as taught by Wu, because it would of the have enabled a simple recovery of the heme-containing polypeptide.

As to claim 2, Londer, in view of Wu, as above, teaches that the cell secretes said heme-containing polypeptide from said cell (see Wu abstract), and upon secretion, said signal peptide is removed from said heme-containing polypeptide (Wu abstract; N-terminal sequence determination indicated that the protein has the expected N-terminus with the signal peptide properly processed").

Claims 33-36 lack an inventive step under PCT Article 33(3) as being obvious over Londer, in view of US 2008/0166757 A1 to BRON et al. (hereinafter "Bron").

As to claim 33, Londer teaches a vector (pg 204 fig 2; construct pCK2) comprising:
(a) a polynucleotide sequence encoding a heme-containing polypeptide (pg 204 fig 2; nucleic acid encoding the gene for triheme cytochrome c7) and (b) a polynucleotide sequence encoding a signal peptide (pg 204 fig 2; OmpA signal peptide). Londer does not teach wherein said signal peptide comprises an amino acid sequence having at least 60% amino acid sequence identity to a signal peptide listed in Table 1 [e.g., phoD signal sequence, SEQ ID NO: 55]. However, Bron teaches a signal peptide comprises an amino acid sequence having 96.5% identity to a signal peptide set forth in SEQ ID NO: 55 (SEQ ID NO: 58 54AA 96.5% sequence identity). An artisan of ordinary skill in the art would have known that may signal peptides for use in B. subtilis were well known in the art, including the one provided by Bron, and could be used "off the shelf". Consequently, it would have been obvious for one of ordinary skill in the art to combine a vector comprising a heme containing polypeptide and a signal peptide, as taught by Londer, where the signal peptide sequence comprises an amino acid sequence having at least 60% identity to a signal peptide set forth in SEQ ID NO: 55, as taught by Bron, because it would have enabled secretion into the culture medium from a bacterial host.

As to claim 34, Bron further teaches that signal peptide comprises an amino acid sequence having at least 60% amino acid sequence identity to residues 1-52 of SEQ ID NO:55 (SEQ ID NO: 58 54AA 100.0% sequence identity).

As to claim 35, Bron further teaches that the signal peptide comprises the amino acid sequence of residues 1-52 of SEQ ID NO:55 (SEQ ID NO: 58 54AA 100.0% sequence identity).

As to claim 36, Londer further teaches that the polynucleotide sequence encoding said heme-containing polypeptide is operably linked to a promoter (pg 204 fig 2; lac promoter in vector pCK2).

Claim 53 lacks an inventive step under PCT Article 33(3) as being obvious over Londer, in view of WO 2012/083424 A1 to Li et al. (hereinafter "Li").

As to claim 53, Londer teaches a vector comprising a polynucleotide sequence encoding a His tag and a sequence encoding a heme-containing polypeptide (pg 204 fig 1). Londer does not teach the vector further comprises a) an amino acid linker between said sequence encoding said tag and said sequence encoding said heme-containing polypeptide. However, Li teaches a linker sequence between a His-tag and a protein-of-interest (sheet 3 fig 3B; linker sequence ATKASK between His-tag and protein of interest in expression vector [901]). An artisan of ordinary skill in the art would have recognized that linker sequences were sometimes included in fusion constructs to prevent interaction between two fusion components, or to impart a third functional attribute, and could have been easily incorporated into the vector of Londer. Consequently, it would have been obvious for one of ordinary skill in the art to combine a vector comprising a polynucleotide sequence encoding a His tag and a sequence encoding a heme-containing polypeptide, as taught by Londer, with an amino acid linker between said sequence encoding said tag and said sequence encoding said heme-containing polypeptide, as taught by Li. In view of the perspective of an artisan of ordinary skill, because it would have enabled preventing interaction between two fusion components or imparting a third functional attribute.

-----continued in next Supplemental Box-----

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of
Box V-2 and previous Supplemental Box

Claims 3 and 4 lack an inventive step under PCT Article 33(3) as being obvious over Londer, in view of Wu, in further view of Bron.

As to claim 3 Wu teaches a sequence for a signal peptide (pg 72 Fig 1B; MNIKKFAKQ...etc.). Neither Londer nor Wu specifically teaches signal peptide comprises an amino acid sequence having at least 60% identity to a signal peptide set forth in SEQ ID NO: 55. However, Bron teaches a signal peptide comprises an amino acid sequence having 96.5% identity to a signal peptide set forth in SEQ ID NO: 55 (SEQ ID NO: 58 54AA 96.5% sequence identity). An artisan of ordinary skill in the art would have known that may signal peptides for use in B. subtilis were well known in the art, including the one provided by Bron, and could be used "off the shelf". Consequently, it would have been obvious for one of ordinary skill in the art to combine a signal peptide, as taught by Wu, where the signal peptide sequence comprises an amino acid sequence having at least 60% identity to a signal peptide set forth in SEQ ID NO: 55, as taught by Bron, because it would have enabled secretion into the culture medium from a bacterial host.

As to claim 4 Wu teaches a sequence for a signal peptide (pg 72 Fig 1B; MNIKKFAKQ...etc.). Neither Londer nor Wu specifically teaches signal peptide comprises an amino acid sequence having at least 60% identity to a signal peptide set forth in SEQ ID NO: 55 or to residues 1-52 of SEQ ID NO:55. However, Bron teaches a signal peptide comprises an amino acid sequence having 100% identity to a signal peptide set forth to amino acids 1-52 of SEQ ID NO: 55 (SEQ ID NO: 58 54AA 100.0% sequence identity). An artisan of ordinary skill in the art would have known that may signal peptides for use in B. subtilis were well known in the art, including the one provided by Bron, and could be used "off the shelf". Consequently, it would have been obvious for one of ordinary skill in the art to combine a signal peptide, as taught by Wu, where the signal peptide sequence comprises an amino acid sequence having at least 60% identity to a signal peptide of residues 1-52 of SEQ ID NO: 55, as taught by Bron, because it would have enabled secretion into the culture medium from a bacterial host.

Claims 1-4, 33-36 and 49-53 have industrial applicability as defined by PCT Article 33(4), because the subject matter can be made or used by industry.

SEARCH HISTORY

Application Number	PCT/US 14/55227
Search Conducted By	DAM
Search Approved By	DAM

USPC/IPC/CPC Classifications Searched	IPC(8): C12P 21/04; C12P 21/06; C12N 15/00; C12N 1/20 (2015.01) CPC: C07K 2319/02; C12N 15/75 USPC: 435/69.1, 69.7, 320.1, 252.3, 252.31
Date Conducted	02 March 2015

Documentation Searched	CPC: C07K 2319/02; C12N 15/75 (text search) USPC: 435/69.1, 69.7, 320.1, 252.3, 252.31; 536/23.4 (text search)
Search Terms Used	heme-containing polypeptide, vector, signal or leader sequence, His-tag or affinity tag, secretion, periplasmic space or cell medium, B. subtilis, globin, cytochrome,
Date Conducted	02 March 2015

Electronic Database Searched	PatBase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	02 March 2015
Search Logic:	

Search 1: IC=(C12P21/04 or C12P21/06 or C12N15/00 or C12N1/20) (Results 73079)

Search 2: CPC=(C07K2319/02 or C12N15/75) (Results 2519)

Search 3: UC=(435/69.1 or 435/69.7 or 435/320.1 or 435/252.3 or 435/252.31 or 536/23.4) (Results 43750)

Search 4: 1 or 2 or 3 (Results 94847)

Search 5: 4 and FT=((heme protein) or cytochrome or globin) (Results 11661)

Search 6: 5 and FT=(secretion W4 vector) (Results 676)

Search 7: 6 and FT=(Bacillus subtilis) (Results 205)

Search 8: 7 and TAC=((heme protein) or cytochrome or globin) (Results 3)

Search 9: 6 and FT=(((heme protein) or cytochrome or globin) W6 periplas*) (Results 0)

Search 10: 6 and FT=((signal peptide) or (leader peptide)) (Results 612)

Search 11: 7 and FT=((signal peptide) or (leader peptide)) (Results 197)

Search 12: 11 and FT=(His-tag) (Results 78)

Search 13: FT=((heme protein) or cytochrome or globin) (Results 44293)

Search 14: 13 and FT=((secretion W4 vector) and bacterial and recombinant) (Results 963)

Search 15: 14 and FT=(Bacillus subtilis) (Results 295)

Search 16: 15 and TAC=((heme protein) or cytochrome or globin) (Results 3)

Search 17: 14 and TAC=((heme protein) or cytochrome or globin) (Results 14)

Search 18: INV=(Fraser Rachel) (Results 1)

Search 19: FT=(heme) and INV=(Davis Simon) (Results 1)

Search 20: FT=(heme) and PA=(Impossible Foods) (Results 2)

Electronic Database Searched	GenCore ver 6.4.1
Files Searched	Published_Applications_NA_Main Published_Applications_NA_New Issued_Patents_AA

[redacted]	PIR_80 UniProt_201407 A_Geneseq_201415
Date Conducted	02 March 2015
Search Logic:	
SEQ ID NOs: 1, 4, 33, 35, 55	

Electronic Database Searched	Google
Files Searched	Google Patents
Date Conducted	02 March 2015
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Electronic Database Searched	Google
Files Searched	Google Scholar
Date Conducted	02 March 2015
Search Logic:	
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Electronic Acknowledgement Receipt

EFS ID:	30096758
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Shannon Glaeser
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	16-AUG-2017
Filing Date:	
Time Stamp:	15:09:44
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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01 FC : 1051 140.00 DA

DocCode - SCORE

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Application Number: 15678342 Document Date: 08/16/2017

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Form Revision Date: September 30, 2013

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Sequence Listing was accepted.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Anjum, Durreshwar
Timestamp: [year=2017; month=8; day=18; hr=9; min=45; sec=10; ms=108; ]
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Validated By CRFValidator v 1.0.5

Application No: 15678342 Version No: 1.0

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Total Errors: 0
No. of SeqIDs Defined: 58
Actual SeqID Count: 58

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SEQUENCE LISTING

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<120> EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

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Arg Lys Phe Ser Arg Arg Asp Leu Leu Leu Arg His Ala Gln Lys Leu
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His Ser Asn Cys Ser Asp Ala Ala Ile Thr Arg Leu Arg Arg Lys Ala
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Thr Arg Arg Ser Ser Asn Ala Ala Gly Ser Ile Ser Gly Ser Thr Pro
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Val Thr Thr Pro Asn Thr Met Gly Thr Pro Glu Asp Gly Glu Lys Arg
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Lys Val Gln Lys Leu Ala Gly Arg Arg Asp Ser Asn Glu Gln Lys Leu
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Gln Leu Gln Gln Gln His Leu Gln Gln Gln Pro Gln Leu Gln Tyr Gln
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Gln Ser Leu Lys Gln His Glu Asn Gln Val Gln Gln Pro Asp Gln Asp
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Pro Leu Ile Ser Pro Arg Met Gln Leu Phe Asn Asp Ser Asn His His
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Val Asn Asn Leu Phe Asp Leu Gly Leu Arg Arg Ala Ser Phe Ser Ala
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Val Ser Gly Asn Asn Tyr Ala His Tyr Val Asn Asn Phe Gln Gln Asp
225                 230                 235                 240

Ala Ser Ser Thr Asn Pro Asn Gln Asp Ser Asn Asn Ala Glu Phe Glu
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Asn Ile Glu Phe Ser Thr Pro Gln Met Met Pro Val Glu Asp Ala Glu
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Thr Trp Met Asn Asn Met Gly Pro Ile Pro Asn Phe Ser Leu Asp Val
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Glu Pro Ile Ile Ser Glu Pro Pro Lys Asp Thr Ala Pro Asn Asp Lys
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Lys Leu Asn Gly Tyr Ser Phe Tyr Glu Ala Pro Ile Lys Pro Leu Glu
                325                 330                 335

Ser Leu Phe Ser Val Arg Asn Thr Lys Arg Asn Lys Tyr Lys Thr Asn
            340                 345                 350

Asp Asp Ser Pro Asp Thr Val Asp Asn Asn Ser Ala Pro Ala Ala Asn
        355                 360                 365

Thr Ile Gln Glu Leu Glu Ser Ser Leu Asn Ala Ser Lys Asn Phe Cys
    370                 375                 380

Leu Pro Thr Gly Tyr Ser Phe Tyr Gly Asn Leu Asp Gln Gln Thr Phe
385                 390                 395                 400

Ser Asn Thr Leu Ser Cys Thr Ser Ser Asn Ala Thr Ile Ser Pro Ile
                405                 410                 415

Leu Leu Asp Asn Ser Ile Asn Asn Asn Ser Thr Ser Asp Val Arg Pro
            420                 425                 430

Glu Phe Arg Thr Gln Ser Val Thr Ser Glu Met Ser Gln Ala Pro Pro
        435                 440                 445

Pro Pro Gln Lys Asn Asn Ser Lys Tyr Ser Thr Glu Val Leu Phe Thr
    450                 455                 460

Ser Asn Met Arg Ser Phe Ile His Tyr Ala Leu Ser Lys Tyr Pro Phe
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Ile Gly Val Pro Thr Pro Thr Leu Pro Glu Asn Glu Arg Leu Asn Glu
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Tyr Ala Asp Ser Phe Thr Asn Arg Phe Leu Asn His Tyr Pro Phe Ile
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Ala Ser Arg Arg Cys Ile His Val Tyr Leu Asp Ser Arg Lys Lys Ile
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Pro Thr Ser Leu Ser Ala Asn Asn Asn Asp Ser Pro Leu Trp Leu Ile
        595                 600                 605

Gln Ser Leu Thr Leu Ser Val Met Tyr Gly Leu Phe Ala Asp Asn Asp
    610                 615                 620

Ile Ser Leu Asn Val Val Ile Arg Gln Val Asn Ala Leu Asn Ser Leu
625                 630                 635                 640

Val Lys Thr Ser Gly Leu Asn Arg Thr Ser Ile Ile Asp Leu Phe Asn
                645                 650                 655

Ile Asn Lys Pro Leu Asp Asn Glu Leu Trp Asn Gln Phe Val Lys Ile
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Glu Ser Thr Val Arg Thr Ile His Thr Ile Phe Gln Ile Ser Ser Asn
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Leu Ser Ala Leu Tyr Asn Ile Ile Pro Ser Leu Lys Ile Asp Asp Leu
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Met Ile Thr Leu Pro Val Pro Thr Thr Leu Trp Gln Ala Asp Ser Phe
705                 710                 715                 720

Val Lys Phe Lys Ser Leu Ser Tyr Gly Asn Gln Ile Pro Phe Gln Tyr
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Thr Arg Val Leu Gln Asn Leu Ile Asp Tyr Asn Gln Pro Leu Ser Asp
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Val Asn Asn Arg Glu Asp Ala Leu Phe Thr Lys Val Val Asn Ser Leu
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His Ser Trp Asp Arg Met Ile Ser Asn Ser Asp Leu Phe Pro Lys Lys
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Ile Tyr Gln Gln Ser Cys Leu Ile Leu Asp Ser Lys Leu Leu Asn Asn
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Phe Leu Ile Val Lys Ser Ser Leu Lys Val Ser Thr Gly Asp Val Ser
        835                 840                 845

Ser Leu Asn Lys Leu Lys Glu Asn Val Trp Leu Lys Asn Trp Asn Gln
    850                 855                 860

Val Cys Ala Ile Tyr Tyr Asn Ser Phe Met Asn Ile Pro Ala Pro Ser
865                 870                 875                 880

Ile Gln Lys Lys Tyr Asn Asp Ile Glu Phe Val Asp Asp Met Ile Asn
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Leu Ser Leu Ile Ile Ile Lys Ile Met Lys Leu Ile Phe Tyr Asn Asn
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Val Lys Asp Asn Tyr Glu Asp Glu Asn Asp Phe Lys Leu Gln Glu Leu
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Asn Leu Thr Phe Asp Asn Phe Asp Glu Lys Ile Ser Leu Asn Leu Thr
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Ile Leu Phe Asp Ile Phe Leu Met Ile Tyr Lys Ile Ile Thr Asn Tyr
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Tyr Leu  Asp Leu Glu Lys Phe  Met Lys Phe Lys Phe  Asn Tyr His
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Asp Phe  Glu Thr Glu Phe Ser  Ser Leu Ser Ile Ser  Asn Ile Leu
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Asp Ser  Asp Arg Leu Phe Met  Leu Asn Glu Leu Ile  Asn Phe Glu
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Val Gly  Leu Lys Phe Leu Lys  Ile Gly Glu Ser Phe  Phe Asp Phe
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Leu Tyr  Glu Asn Asn Tyr Lys  Phe Ile His Phe Lys  Asn Leu Asn
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Ser Asn Gly Val Asp Pro Ser Asn Pro Lys Leu Thr Gly His Ala Glu
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Lys Leu Phe Gly Leu Val Arg Asp Ser Ala Gly Gln Leu Lys Ala Asn
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Gly Thr Val Val Ala Asp Ala Ala Leu Gly Ser Ile His Ala Gln Lys
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Thr Ile Lys Glu Ala Val Gly Asp Lys Trp Ser Asp Glu Leu Ser Ser
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Phe
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United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/678,342	08/16/2017	1636	1740	38767-0048002	13	1

CONFIRMATION NO. 1018

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

FILING RECEIPT



Date Mailed: 08/30/2017

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Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney: The patent practitioners associated with Customer Number 26191

Domestic Priority data as claimed by applicant

This application is a CON of PCT/US2016/031797 05/11/2016
which claims benefit of 62/313,491 03/25/2016
and claims benefit of 62/236,506 10/02/2015
and claims benefit of 62/222,388 09/23/2015
and claims benefit of 62/220,366 09/18/2015
and claims benefit of 62/203,052 08/10/2015
and claims benefit of 62/185,921 06/29/2015
and claims benefit of 62/183,074 06/22/2015
and claims benefit of 62/159,899 05/11/2015

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 08/29/2017
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/678,342**
Projected Publication Date: 12/07/2017
Non-Publication Request: No
Early Publication Request: No
Title

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative,

this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/678,342

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	280
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	600
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	720
TOTAL CLAIMS (37 CFR 1.16(i))	13 minus 20 =	*			OR	x 80 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 420 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1600

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/678,342	08/16/2017	Smita Shankar	38767-0048002

CONFIRMATION NO. 1018

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

INFORMAL NOTICE

OC000000093768648

Date Mailed: 08/30/2017

INFORMATIONAL NOTICE TO APPLICANT

Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.53(f).

The item(s) indicated below are also required and should be submitted with any reply to this notice to avoid further processing delays.

- A properly executed inventor's oath or declaration has not been received for the following inventor(s):
  Smita Shankar
  Martin Andrew Hoyt

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/erimando/

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	Unknown
Serial No.	:	15/678,342	Examiner	:	Unknown
Filed	:	August 16, 2017	Conf. No.	:	1018

Title : EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

MAIL STOP MISSING PARTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SUBMISSION OF MISSING PARTS OF APPLICATION

In order to complete the above-indicated application, Applicant, as a Large Entity, submits herewith a Declaration in compliance with 37 CFR § 1.63.

No fees are believed due. If this is incorrect, please apply any charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Respectfully submitted,

Date: /August 30, 2017/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61555057.doc

Attorney Docket No.: 38767-0048002

PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

As the below named inventor, I hereby declare that:

This declaration is directed to:

[ ] The attached application, or

[X] United States application or PCT international application number 15/678,342

filed on August 16, 2017

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Smita Shankar Date (Optional): 08|23|17

Signature: Smita Shankar

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

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Attorney Docket No.: 38767-0048002

PTO/AIA/01 (06-12)
Approved for use through 01/31/2014. OMB 0651-0032
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DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

As the below named inventor, I hereby declare that:

This declaration is directed to:

[ ] The attached application, or

[X] United States application or PCT international application number 15/678,342

filed on August 16, 2017

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Martin Andrew Hoyt Date (Optional): 6/22/2017

Signature:

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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61549181.doc

Electronic Acknowledgement Receipt

EFS ID:	30230403
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	30-AUG-2017
Filing Date:	16-AUG-2017
Time Stamp:	17:09:07
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	Submission.pdf	72149 4a99e3e93da16906cce391119a19d57f3647e370	no	1

Warnings:

Information:					
2	Oath or Declaration filed	EXECUTED_Declaration.pdf	1102404 f9649c7464ab2554a12102896156043daeff9a8b	no	2

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Information:

Total Files Size (in bytes):	1174553

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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Alexandria, Virginia 22313-1450
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

26191 7590 09/06/2017
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
ART UNIT	PAPER NUMBER
1636	

NOTIFICATION DATE	DELIVERY MODE
09/06/2017	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.:15/678,342

1. THE REQUEST FILED August 16, 2017 IS **GRANTED.**

The above-identified application has met the requirements for prioritized examination
- A. ☒ for an original nonprovisional application (Track I).
- B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

- A. filing a **petition for extension of time** to extend the time period for filing a reply;
- B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;
- C. filing a **request for continued examination**;
- D. filing a notice of appeal;
- E. filing a request for suspension of action;
- F. mailing of a notice of allowance;
- G. mailing of a final Office action;
- H. completion of examination as defined in 37 CFR 41.102; or
- I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to Vanitha Elgart at 571-272-7395.

/Vanitha Elgart/
Vanitha Elgart
[Signature]

Petitions Examiner, Office of Petitions
(Title)

U.S. Patent and Trademark Office
PTO-2298 (Rev. 02-2012)

Office of Petitions: Decision Count Sheet

Mailing Month

Application No. 15678342

15678342

For US serial numbers: enter number only, no slashes or commas. Ex: 10123456
For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: ELGART, VANITHA

Count (1) - Palm Credit 15/678,342

Decision: GRANT

FINANCE WORK NEEDED
☐ Select Check Box for YES

GRANT

Decision Type: 643 - Track One request

643

Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

Initials of Approving Official (if required)

If more than 3 decisions, attach 2nd count sheet & mark this box ☐

Printed on:

Office of Petitions Internal Document - Ver. 5.0

Office of Petitions: Routing Sheet

4700

Application No. 15/678,342

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

- [X] **GRANTED**
- [ ] **DISMISSED**
- [ ] **DENIED**

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Serial No.	:	15/678,342	Examiner	:	Unknown
Filed	:	August 16, 2017	Conf. No.	:	1018
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-1449 Form. Foreign patent documents and non-patent literature are enclosed; U.S. patents and patent application publications will be provided upon request.

This statement is being filed before the receipt of a first Office Action on the merits. Therefore, no fees are believed due. If this is incorrect, please apply any charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /October 31, 2017/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61584819.doc

Sheet 1 of 1

Substitute Disclosure Form (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
		Filing Date August 16, 2017	Group Art Unit 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	4,886,753	12/01/1989	Marcker			
	2.	2007/0031832	02/08/2007	Watt			
	3.	2017/0188612	07/06/2017	Varadan			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No
	4.	WO 2009/009142	01/01/2009	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	5.	Lin-Cereghino et al., "Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in Pichia pastoris," Molecular and Cellular Biology, 26:883-897 (February 2006)
	6.	Sievers et al., "The Primary Structure of Soybean (Glycine max) Leghemoglobin c*," Acta Chemica Scandinavico B, 32:380-386 (1978)

Examiner Signature	Date Considered
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 January 2009 (15.01.2009)

PCT

(10) International Publication Number
WO 2009/009142 A2

(51) International Patent Classification:
A01H 1/00 (2006.01) *C12N 5/04* (2006.01)
C12N 15/82 (2006.01)

(21) International Application Number:
PCT/US2008/008557

(22) International Filing Date: 10 July 2008 (10.07.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/958,909 10 July 2007 (10.07.2007) US

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(54) Title: TRANSGENIC PLANTS WITH ENHANCED AGRONOMIC TRAITS

(57) Abstract: This invention provides recombinant DNA constructs, transgenic plant nuclei and cells with such recombinant DNA construct for expression of proteins that are useful for imparting enhanced agronomic trait(s) to transgenic crop plants. This invention also provides transgenic plants and progeny seed comprising the transgenic plant cells where the plants are selected for having an enhanced trait selected from the group of traits consisting of enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil. Also disclosed are methods for manufacturing transgenic seed and plants with enhanced traits.

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

Published:

— *without international search report and to be republished upon receipt of that report*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

Transgenic Plants With Enhanced Agronomic Traits

Cross Reference To Related Applications

This application claims benefit under 35USC § 119(e) of United States provisional application Serial No. 60/958,909, filed 07/10/2007 herein incorporated by reference in its entirety.

Incorporation of Sequence Listing

Two copies of the sequence listing (Copy 1 and Copy 2) and a computer readable form (CRF) of the sequence listing, all on CD-Rs, each containing the text file named 38-21(54973)A_seqListing.txt, which is 105,877,504 bytes (measured in MS-WINDOWS), were created on July 10, 2008 and are herein incorporated by reference.

Field of The Invention

Disclosed herein are recombinant DNA useful for providing enhanced traits to transgenic plants, seeds, pollen, plant cells and plant nucleui of such transgenic plants, methods of making and using such recombinant DNA, plants, seeds, pollen, plant cells and plant nuclei. Also disclosed are methods of producing hybrid seed comprising such recombinant DNA.

Summary of The Invention

This invention employs recombinant DNA for expression of proteins that are useful for imparting enhanced agronomic traits to the transgenic plants. Recombinant DNA in this invention is provided in a construct comprising a promoter that is functional in plant cells and that is operably linked to a DNA segment that encodes a protein. In some embodiments of the invention, such protein defined by protein domains e.g. a "Pfam domain module" (as defined herein below) from the group of Pfam domain modules identified in Table 11. In other embodiments of the invention, e.g. where a Pfam domain module is not available, such protein is defined a consensus amino acid sequence of an encoded protein that is targeted for production e.g. a protein having amino acid sequence with at least 90% identity to a consensus amino acid sequence in the group of SEQ ID NO: 30526 through SEQ ID NO: 30550. In more specific embodiments of the invention the protein expressed in plant cells is a protein selected from the group of proteins identified in Table 2 and their homologs identified in Table 8.

Other aspects of the invention are specifically directed to plant cell nuclei and transgenic cells comprising the recombinant DNA construct of the invention, transgenic plants comprising a plurality of such plant cells, progeny transgenic seed, embryo and transgenic pollen from such plants. Such transgenic plants are selected from a population of transgenic plants regenerated from plant cells transformed with recombinant DNA construct and expressed the protein by screening transgenic plants in the population for an enhanced trait as compared to control plants that do not have the recombinant DNA construct, where the enhanced trait is selected from group of enhanced traits consisting of enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil.

In yet another aspect of the invention the plant cell nuclei, plant cells, transgenic plants, seeds, and pollen further comprise recombinant DNA expressing a protein that provides tolerance from exposure to an herbicide applied at levels that are lethal to a wild type plant cell. Such tolerance is especially useful not only as an advantageous trait in such plants but is also useful in a selection step in the methods of the invention. In aspects of the invention such herbicide is a glyphosate, dicamba, or glufosinate compound.

Yet other aspects of the invention provide transgenic plants which are homozygous for the recombinant DNA and transgenic seed of the invention from corn, soybean, cotton, canola, alfalfa, wheat or rice plants.

This invention also provides methods for manufacturing non-natural, transgenic seed that can be used to produce a crop of transgenic plants with an enhanced trait resulting from expression of stably-integrated, recombinant DNA construct provided by herein. More specifically the method comprises (a) screening a population of plants for an enhanced trait and a recombinant DNA construct of the invention, where individual plants in the population can exhibit the trait at a level less than, essentially the same as or greater than the level that the trait is exhibited in control plants which do not express the recombinant DNA, (b) selecting from the population one or more plants that exhibit the trait at a level greater than the level that said trait is exhibited in control plants, and (c) collecting seed from a selected plant. The method further comprises (d) verifying that the recombinant DNA construct is stably integrated in said selected plants, and (e) analyzing tissue of a selected plant to determine the production of a protein having the function of a protein selected from SEQ ID NO: 299 through SEQ ID NO: 30468. In one aspect of the invention the plants in the population further comprise DNA expressing a protein that provides tolerance to exposure to a herbicide applied at levels that are lethal to wild type plant cells and the selecting is affected

by treating the population with the herbicide, e.g. a glyphosate, dicamba, or glufosinate compound. In another aspect of the invention the plants are selected by identifying plants with the enhanced trait. The methods are especially useful for manufacturing corn, soybean, cotton, canola, alfalfa, wheat or rice seed.

Another aspect of the invention provides a method of producing hybrid corn seed comprising acquiring hybrid corn seed from a herbicide tolerant corn plant which also has stably-integrated, recombinant DNA construct comprising a promoter that is (a) functional in plant cells and (b) is operably linked to DNA that encodes a protein provided by the invention. The methods further comprise producing corn plants from the hybrid corn seed, wherein a fraction of the plants produced from said hybrid corn seed is homozygous for said recombinant DNA, a fraction of the plants produced from said hybrid corn seed is hemizygous for the recombinant DNA construct, and a fraction of the plants produced from said hybrid corn seed has none of the recombinant DNA construct; selecting corn plants which are homozygous and hemizygous for the recombinant DNA construct by treating with an herbicide; collecting seed from herbicide-treated-surviving corn plants and planting the seed to produce further progeny corn plants; repeating the selecting and collecting steps at least once to produce an inbred corn line; and crossing the inbred corn line with a second corn line to produce hybrid seed.

Brief Description Of The Drawsings

Figure 1 is a consensus amino acid sequence of SEQ ID NO: 301 and its homologs.

Figures 2-4 are plasmid maps.

Detailed Description Of The Invention

In the attached sequence listing:

SEQ ID NO:1-298 are nucleotide sequences of the coding strand of DNA for "genes" used in the recombinant DNA imparting an enhanced trait in plant cells, i.e. each represents a coding sequence for a protein;

SEQ ID NO: 299-596 are amino acid sequences of the cognate protein of the "genes" with nucleotide coding sequences 1-298;

SEQ ID NO: 597-30468 are amino acid sequences of homologous proteins;

SEQ ID NO: 30469-30520 are nucleotide sequences of the elements in base plasmid vectors

SEQ ID NO: 30521 is a nucleotide sequence of a base plasmid vector useful for corn transformation;

SEQ ID NO: 30522 is a nucleotide sequence of a base plasmid vector useful for soybean and canola transformation;

SEQ ID NO: 30523 is a nucleotide sequence of a base plasmid vector useful for cotton transformation;

SEQ ID NO: 30524 is a nucleotide sequence of a Sphas1 promoter from soybean;

SEQ ID NO: 30525 is a nucleotide sequence of a Sphas1 leader from soybean.

SEQ ID NO: 30526-30550 are consensus sequences.

Table 1 lists the protein SEQ ID NOs and their corresponding consensus SEQ ID NOs.

Table 1.

Gene ID	PEP SEQ ID NO	Consensus SEQ ID NO
PHE0006007_18714	301	30526
PHE0006460_15962	453	30527
PHE0006657_16192	334	30528
PHE0006712_16273	415	30529
PHE0006859_16873	376	30530
PHE0006907_16797	594	30531
PHE0006936_16828	348	30532
PHE0006969_16871	428	30533
PHE0007578_17852	446	30534
PHE0007650_18175	358	30535
PHE0008172_18471	397	30536
PHE0008308_18841	392	30537
PHE0008340_19155	410	30538
PHE0008422_18842	412	30539
PHE0008698_24421	364	30540
PHE0010197_21429	569	30541
PHE0010201_21433	576	30542
PHE0010201_21768	574	30543
PHE0010223_21491	545	30544
PHE0010397_21762	538	30545
PHE0010398_21763	539	30546
PHE0010615_22400	510	30547
PHE0010838_22702	575	30548
PHE0011447_23660	586	30549
PHE0012170_24424	572	30550

As used herein a "plant cell" means a plant cell that is transformed with stably-integrated, non-natural, recombinant DNA construct, e.g. by *Agrobacterium*-mediated transformation or by baombardment using microparticles coated with recombinant DNA

construct or other means. A plant cell of this invention can be an originally-transformed plant cell that exists as a microorganism or as a progeny plant cell that is regenerated into differentiated tissue, e.g. into a transgenic plant with stably-integrated, non-natural recombinant DNA, or seed or pollen derived from a progeny transgenic plant.

As used herein a "transgenic plant" means a plant whose genome has been altered by the stable integration of recombinant DNA construct. A 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" means DNA which has been a genetically engineered and constructed outside of a cell including DNA containing naturally occurring DNA or cDNA or synthetic DNA.

As used herein "consensus sequence" means an artificial sequence of amino acids in a conserved region of an alignment of amino acid sequences of homologous proteins, e.g. as determined by a CLUSTALW alignment of amino acid sequence of homolog proteins.

As used herein a "homolog" means a protein in a group of proteins that perform the same biological function, e.g. proteins that belong to the same Pfam protein family and that provide a common enhanced trait in transgenic plants of this invention. Homologs are expressed by homologous genes. Homologous genes include naturally occurring alleles and artificially-created variants. Degeneracy of the genetic code provides the possibility to substitute at least one base of the protein encoding sequence of a gene with a different base without causing the amino acid sequence of the polypeptide produced from the gene to be changed. Hence, a polynucleotide useful in the present invention may have any base sequence that has been changed from SEQ ID NO:1 through SEQ ID NO: 298 substitution in accordance with degeneracy of the genetic code. Homologs are proteins that, when optimally aligned, have at least 60% identity, more preferably about 70% or higher, more preferably at least 80% and even more preferably at least 90% identity over the full length of a protein identified as being associated with imparting an enhanced trait when expressed in plant cells. Homologs include proteins with an amino acid sequence that has at least 90% identity to a consensus amino acid sequence of proteins and homologs disclosed herein.

Homologs are identified by comparison of amino acid sequence, e.g. manually or by use of a computer-based tool using known homology-based search algorithms such as those commonly known and referred to as BLAST, FASTA, and Smith-Waterman. A local sequence alignment program, e.g. BLAST, can be used to search a database of sequences to find similar sequences, and the summary Expectation value (E-value) used to measure the

sequence base similarity. As a protein hit with the best E-value for a particular organism may not necessarily be an ortholog or the only ortholog, a reciprocal query is used in the present invention to filter hit sequences with significant E-values for ortholog identification. The reciprocal query entails search of the significant hits against a database of amino acid sequences from the base organism that are similar to the sequence of the query protein. A hit is a likely ortholog, when the reciprocal query's best hit is the query protein itself or a protein encoded by a duplicated gene after speciation. A further aspect of the invention comprises functional homolog proteins that differ in one or more amino acids from those of disclosed protein as the result of conservative amino acid substitutions, for example substitutions are among: acidic (negatively charged) amino acids such as aspartic acid and glutamic acid; basic (positively charged) amino acids such as arginine, histidine, and lysine; neutral polar amino acids such as glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; neutral nonpolar (hydrophobic) amino acids such as alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; amino acids having aliphatic side chains such as glycine, alanine, valine, leucine, and isoleucine; amino acids having aliphatic-hydroxyl side chains such as serine and threonine; amino acids having amide-containing side chains such as asparagine and glutamine; amino acids having aromatic side chains such as phenylalanine, tyrosine, and tryptophan; amino acids having basic side chains such as lysine, arginine, and histidine; amino acids having sulfur-containing side chains such as cysteine and methionine; naturally conservative amino acids such as valine-leucine, valine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, aspartic acid-glutamic acid, and asparagine-glutamine. A further aspect of the homologs encoded by DNA useful in the transgenic plants of the invention are those proteins that differ from a disclosed protein as the result of deletion or insertion of one or more amino acids in a native sequence.

"Percent identity" describes the extent to which the sequences of DNA or protein segments are invariant throughout a window of alignment of nucleotide or amino acid sequences. An "identity fraction" for a sequence aligned with a reference sequence is the number of identical components which are shared by the sequences, divided by a length of the window of alignment, wherein the length does not include gaps introduced by an alignment algorithm. "Percent identity" ("% identity") is the identity fraction times 100. The alignment algorithm is preferably a local alignment algorithm, such as BLASTp. As used herein, sequences are "aligned" when the alignment produced by BLASTp has a minimal e-value.

"Pfam" database is a large collection of multiple sequence alignments and hidden

Markov models covering many common protein families, e.g. Pfam version 19.0 (December 2005) contains alignments and models for 8183 protein families and is based on the Swissprot 47.0 and SP-TrEMBL 30.0 protein sequence databases. See S.R. Eddy, "Profile Hidden Markov Models", *Bioinformatics* 14:755-763, 1998. The Pfam database is currently maintained and updated by the Pfam Consortium. The alignments represent some evolutionary conserved structure that has implications for the protein's function. Profile hidden Markov models (profile HMMs) built from the protein family alignments are useful for automatically recognizing that a new protein belongs to an existing protein family even if the homology by alignment appears to be low.

Protein domains are identified by querying the amino acid sequence of a protein against Hidden Markov Models which characterize protein family domains ("Pfam domains") using HMMER software, a current version of which is publicly available from the Pfam Consortium. A protein domain meeting the gathering cutoff for the alignment of a particular Pfam domain is considered to contain the Pfam domain.

A "Pfam domain module" is a representation of Pfam domains in a protein, in order from N terminus to C terminus. In a Pfam domain module individual Pfam domains are separated by double colons "::". The order and copy number of the Pfam domains from N to C terminus are attributes of a Pfam domain module. Although the copy number of repetitive domains is important, varying copy number often enables a similar function. Thus, a Pfam domain module with multiple copies of a domain should define an equivalent Pfam domain module with variance in the number of multiple copies. A Pfam domain module is not specific for distance between adjacent domains, but contemplates natural distances and variations in distance that provide equivalent funtion. The Pfam database contains both narrowly- and broadly-defined domains, leading to identification of overlapping domains on some proteins. A Pfam domain module is characterized by non-overlapping domains. Where there is overlap, the domain having a function that is more closely associated with the function of the protein (based on the E value of the Pfam match) is selected.

Once one DNA is identified as encoding a protein which imparts an enhanced trait when expressed in transgenic plants, other DNA encoding proteins with the same Pfam domain module are identified by querying the amino acid sequence of protein encoded by candidate DNA against the Hidden Markov Models which characterizes the Pfam domains using HMMER software. Candidate proteins meeting the same Pfam domain module are in the protein family and have cognate DNA that is useful in constructing recombinant DNA for the use in the plant cells of this invention. Hidden Markov Model databases for use with

HMMER software in identifying DNA expressing protein with a common Pfam domain module for recombinant DNA in the plant cells of this invention are also publicly available from the Pfam Consortium.

The HMMER software and Pfam databases are version 19.0 and were used to identify known domains in the proteins corresponding to amino acid sequence of SEQ ID NO: 299 through SEQ ID NO: 596. All DNA encoding proteins that have scores higher than the gathering cutoff disclosed in Table 14 by Pfam analysis disclosed herein can be used in recombinant DNA of the plant cells of this invention, e.g. for selecting transgenic plants having enhanced agronomic traits. The relevant Pfams modules for use in this invention, as more specifically disclosed below, are zf-CCCH, PALP, GAF::HisKA::HATPase_c, TPR_2::TPR_1::TPR_2::TPR_1::TPR_4::TPR_2::TPR_1, efhand::efhand, Spermine_synth, ELFV_dehydrog_N::ELFV_dehydrog, PFK, PAS_3::PAS_3::Pkinase, S1, GDC-P, SWIM, B12-binding::Radical_SAM, S1, LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1, Chloroa_b-bind, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase, WD40::WD40, YABBY, Ldh_1_N::Ldh_1_C, Sina, Na_H_antiport_1, ParBc, YABBY, Histone, Fe_bilin_red, Tryp_alpha_amyl, Pyr_redox_2::Thioredoxin, E2F_TDP, CN_hydrolase, YDG_SRA::Pre-SET::SET, APC8::TPR_1::TPR_1::TPR_1, Ras, tRNA_anti::tRNA-synt_2, Auxin_inducible, PGI, S1, Chloroa_b-bind, Bac_globin, Glyco_hydro_17, MGS, Spermine_synth, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase, Aa_trans, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr, Gln-synt_N::Gln-synt_C, SAP18, TPP_enzyme_N::TPP_enzyme_M::TPP_enzyme_C, zf-C3HC4, eIF-1a, RPE65, PBP, Pkinase, AA_permease, F-box::LRR_1::LRR_2, zf-CCCH::zf-CCCH, Lactamase_B::Flavodoxin_1::Flavin_Reduct, Bac_globin, DSPc, adh_short, Tim17, Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1, ubiquitin::ubiquitin::ubiquitin::ubiquitin, MBD, CXC::CXC, HSF_DNA-bind, Spermine_synth, AP2, Peptidase_S10, PALP, EIN3, Gln-synt_N::Gln-synt_C, 2OG-FeII_Oxy, Glyco_hydro_9, GDC-P, B3, PTPA, Acyltransferase, Isochorismatase, FMO-like, Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD, Lir1, Prismane, Fer2, DEAD::Helicase_C, Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD, KNOX1::KNOX2::ELK, Glyoxalase, Sad1_UNC, Bac_globin, VPS28, PP2C,

Pkinase::efhand::efhand::efhand, LEA_3, Peptidase_S10, Pkinase, CBFD_NFYB_HMF, Gln-synt_N::Gln-synt_C, Pyr_redox_2::Fer2_BFD::NIR_SIR_ferr::NIR_SIR, NUDIX::NUDIX, FAD_binding_3, GST_N::GST_C, SAM_decarbox, Acyltransferase, NTP_transferase, G-patch, 2OG-FeII_Oxy, Gln-synt_N::Gln-synt_C, AAA::Vps4_C, Histone, Pkinase, TPR_1, F-box::Kelch_1::Kelch_1, Spermine_synth, Bac_globin, Bac_globin, zf-UBR, Homeobox::HALZ, Whirly, NAD_binding_1, PTR2, EIN3, 4HBT, adh_short, 2OG-FeII_Oxy, P-II, Myb_DNA-binding::Myb_DNA-binding, DAGK_cat, AP2, MFS_1, Chloroa_b-bind, DUF716, zf-Dof, CCT, Homeobox::HALZ, Histone, 2OG-FeII_Oxy, Globin, Pyr_redox_2::Fer2_BFD::NIR_SIR_ferr::NIR_SIR, Whirly, PsbP, bZIP_1, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr, Phi_1, BURP, Sterol_desat, DSPc, SNF5, Acyltransferase, GATase_2::Asn_synthase, adh_short, Homeobox::START, Pkinase, ParBc, SOUL, DNA_photolyase::FAD_binding_7, Pkinase_Tyr, NAD_Gly3P_dh_N::NAD_Gly3P_dh_C, Na_H_Exchanger, peroxidase, Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1, Hexokinase_1::Hexokinase_2, DUF716, S10_plectin, Thi4, p450, CCT, adh_short, PSI_PSAK, DUF640, Thioredoxin, Globin, Ank::Pkinase, DAGAT, RPE65, Ank::Pkinase, GSHPx, Gln-synt_N::Gln-synt_C, MtN3_slv::MtN3_slv, Allene_ox_cyc, IGPD, MBD, CorA, Response_reg, Histone, AAA, Ribosomal_L10e, Pkinase, DUF26::DUF26::Pkinase, p450, mTERF, AA_kinase, PBP, GUN4, Lactamase_B::Flavodoxin_1::Rubredoxin, C2, RRM_1::RRM_1, Histone, Alpha-amylase, HLH, Thioredoxin, Histone_HNS, Myb_DNA-binding::Myb_DNA-binding, Cytochrom_C552, AP2::AP2, MtN3_slv::MtN3_slv, SHMT, ParBc, Mit_rib_S27, Ribosomal_S2, KNOX1::KNOX2::ELK, MFS_1, Glyco_transf_5::Glycos_transf_1, Cellulase, Ribosomal_L10e, Spermine_synth, Glyco_hydro_2_N::Glyco_hydro_2::Glyco_hydro_2_C, TP_methylase, AP2::AP2, Histone, Response_reg::CCT, Histone_HNS, DUF1716, p450, GATA, Pkinase, Sugar_tr, Aa_trans, Pribosyltran, Ribosomal_L10e, HLH, PMSR, DnaJ::DnaJ_CXXCXGXG::DnaJ_C, DUF1005, Glyco_transf_5::Glycos_transf_1, Spermine_synth, S1::EIF_2_alpha, RGS, Na_sulph_symp, S1, MtN3_slv::MtN3_slv, Lactamase_B::Flavin_Reduct, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase, Chloroa_b-bind, PTR2, Agglutinin, PLATZ, NPH3, Auxin_inducible, PTR2, GAF::HisKA::Response_reg, PsbQ, GSH_synth_ATP, GATase_2::Asn_synthase, PHP, FtsJ,

DUF6::TPT, Proteasome, PsbW_2, Glyco_hydro_9, NAD_binding_2::6PGD, S1::EIF_2_alpha, Homeobox::START::MEKHLA, S1, Isoamylase_N::Alpha-amylase, E2F_TDP, and Rieske::PaO.

As used herein "promoter" means regulatory DNA for initializing transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells whether or not its origin is a plant cell, e.g. is it well known that *Agrobacterium* promoters are functional in plant cells. Thus, plant promoters include promoter DNA obtained from plants, plant viruses and bacteria such as *Agrobacterium* and *Bradyrhizobium* bacteria. 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". Promoters that initiate transcription only in certain tissues are referred to as "tissue specific". A "cell type" specific promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An "inducible" or "repressible" promoter is a promoter which is under environmental control. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions, or certain chemicals, or the presence of light. Tissue specific, tissue preferred, cell type specific, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter which is active under most conditions.

As used herein "operably linked" means the association of two or more DNA fragments in a DNA construct so that the function of one, e.g. protein-encoding DNA, is controlled by the other, e.g. a promoter.

As used herein "expressed" means produced, e.g. a protein is expressed in a plant cell when its cognate DNA is transcribed to mRNA that is translated to the protein.

As used herein a "control plant" means a plant that does not contain the recombinant DNA that expressed a protein that impart an enhanced trait. A control plant is to identify and select a transgenic plant that has an enhance trait. A suitable control plant can be a non-transgenic plant of the parental line used to generate a transgenic plant, i.e. devoid of recombinant DNA. A suitable control plant may in some cases be a progeny of a hemizygous transgenic plant line that is does not contain the recombinant DNA, known as a negative segregant.

As used herein an "enhanced trait" means a characteristic of a transgenic plant that includes, but is not limited to, an enhance agronomic trait characterized by enhanced plant morphology, physiology, growth and development, yield, nutritional enhancement, disease or pest resistance, or environmental or chemical tolerance. In more specific aspects of this

invention enhanced trait is selected from group of enhanced traits consisting of enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil. In an important aspect of the invention the enhanced trait is enhanced yield including increased yield under non-stress conditions and increased yield under environmental stress conditions. Stress conditions may include, for example, drought, shade, fungal disease, viral disease, bacterial disease, insect infestation, nematode infestation, cold temperature exposure, heat exposure, osmotic stress, reduced nitrogen nutrient availability, reduced phosphorus nutrient availability and high plant density. "Yield" can be affected by many properties including without limitation, plant height, pod number, pod position on the plant, number of internodes, incidence of pod shatter, grain size, efficiency of nodulation and nitrogen fixation, efficiency of nutrient assimilation, resistance to biotic and abiotic stress, carbon assimilation, plant architecture, resistance to lodging, percent seed germination, seedling vigor, and juvenile traits. Yield can also be affected by efficiency of germination (including germination in stressed conditions), growth rate (including growth rate in stressed conditions), ear number, seed number per ear, seed size, composition of seed (starch, oil, protein) and characteristics of seed fill.

Increased yield of a transgenic plant of the present invention can be measured in a number of ways, including test weight, seed number per plant, seed weight, seed number per unit area (i.e. seeds, or weight of seeds, per acre), bushels per acre, tonnes per acre, tons per acre, kilo per hectare. For example, maize yield may be measured as production of shelled corn kernels per unit of production area, for example in bushels per acre or metric tons per hectare, often reported on a moisture adjusted basis, for example at 15.5 percent moisture. Increased yield may result from improved utilization of key biochemical compounds, such as nitrogen, phosphorous and carbohydrate, or from improved responses to environmental stresses, such as cold, heat, drought, salt, and attack by pests or pathogens. Recombinant DNA used in this invention can also be used to provide plants having improved growth and development, and ultimately increased yield, as the result of modified expression of plant growth regulators or modification of cell cycle or photosynthesis pathways. Also of interest is the generation of transgenic plants that demonstrate enhanced yield with respect to a seed component that may or may not correspond to an increase in overall plant yield. Such properties include enhancements in seed oil, seed molecules such as tocopherol, protein and starch, or oil particular oil components as may be manifest by an alterations in the ratios of seed components.

A subset of the nucleic molecules of this invention includes fragments of the disclosed recombinant DNA consisting of oligonucleotides of at least 15, preferably at least 16 or 17, more preferably at least 18 or 19, and even more preferably at least 20 or more, consecutive nucleotides. Such oligonucleotides are fragments of the larger molecules having a sequence selected from the group consisting of SEQ ID NO:1 through SEQ ID NO: 298, and find use, for example as probes and primers for detection of the polynucleotides of the present invention.

In some embodiments of the invention a constitutively active mutant, e.g. SEQ ID NO : 203, is constructed to achieve the desired effect. In other embodiments of the invention, a dominant negative gene is constructed to adversely affect the normal, wild-type gene product within the same cell.

DNA constructs are assembled using methods well known to persons of ordinary skill in the art and typically comprise a promoter operably linked to DNA, the expression of which provides the enhanced agronomic trait. Other construct components may include additional regulatory elements, such as 5' leasders and introns for enhancing transcription, 3' untranslated regions (such as polyadenylation signals and sites), DNA for transit or signal peptides.

Numerous promoters that are active in plant cells have been described in the literature. These include promoters present in plant genomes as well as promoters from other sources, including nopaline synthase (NOS) promoter and octopine synthase (OCS) promoters carried on tumor-inducing plasmids of *Agrobacterium tumefaciens* and the CaMV35S promoters from the cauliflower mosaic virus as disclosed in US Patents No. 5,164,316 and 5,322,938. Useful promoters derived from plant genes are found in U.S. Patent 5,641,876, which discloses a rice actin promoter, U.S. Patent No. 7,151,204, which discloses a maize chloroplast aldolase promoter and a maize aldolase (FDA) promoter, and U.S. Patent Application Publication 2003/0131377 A1, which discloses a maize nicotianamine synthase promoter, all of which are incorporated herein by reference. These and numerous other promoters that function in plant cells are known to those skilled in the art and available for use in recombinant polynucleotides of the present invention to provide for expression of desired genes in transgenic plant cells.

In other aspects of the invention, preferential expression in plant green tissues is desired. Promoters of interest for such uses include those from genes such as Arabidopsis thaliana ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit (Fischhoff *et al.* (1992) *Plant Mol Biol.* 20:81-93), aldolase and pyruvate orthophosphate dikinase (PPDK)

(Taniguchi *et al.* (2000) *Plant Cell Physiol.* 41(1):42-48).

Furthermore, the promoters may be altered to contain multiple "enhancer sequences" to assist in elevating gene expression. Such enhancers are known in the art. By including an enhancer sequence with such constructs, the expression of the selected protein may be enhanced. These enhancers often are found 5' to the start of transcription in a promoter that functions in eukaryotic cells, but can often be inserted upstream (5') or downstream (3') to the coding sequence. In some instances, these 5' enhancing elements are introns. Particularly useful as enhancers are the 5' introns of the rice actin 1 (see US Patent 5,641,876) and rice actin 2 genes, the maize alcohol dehydrogenase gene intron, the maize heat shock protein 70 gene intron (U.S. Patent 5,593,874) and the maize shrunken 1 gene.

In other aspects of the invention, sufficient expression in plant seed tissues is desired to affect improvements in seed composition. Exemplary promoters for use for seed composition modification include promoters from seed genes such as napin (U.S. 5,420,034), maize L3 oleosin (U.S. 6,433,252), zein Z27 (Russell *et al.* (1997) *Transgenic Res.* 6(2):157-166), globulin 1 (Belanger *et al* (1991) *Genetics* 129:863-872), glutelin 1 (Russell (1997) *supra*), and peroxiredoxin antioxidant (Per1) (Stacy *et al.* (1996) *Plant Mol Biol.* 31(6):1205-1216).

Recombinant DNA constructs prepared in accordance with the invention will also generally include a 3' element that typically contains a polyadenylation signal and site. Well-known 3' elements include those from *Agrobacterium tumefaciens* genes such as *nos 3'*, *tml 3'*, *tmr 3'*, *tms 3'*, *ocs 3'*, *tr7 3'*, for example disclosed in U.S. 6,090,627, incorporated herein by reference; 3' elements from plant genes such as wheat (*Triticum aesevitum*) heat shock protein 17 (*Hsp17 3'*), a wheat ubiquitin gene, a wheat fructose-1,6-biphosphatase gene, a rice glutelin gene, a rice lactate dehydrogenase gene and a rice beta-tubulin gene, all of which are disclosed in U.S. published patent application 2002/0192813 A1, incorporated herein by reference; and the pea (*Pisum sativum)* ribulose biphosphate carboxylase gene (*rbs 3'*), and 3' elements from the genes within the host plant.

Constructs and vectors may also include a transit peptide for targeting of a gene to a plant organelle, particularly to a chloroplast, leucoplast or other plastid organelle. For descriptions of the use of chloroplast transit peptides see U.S. Patent 5, 188,642 and U.S. Patent No. 5,728,925, incorporated herein by reference. For description of the transit peptide region of an Arabidopsis EPSPS gene useful in the present invention, see Klee, H.J. *et al* (*MGG* (1987) 210:437-442).

Transgenic plants comprising or derived from plant cells of this invention transformed with recombinant DNA construct can be further enhanced with stacked traits, e.g. a crop plant having an enhanced trait resulting from expression of DNA disclosed herein in combination with herbicide and/or pest resistance traits. For example, genes of the current invention can be stacked with other traits of agronomic interest, such as a trait providing herbicide resistance, or insect resistance, such as using a gene from *Bacillus thuringensis* to provide resistance against lepidopteran, coliopteran, homopteran, hemiopteran, and other insects. Herbicides for which transgenic plant tolerance has been demonstrated and the method of the present invention can be applied include, but are not limited to, glyphosate, dicamba, glufosinate, sulfonylurea, bromoxynil and norflurazon herbicides. Polynucleotide molecules encoding proteins involved in herbicide tolerance are well-known in the art and include, but are not limited to, a polynucleotide molecule encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) disclosed in U.S. Patent 5,094,945; 5,627,061; 5,633,435 and 6,040,497 for imparting glyphosate tolerance; polynucleotide molecules encoding a glyphosate oxidoreductase (GOX) disclosed in U.S. Patent 5,463,175 and a glyphosate-N-acetyl transferase (GAT) disclosed in U.S. Patent Application publication 2003/0083480 A1 also for imparting glyphosate tolerance; dicamba monooxygenase disclosed in U.S. Patent Application publication 2003/0135879 A1 for imparting dicamba tolerance; a polynucleotide molecule encoding bromoxynil nitrilase (*Bxn)* disclosed in U.S. Patent 4,810,648 for imparting bromoxynil tolerance; a polynucleotide molecule encoding phytoene desaturase (*crtI*) described in Misawa et al, (1993) *Plant J.* 4:833-840 and in Misawa et al, (1994) *Plant J.* 6:481-489 for norflurazon tolerance; a polynucleotide molecule encoding acetohydroxyacid synthase (AHAS, *aka* ALS) described in Sathasiivan *et al.* (1990) *Nucl. Acids Res.* 18:2188-2193 for imparting tolerance to sulfonylurea herbicides; polynucleotide molecules known as *bar* genes disclosed in DeBlock, *et al.* (1987) *EMBO J.* 6:2513-2519 for imparting glufosinate and bialaphos tolerance; polynucleotide molecules disclosed in U.S. Patent Application Publication 2003/010609 A1 for imparting N-amino methyl phosphonic acid tolerance; polynucleotide molecules disclosed in U.S. Patent 6,107,549 for impartinig pyridine herbicide resistance; molecules and methods for imparting tolerance to multiple herbicides such as glyphosate, atrazine, ALS inhibitors, isoxoflutole and glufosinate herbicides are disclosed in U.S. Patent 6,376,754 and U.S. Patent Application Publication 2002/0112260, all of said U.S. Patents and Patent Application Publications are incorporated herein by reference. Molecules and methods for imparting insect/nematode/virus resistance

are disclosed in U.S. Patents 5,250,515; 5,880,275; 6,506,599; 5,986,175 and U.S. Patent Application Publication 2003/0150017 A1, all of which are incorporated herein by reference.

Plant Cell Transformation Methods

Numerous methods for transforming chromosomes in a plant cell nucleus with recombinant DNA are known in the art and are used in methods of preparing a transgenic plant cell nucleus cell, and plant. Two effective methods for such transformation are *Agrobacterium*-mediated transformation and microprojectile bombardment. Microprojectile bombardment methods are illustrated in U.S. Patents 5,015,580 (soybean); 5,550,318 (corn); 5,538,880 (corn); 5,914,451 (soybean); 6,160,208 (corn); 6,399,861 (corn); 6,153,812 (wheat) and 6,365,807 (rice) and *Agrobacterium*-mediated transformation is described in U.S. Patents 5,159,135 (cotton); 5,824,877 (soybean); 5,463,174 (canola); 5,591,616 (corn); 6,384,301 (soybean), 7,026,528 (wheat) and 6,329,571 (rice), all of which are incorporated herein by reference. Transformation of plant material is practiced in tissue culture on a nutrient media, i.e. a mixture of nutrients that will allow cells to grow *in vitro*. Recipient cell targets include, but are not limited to, meristem cells, hypocotyls, calli, immature embryos and gametic cells such as microspores, pollen, sperm and egg cells. Callus may be initiated from tissue sources including, but not limited to, immature embryos, hypocotyls, seedling apical meristems, microspores and the like. Cells containing a transgenic nucleus are grown into transgenic plants.

In addition to direct transformation of a plant material with a recombinant DNA, a transgenic plant cell nucleus can be prepared by crossing a first plant having cells with a transgenic nucleus with recombinant DNA with a second plant lacking the trangenci nucleus. For example, recombinant DNA can be introduced into a nucleus from a first plant line that is amenable to transformation to transgenic nucleus in cells that are grown into a transgenic plant which can be crossed with a second plant line to introgress the recombinant DNA into the second plant line. A transgenic plant with recombinant DNA providing an enhanced trait, e.g. enhanced yield, can be crossed with transgenic plant line having other recombinant DNA that confers another trait, for example herbicide resistance or pest resistance, to produce progeny plants having recombinant DNA that confers both traits. Typically, in such breeding for combining traits the transgenic plant donating the additional trait is a male line and the transgenic plant carrying the base traits is the female line. The progeny of this cross will segregate such that some of the plants will carry the DNA for both parental traits and some will carry DNA for one parental trait; such plants can be identified by markers associated

with parental recombinant DNA, e.g. marker identification by analysis for recombinant DNA or, in the case where a selectable marker is linked to the recombinant, by application of the selecting agent such as a herbicide for use with a herbicide tolerance marker, or by selection for the enhanced trait. Progeny plants carrying DNA for both parental traits can be crossed back into the female parent line multiple times, for example usually 6 to 8 generations, to produce a progeny plant with substantially the same genotype as one original transgenic parental line but for the recombinant DNA of the other transgenic parental line

In the practice of transformation DNA is typically introduced into only a small percentage of target plant cells in any one transformation experiment. Marker genes are used to provide an efficient system for identification of those cells that are stably transformed by receiving and integrating a recombinant DNA molecule into their genomes. Preferred marker genes provide selective markers which confer resistance to a selective agent, such as an antibiotic or a herbicide. Any of the herbicides to which plants of this invention may be resistant are useful agents for selective markers. Potentially transformed cells are exposed to the selective agent. In the population of surviving cells will be those cells where, generally, the resistance-conferring gene is integrated and expressed at sufficient levels to permit cell survival. Cells may be tested further to confirm stable integration of the exogenous DNA. Commonly used selective marker genes include those conferring resistance to antibiotics such as kanamycin and paromomycin (*nptII*), hygromycin B (*aph IV*), spectinomycin (*aad*A) and gentamycin (*aac3* and *aac*C4) or resistance to herbicides such as glufosinate (*bar* or *pat*), dicamba (DMO) and glyphosate (*aroA* or EPSPS). Examples of such selectable markers are illustrated in U.S. Patents 5,550,318; 5,633,435; 5,780,708 and 6,118,047, all of which are incorporated herein by reference. Selectable markers which provide an ability to visually identify transformants can also be employed, for example, a gene expressing a colored or fluorescent protein such as a luciferase or green fluorescent protein (GFP) or a gene expressing a *beta*-glucuronidase or *uid*A gene (GUS) for which various chromogenic substrates are known.

Plant cells that survive exposure to the selective agent, or plant cells that have been scored positive in a screening assay, may be cultured in regeneration media and allowed to mature into plants. Developing plantlets regenerated from transformed plant cells can be transferred to plant growth mix, and hardened off, for example, in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO_2, and 25-250 microeinsteins $m^{-2} s^{-1}$ of light, prior to transfer to a greenhouse or growth chamber for maturation. Plants are regenerated from about 6 weeks to 10 months after a transformant is identified, depending on

the initial tissue, and plant species. Plants may be pollinated using conventional plant breeding methods known to those of skill in the art and seed produced, for example self-pollination is commonly used with transgenic corn. The regenerated transformed plant or its progeny seed or plants can be tested for expression of the recombinant DNA and selected for the presence of enhanced agronomic trait.

Transgenic Plants and Seeds

Transgenic plants derived from the plant cells of this invention are grown to generate transgenic plants having an enhanced trait as compared to a control plant and produce transgenic seed and pollen of this invention. Such transgenic plants with enhanced traits are identified by selection of transformed plants or progeny seed for the enhanced trait. For efficiency a selection method is designed to evaluate multiple transgenic plants (events) comprising the recombinant DNA , for example multiple plants from 2 to 20 or more transgenic events. Transgenic plants grown from transgenic seed provided herein have enhanced agronomic traits that contribute to increased yield or other trait that provides increased plant value, including, for example, improved seed quality. Of particular interest are plants having enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil.

Table 2 provides a list of protein encoding DNA ("genes") that are useful as DNA segament in a recombinant DNA construct for production of transgenic plants with enhanced agronomic trait, the elements of Table 2 are described by reference to:

"PEP SEQ ID NO" identifies an amino acid sequence from SEQ ID NO: 299 to 596.

"NUC SEQ ID NO" identifies a DNA sequence from SEQ ID NO:1 to 298.

"BV id" is a reference to the identifying number of base vectors in Table 3 used for construction of the transformation vectors of the recombinant DNA. Construction of plant transformation constructs is illustrated in Example 1.

"Gene Name" denotes a common name for protein encoded by the recombinant DNA.

"Annotation" refers to a description of the top hit protein obtained from an amino acid sequence query of each PEP SEQ ID NO to GenBank database of the National Center for Biotechnology Information (ncbi). More particularly, "gi" is the GenBank ID number for the top BLAST hit;

"% id" refers to the percentage of identically matched amino acid residues along the length of the portion of the sequences which is aligned by BLAST (-F T) between the sequence of interest provided herein and the hit sequence in GenBank.

Table 2.

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
1	299	PHE0007721_18998	21	Corn Homeotic protein knotted-1	1.00E-152	77	123183	gb|AAP76321.1| homeobox transcription factor KNOTTED1 [Zea mays]
2	300	PHE0007756_18009	33	Arabidopsis 9-cis-epoxycarotenoid dioxygenase	0	90	15231856	dbj|BAB01336.1| 9-cis-epoxycarotenoid dioxygenase [Arabidopsis thaliana]
3	301	PHE0006007_18714	30	Arabidopsis nodulin MtN21 family protein	1.00E-105	86	79607902	ref|NP_974371.2|unknown protein [Arabidopsis thaliana]
4	302	PHE0004988_15925	13	Arabidopsis transport inhibitor response 1	0	100	18412567	gb|AAN71945.1| transport inhibitor response TIR1, AtFBL1 protein [Arabidopsis thaliana]
5	303	PHE0007140_21771	30	Arabidopsis nodulin MtN3 like protein-4	1.00E-121	92	18421965	gb|AAM61405.1| MtN3 like [Arabidopsis thaliana]
5	303	PHE0007140_22049	13	Arabidopsis nodulin MtN3 like protein-4	1.00E-121	92	18421965	gb|AAM61405.1| MtN3 like [Arabidopsis thaliana]
6	304	PHE0006823_16403	13	Arabidopsis class 2 non-symbiotic hemoglobin	3.00E-85	100	15228313	gb|AAM65188.1| Non-symbiotic hemoglobin Hb2 [Arabidopsis thaliana]
7	305	PHE0007440_22555	18	Corn putative GTP-binding protein Rab11	1.00E-107	91	115462975	gb|AAS98506.1| GTP-binding protein Rab11 [Oryza sativa (japonica cultivar-group)]
8	306	PHE0000206_22432	9	Corn CDPK kinase domain	0	87	115450483	sp|P53684|Calcium-dependent protein kinase, isoform 11 (CDPK 11) [Oryza sativa (japonica cultivar-group)]
9	307	PHE0000598_16824	9	Corn B1 regulatory protein	0	91	22195	emb|CAA40544.1| regulatory protein [Zea mays]
10	308	PHE0007590_17883	8	E. coli ribosomal protein S1 domain 3	7.00E-33	56	118740922	ref|ZP_01588962.1 |ribosomal protein S1 [Enterobacter sp. 638]
11	309	PHE0007588_17881	8	E. Coli RNAse E S1 domain	2.00E-45	98	52695516	pdb|1SMX| the S1 domain of Rnase E from E. Coli (Native)
12	310	PHE0007592_17885	8	Yeast pre-mRNA splicing factor RNA helicase S1 domain	1.00E-40	98	6320850	gb|AAB64546.1| pre-mRNA splicing factor RNA helicase [Saccharomyces cerevisiae]
13	311	PHE0007587_17880	8	E. coli Polyribonucleotide nucleotidyltransferase	7.00E-35	100	124525928	ref|ZP_01697933.1| Polyribonucleotide nucleotidyltransferase [Escherichia coli B]
14	312	PHE0007624_17923	8	E. coli translation initiation factor IF-1	8.00E-34	100	15800747	ref|NP_286761.1| translation initiation factor IF-1 [Escherichia coli O157:H7 EDL933]
15	313	PHE0007591_17884	8	E. coli ribosomal protein S1 domain 5	2.00E-20	51	118068082	ref|ZP_01536336.1| ribosomal protein S1 [Serratia proteamaculans 568]
16	314	PHE0007623_17922	8	Arabidopsis N-carbamoylputrescine amidohydrolase	1.00E-172	100	18401429	gb|AAM63266.1| putative nitrilase [Arabidopsis thaliana]
17	315	PHE0007583_17871	6	Rice MtN3 homolog	1.00E-130	100	125526770	gb|EAY74884.1|hypothetical protein OsI_002731 [Oryza sativa (indica cultivar-group)]
18	316	PHE0006618_16146	13	Corn putative ribosomal protein S10	4.00E-49	95	115442401	dbj|BAB63622.1| putative 40S ribosomal protein S10 [Oryza sativa (japonica cultivar-group)]

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
19	317	PHE0006791_16374	9	Arabidopsis GUN4_ARATH Tetrapyrrole-binding protein	1.00E-120	83	15231674	sp|Q9LX31|GUN4_ARATH Tetrapyrrole-binding protein, chloroplast precursor [Arabidopsis thaliana]
20	318	PHE0007620_17918	13	Arabidopsis thylakoid ascorbate peroxidase	0	96	15223971	dbj|BAF01533.1| thylakoid-bound ascorbate peroxidase [Arabidopsis thaliana]
21	319	PHE0006659_16195	13	Soy hypothetical protein	1.00E-112	49	125540608	gb|EAY87003.1|hypothetical protein OsI_008236 [Oryza sativa (indica cultivar-group)]
22	320	PHE0006662_16198	13	Ralstonia metallidurans hypothetical protein	1.00E-06	38	114582111	ref|XP_001161638.1| hypothetical protein isoform 1 [Pan troglodytes]
23	321	PHE0006274_15867	13	Corn Transcription factor E2Fb	0	78	115487438	gb|ABA96515.1| Transcription factor E2F [Oryza sativa (japonica cultivar-group)]
24	322	PHE0006637_16166	14	Corn ABI1 homolog	1.00E-145	78	115437928	ref|NP_001043415.1|Os01g0583100 [Oryza sativa (japonica cultivar-group)]
25	323	PHE0006926_16815	13	Corn putative vacuolar protein sorting-associated protein	1.00E-84	81	115440323	dbj|BAB63580.1| putative vacuolar protein sorting 28 [Oryza sativa (japonica cultivar-group)]
26	324	PHE0006821_16402	13	Corn 1-aminocyclopropane-1-carboxylate deaminase	1.00E-176	79	115448975	dbj|BAD16875.1| 1-aminocyclopropane-1-carboxylate deaminase [Oryza sativa (japonica cultivar-group)]
27	325	PHE0007687_18057	29	Arabidopsis cytochrome P450	0	100	21536828	gb|AAM61160.1| cytochrome P450 homolog [Arabidopsis thaliana]
27	325	PHE0007687_18200	13	Arabidopsis cytochrome P450	0	100	21536828	gb|AAM61160.1| cytochrome P450 homolog, [Arabidopsis thaliana]
28	326	PHE0007642_17999	29	Arabidopsis Non-phototropic hypocotyl protein 1	0	94	15231245	sp|O48963| Phototropin-1 (Root phototropism protein 1) [Arabidopsis thaliana]
29	327	PHE0007677_18052	29	Arabidopsis oxidoreductase	0	100	15239022	ref|NP_196694.1|oxidoreductase [Arabidopsis thaliana]
29	327	PHE0007677_18179	13	Arabidopsis oxidoreductase	0	100	15239022	ref|NP_196694.1|oxidoreductase [Arabidopsis thaliana]
30	328	PHE0007644_18001	29	Arabidopsis receptor protein kinase	0	88	15220455	ref|NP_176918.1| protein serine/ threonine kinase [Arabidopsis thaliana]
30	328	PHE0007644_18169	13	Arabidopsis receptor protein kinase	0	88	15220455	ref|NP_176918.1| protein serine/threonine kinase [Arabidopsis thaliana]
31	329	PHE0007640_17992	29	Soy magnesium transporter mrs2-1-like	1.00E-165	80	92889086	gb|ABE89684.1| Mg2+ transporter protein, CorA-like [Medicago truncatula]
32	330	PHE0007678_18053	29	Arabidopsis acyl-CoA thioesterase	1.00E-107	100	18399594	ref|NP_564457.1| acyl-CoA thioesterase [Arabidopsis thaliana]
32	330	PHE0007678_18180	13	Arabidopsis acyl-CoA thioesterase	1.00E-107	100	18399594	ref|NP_564457.1| acyl-CoA thioesterase [Arabidopsis thaliana]
33	331	PHE0007649_18006	29	Arabidopsis putative ankyrin protein kinase	0	99	42566072	ref|NP_191542.2| protein kinase/threonine kinase/ protein-tyrosine kinase [Arabidopsis thaliana]
34	332	PHE0006726_16298	25	Arabidopsis putative ARP2/3 protein complex subunit p41	0	97	15224586	gb|AAC20725.1| putative ARP2/3 protein complex subunit p41 [Arabidopsis thaliana]

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35	333	PHE0006218_8776	25	Arabidopsis thioredoxin-disulfide reductase	0	85	18405775	ref|NP_565954.1|disulfide oxidoreductase/ thioredoxin-disulfide reductase [Arabidopsis thaliana]
36	334	PHE0006657_16192	13	Corn putative ATP-binding protein	2.00E-83	71	115462871	gb|AAV31386.1| putative ATP-binding protein [Oryza sativa (japonica cultivar-group)]
37	335	PHE0002554_17876	13	Corn Chlorophyll a/b-binding protein CP29 precursor	1.00E-158	95	2326947	emb|CAA90681.1| Chlorophyll a/b-binding protein CP29 precursor [Zea mays]
37	335	PHE0002554_23104	9	Corn Chlorophyll a/b-binding protein CP29 precursor	1.00E-158	95	2326947	emb|CAA90681.1| Chlorophyll a/b-binding protein CP29 precursor [Zea mays]
38	336	PHE0006807_16388	13	Corn transcription factor APRR9 like	1.00E-140	64	115484281	gb|ABG22372.1| CCT motif family protein [Oryza sativa (japonica cultivar-group)]
39	337	PHE0003359_8487	9	Corn dwf4-like protein	0	86	60677681	dbj|BAD90972.1| cytochrome P450 [Oryza sativa (japonica cultivar-group)]
40	338	PHE0006542_15765	9	Arabidopsis methionine sulfoxide reductase	1.00E-149	100	15234942	ref|NP_194243.1| protein-methionine-S-oxide reductase [Arabidopsis thaliana]
41	339	PHE0007622_17921	8	Arabidopsis spermine synthase	0	100	18419941	gb|AAF01311.1| spermine synthase [Arabidopsis thaliana]
42	340	PHE0007630_17956	3	Corn GA3ox1	1.00E-151	75	125544881	gb|EAY91020.1|hypothetical protein OsI_012253 [Oryza sativa (indica cultivar-group)]
43	341	PHE0007593_17886	8	Corn spermidine synthase	1.00E-169	92	115471679	dbj|BAD30581.1| spermidine synthase 1 [Oryza sativa (japonica cultivar-group)]
44	342	PHE0006475_15588	9	Arbidopsis PsbQ_like	9.00E-89	100	42571459	ref|NP_973820.1| calcium ion binding [Arabidopsis thaliana]
45	343	PHE0007619_17915	13	Soy methyl-CpG-binding domain-containing protein	1.00E-44	43	18394229	ref|NP_563971.1|MBD10; DNA binding [Arabidopsis thaliana]
46	344	PHE0006846_16447	13	Soy cuticle protein	0	70	9758349	dbj|BAB08850.1| lipid transfer protein; glossy1 homolog [Arabidopsis thaliana]
47	345	PHE0007584_17874	13	Arabidopsis methyl-CpG-binding domain-containing protein	1.00E-124	65	18394229	ref|NP_563971.1 |MBD10; DNA binding [Arabidopsis thaliana]
48	346	PHE0003695_17913	17	Arabidopsis Erecta	0	95	15225286	gb|AAC49302.1| ERECTA, receptor protein kinase [Arabidopsis thaliana]
48	346	PHE0011554_23650	32	Arabidopsis Erecta	0	95	15225286	gb|AAC49302.1| ERECTA, receptor protein kinase [Arabidopsis thaliana]
49	347	PHE0003695_17879	8	Arabidopsis Erecta	0	95	15225286	gb|AAK59615.1| receptor protein kinase, ERECTA [Arabidopsis thaliana]
49	347	PHE0003695_23448	22	Arabidopsis Erecta	0	95	15225286	gb|AAK59615.1| receptor protein kinase, ERECTA [Arabidopsis thaliana]
50	348	PHE0006936_16828	29	Soy GNS1/SUR4 membrane protein	2.00E-91	66	92868497	gb|ABE78502.1|GNS1/SUR4 membrane protein [Medicago truncatula]
51	349	PHE0007645_18002	29	Soy DNA binding protein	2.00E-26	32	21617964	gb|AAM67014.1| DNA binding protein-like [Arabidopsis thaliana]
52	350	PHE0006784_16366	6	Corn cysteine synthase	1.00E-165	93	2829688	emb|CAA59798.1| O-acetylserine (thiol) lyase; cysteine synthase [Zea mays]

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53	351	PHE0006171_16491	13	Sorghum bicolor Glyoxalase	6.00E-91	86	115463027	ref|NP_001055113.1|Os05g0295800 [Oryza sativa (japonica cultivar-group)]
54	352	PHE0006868_16682	13	Arabidopsis ME01451	3.00E-05	100	21592593	gb|AAM64542.1| unknown [Arabidopsis thaliana]
55	353	PHE0006906_16796	13	Corn 20S proteasome subunit beta-6	1.00E-121	96	115480019	dbj|BAA28276.1| beta 6 subunit of 20S proteasome [Oryza sativa (japonica cultivar-group)]
56	354	PHE0006649_16180	13	Wheat putative DRE binding factor 2	5.00E-62	64	60547461	gb|AAX23704.1|HvCBF7 [Hordeum vulgare subsp. vulgare]
57	355	PHE0006809_16390	13	E.coli hydroxylamine reductase	0	97	89107724	ref|NP_415394.4| hydroxylamine reductase [Escherichia coli K12]
58	356	PHE0006918_16808	13	Corn putative dihydropyrimidine dehydrogenase	0	82	125571037	gb|EAZ12552.1|hypothetical protein OsJ_002377 [Oryza sativa (japonica cultivar-group)]
59	357	PHE0007813_18219	6	Rice MtN3 homolog	1.00E-119	86	115445683	dbj|BAD23335.1| putative NEC1 [Oryza sativa (japonica cultivar-group)]
60	358	PHE0007650_18175	13	Arabidopsis Kin17 protein	0	83	18405389	AAD10649.1| Kin17 protein [Arabidopsis thaliana]
61	359	PHE0006825_16405	13	E. coli cytochrome c nitrite reductase	0	100	15834306	ref|ZP_01699144.1| Nitrite reductase [Escherichia coli B]
62	360	PHE0006964_16864	9	Corn asparaginyl-tRNA synthetase	0	84	115436616	dbj|BAB61140.1| putative asparagine-tRNA ligase [Oryza sativa (japonica cultivar-group)]
62	360	PHE0006964_16865	13	Corn asparaginyl-tRNA synthetase	0	84	115436616	dbj|BAB61140.1| putative asparagine-tRNA ligase [Oryza sativa (japonica cultivar-group)]
63	361	PHE0008105_18407	13	Yeast transcriptional activator HAP5	1.00E-116	88	6324934	sp|Q02516| Transcriptional activator HAP5 [Saccharomyces cerevisiae]
63	361	PHE0008105_18733	30	Yeast transcriptional activator HAP5	1.00E-116	88	6324934	sp|Q02516| Transcriptional activator HAP5 [Saccharomyces cerevisiae]
64	362	PHE0006630_16159	13	Corn malate dehydrogenase	0	92	126888	sp|P15719 |MDHP_maize Malate dehydrogenase [NADP]
65	363	PHE0006966_16868	29	Arabidopsis Cryptochrome 1	0	95	18413170	ref|NP_567341.1|Cryptochrome 1 [Arabidopsis thaliana]
66	364	PHE0008698_24421	3	Corn expressed protein	9.00E-06	38	115484935	gb|AAX96767.1| expressed protein [Oryza sativa (japonica cultivar-group)]
67	365	PHE0006608_16123	25	Soy glutathione peroxidase	2.00E-82	90	21068666	emb|CAD31839.1|putative phospholipid hydroperoxide glutathione peroxidase [Cicer arietinum]
68	366	PHE0010462_21459	na	Deinococcus radiodurans nudixhydrolase	1.00E-155	86	15805358	ref|NP_294052.1|MutT/nudix family protein [Deinococcus radiodurans R1]
69	367	PHE0009211_21774	30	Arabidopsis PF02519-auxin inducible-3	5.00E-50	89	15236198	ref|NP_195205.1|unknown protein [Arabidopsis thaliana]
70	368	PHE0006256_8775	27	Arabidopsis 9-cis-epoxycarotenoid dioxygenase	0	92	15231856	dbj|BAB01336.1| 9-cis-epoxycarotenoid dioxygenase [Arabidopsis thaliana]
71	369	PHE0006989_16918	29	Arabidopsis putative protein kinase	0	79	15231840	dbj|BAB01042.1| protein kinase [Arabidopsis thaliana]

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72	370	PHE0007649_18166	13	Arabidopsis putative ankyrin protein kinase	0	100	42566072	ref|NP_191542.2| protein kinase/serine/threonine kinase/ tyrosine kinase [Arabidopsis thaliana]
73	371	PHE0006708_16264	29	Soy DNA helicase 45	0	89	3097266	emb|CAA76677.1| translation initiation factor [Pisum sativum]
74	372	PHE0001117_19094	4	Corn transcription factor G1543-like	1.00E-101	84	125527033	gb|EAY75147.1|hypothetical protein OsI_002994 [Oryza sativa (indica cultivar-group)]
74	372	PHE0001117_19095	9	Corn transcription factor G1543-like	1.00E-101	84	125527033	gb|EAY75147.1|hypothetical protein OsI_002994 [Oryza sativa (indica cultivar-group)]
75	373	PHE0006266_8814	9	corn phytochromobilin synthase	1.00E-164	100	49472889	gb|AAT66259.1| phytochromobilin synthase [Zea mays]
75	373	PHE0006266_15483	4	corn phytochromobilin synthase	1.00E-164	100	49472889	gb|AAT66259.1| phytochromobilin synthase [Zea mays]
76	374	PHE0006795_16378	13	E. coli pH-dependent sodium/proton antiporter	1.00E-172	82	16128013	ref|NP_414560.1| pH-dependent sodium/proton antiporter [Escherichia coli K12]
77	375	PHE0006956_16853	13	Agrobacterium nitrate reductase	0	98	15891062	ref|NP_356734.1|hypothetical protein AGR_L_1895 [Agrobacterium tumefaciens str. C58]
78	376	PHE0006859_16873	9	Corn bHLH protein family	2.00E-31	92	115451023	gb|EAZ25749.1| hypothetical protein OsJ_009232 [Oryza sativa (japonica cultivar-group)]
79	377	PHE0006824_16404	13	Corn Nonlegume hemoglobin-like	6.00E-86	98	3913789	gb|AAA19576.1| haemoglobin apoprotein [Hordeum vulgare]
80	378	PHE0006812_16393	13	Nostoc 2-on-2 hemoglobin	1.00E-61	100	23130505	ref|ZP_00112318.1| Truncated hemoglobins [Nostoc punctiforme PCC 73102]
81	379	PHE0006815_16396	13	Rice Trb-like	2.00E-98	100	115468746	dbj|BAD32857.1| putative 2-on-2 hemoglobin [Oryza sativa (japonica cultivar-group)]
82	380	PHE0006806_16387	13	Corn Trb-like	7.00E-80	83	14165165	gb|AAK55410.1|AF376063_12 -on-2 hemoglobin [Hordeum vulgare]
83	381	PHE0006624_16153	13	Corn putative cytosolic 6-phosphogluconate dehydrogenase	0	98	3342802	gb|AAC27703.1| putative cytosolic 6-phosphogluconate dehydrogenase [Zea mays]
84	382	PHE0005006_15823	13	Corn chlorophyll a/b-binding apoprotein CP24 precursor	1.00E-112	80	115458738	gb|EAY94374.1| hypothetical protein OsI_015607 [Oryza sativa (indica cultivar-group)]
85	383	PHE0003991_16771	13	Corn AfMONFEED000559 Protamine P1	-	-	-	-
86	384	PHE0006683_16229	13	Corn response regulator 4	1.00E-102	85	12060384	dbj|BAB20579.1| response regulator 4 [Zea mays]
87	385	PHE0006884_16703	7	Corn granule-bound starch synthase precursor	0	89	33321047	gb|AAQ06291.1| granule-bound starch synthase precursor [Zea mays]
88	386	PHE0006691_16237	3	E. coli NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	0	100	15804152	ref|NP_290191.1| NAD(P)H-dependent glycerol-3-phosphate dehydrogenase [Escherichia coli O157:H7 EDL933]
89	387	PHE0006790_16372	9	Arabidopsis chlorophyll A oxygenase	0	95	15219408	ref|NP_175088.1| chlorophyll a oxygenase [Arabidopsis thaliana]

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90	388	PHE0009143_19932	30	E. coli StpA	2.00E-57	86	83569970	ref|ZP_00921418.1| DNA-binding protein H-NS [Shigella dysenteriae 1012]
91	389	PHE0008406_18830	30	Yeast general amino acid permease	0	98	14318464	sp|P43548| Yeast general amino acid permease AGP3
92	390	PHE0008279_18708	30	Corn hypothetical protein	5.00E-25	73	115462095	gb|EAY96551.1| hypothetical protein OsI_017784 [Oryza sativa (indica cultivar-group)]
93	391	PHE0009142_19931	30	E. coli DNA-binding protein H-NS	3.00E-45	72	15801465	ref|NP_415753.1| global DNA-binding transcriptional dual regulator H-NS [Escherichia coli K12]
94	392	PHE0008308_18841	30	Corn hypothetical protein	5.00E-41	89	125537055	gb|EAY83543.1| hypothetical protein OsI_037502 [Oryza sativa (indica cultivar-group)]
95	393	PHE0007639_17991	29	Chlorella optimized beta glutamate dehydrogenase	0	99	118546	emb|CAA41636.1| glutamate dehydrogenase (NADP+) [Chlorella sorokiniana]
96	394	PHE0007646_18170	13	Arabidopsis kelch repeat-containing F-box family protein	0	94	116831441	gb|ABK28673.1| unknown [Arabidopsis thaliana]
97	395	PHE0007765_18182	13	Arabidopsis glutathione transferase	1.00E-130	100	15218872	ref|NP_176178.1| glutathione transferase [Arabidopsis thaliana]
98	396	PHE0008167_18465	5	Arabidopsis protein kinase	1.00E-163	95	15233846	ref|NP_194179.1| protein kinase/serine/threonine kinase/ protein-tyrosine kinase [Arabidopsis thaliana]
99	397	PHE0008172_18471	5	Arabidopsis putative thioredoxin H	2.00E-46	97	4895214	gb|AAD32800.1| putative thioredoxin H [Arabidopsis thaliana]
100	398	PHE0008183_18479	5	Arabidopsis receptor protein kinase	0	98	15239123	ref|NP_201371.1| protein serine/threonine kinase [Arabidopsis thaliana]
101	399	PHE0007585_17875	6	Arabidopsis drought-induced protein (Di21)	2.00E-42	87	469112	emb|CAA55322.1| Di21 [Arabidopsis thaliana]
101	399	PHE0007585_23902	30	Arabidopsis drought-induced protein (Di21)	2.00E-42	87	469112	emb|CAA55322.1| Di21 [Arabidopsis thaliana]
102	400	PHE0006804_16385	6	Rice small subunit of U2 snRNP auxiliary factor	1.00E-107	100	125553324	gb|EAY99033.1| hypothetical protein OsI_020266 [Oryza sativa (indica cultivar-group)]
103	401	PHE0006703_15751	15	Lactobacillus ATP-dependent phosphofructokinase with ctp	1.00E-164	92	104773899	ref|YP_618879.1| 6-phosphofructokinase [Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842]
104	402	PHE0007410_17653	3	Umbelopsis glycerol-3-phosphate O-acyltransferase	1.00E-144	42	58264662	ref|XP_569487.1| glycerol-3-phosphate O-acyltransferase [Cryptococcus neoformans var. neoformans JEC21]
105	403	PHE0006854_16456	2	Corn kernel specific yabby	1.00E-115	71	32330681	gb|AAP79887.1| yabby10 protein [Zea mays]
106	404	PHE0007721_21293	19	Corn homeobox transcription factor KNOTTED1	1.00E-152	77	123183	gb|AAP76321.1| homeobox transcription factor KNOTTED1 [Zea mays]
107	405	PHE0006563_15990	13	Wheat glutamine-dependent asparagine synthetase	0	97	53680379	gb|AAU89392.1| glutamine-dependent asparagine synthetase [Triticum aestivum]
107	405	PHE0006563_16140	9	Wheat glutamine-dependent asparagine synthetase	0	97	53680379	gb|AAU89392.1| glutamine-dependent asparagine synthetase [Triticum aestivum]

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108	406	PHE0006619_16147	13	Corn Delta 1-pyrroline-5-carboxylate synthetase	0	84	2081612	dbj|BAA19916.1| deltal-pyrroline-5-carboxylate synthetase [Oryza sativa (japonica cultivar-group)]
109	407	PHE0006813_16394	13	Bacillus subtilis Hemoglobin-like protein TrHb	1.00E-67	93	16078221	sp|O31607| Hemoglobin-like protein yjbI (Truncated BHb) (trBHb) [Bacillus Subtilis]
110	408	PHE0006688_16234	13	Corn ribosomal protein S2	1.00E-131	99	11467185	sp|P16037| maize Chloroplast 30S ribosomal protein S2
111	409	PHE0006635_16164	13	E.coli methylglyoxal synthase	9.00E-86	100	15800822	ref|NP_286838.1|methylglyoxal synthase [Escherichia coli O157:H7 EDL933]
112	410	PHE0008340_19155	30	Soy hypothetical protein	1.00E-178	78	92896427	gb|ABE93200.1| conserved hypothetical protein [Medicago truncatula]
113	411	PHE0008416_18838	30	Corn putative oligopeptide transporter	0	58	115480880	ref|NP_001064033.1|Os10g0110600 [Oryza sativa (japonica cultivar-group)]
114	412	PHE0008422_18842	30	Corn unknown protein	1.00E-121	55	115448163	dbj|BAD08083.1| unknown protein [Oryza sativa (japonica cultivar-group)]
115	413	PHE0008599_19166	30	Synechocystis glycogen operon protein	0	95	16330244	ref|NP_440972.1| glycogen operon protein; GlgX [Synechocystis sp. PCC 6803]
116	414	PHE0008375_18732	30	Bacillus hypoxanthine-guanine phosphoribosyltransferase	6.00E-95	99	16077136	sp|P37472| Hypoxanthine-guanine phosphoribosyl-transferase (HGPRTase) [Bacillus subtilis]
117	415	PHE0006712_16273	27	Arabidopsis unknown function	4.00E-92	100	15237778	ref|NP_200700.1| unknown protein [Arabidopsis thaliana]
117	415	PHE0006712_16362	13	Arabidopsis unknown function	4.00E-92	100	15237778	ref|NP_200700.1| unknown protein [Arabidopsis thaliana]
117	415	PHE0006712_16363	9	Arabidopsis unknown function	4.00E-92	100	15237778	ref|NP_200700.1| unknown protein [Arabidopsis thaliana]
117	415	PHE0006712_16364	6	Arabidopsis unknown function	4.00E-92	100	15237778	ref|NP_200700.1| unknown protein [Arabidopsis thaliana]
118	416	PHE0008394_18763	9	Corn altered glutamine synthetase	0	97	121341	sp|P25462| Glutamine synthetase (Glutamate--ammonia ligase) (GS2) [Zea mays]
119	417	PHE0008395_18764	9	Corn altered glutamine synthetase	0	97	121341	sp|P25462| Glutamine synthetase (Glutamate--ammonia ligase) (GS2) [Zea mays]
120	418	PHE0008444_18846	3	Corn glutamine synthetase1	0	100	112490284	dbj|BAA03430.1| glutamine synthetase [Zea mays]
121	419	PHE0006819_16400	13	Synechocystis A-type flavoproteins	0	100	16331685	sp|Q55393| Diflavin flavoprotein A 1 (SsATF573) (NADH:oxygen oxidoreductase) [Synechocystis sp. PCC 6803]
122	420	PHE0007417_17663	15	Corn EIL1	0	76	125543684	gb|EAY89823.1| hypothetical protein OsI_011056 [Oryza sativa (indica cultivar-group)]
123	421	PHE0008443_18845	13	Barley asparagine synthetase1	0	97	13925886	gb|AAK49456.1| glutamine-dependent asparagine synthetase 1 [Hordeum vulgare subsp. vulgare]
124	422	PHE0006818_16399	13	E. coli flavorubredoxin	0	100	16130617	ref|AP003277.1|flavorubredoxin oxidoreductase [Escherichia coli W3110]

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
125	423	PHE0006579_16031	13	Corn thiazole biosynthetic enzyme	0	97	2501189	sp|Q41738| Thiazole biosynthetic enzyme 1-1, chloroplast precursor [Zea mays]
126	424	PHE0006808_16389	13	Rice transcription factor APRR9 like	0	97	115484281	gb|ABG22372.1| CCT motif family protein [Oryza sativa (japonica cultivar-group)]
127	425	PHE0008160_18460	5	Arabidopsis pyruvate decarboxylase	0	100	15234062	ref|NP_195033.1| pyruvate decarboxylase [Arabidopsis thaliana]
128	426	PHE0007571_17834	24	Soy homoglutathione synthetase	0	97	7799808	emb|CAB91078.1| homoglutathione synthetase [Glycine max]
129	427	PHE0007586_17877	13	Arabidopsis Chlorophyll a-b binding protein	1.00E-151	96	15225630	gb|AAD32843.1| putative chlorophyll a/b binding protein [Arabidopsis thaliana]
130	428	PHE0006969_16871	13	Rice hypothetical protein	4.00E-34	100	115451023	gb|EAZ25749.1| hypothetical protein OsJ_009232 [Oryza sativa (japonica cultivar-group)]
131	429	PHE0009729_21717	13	Arabidopsis bHLH	1.00E-91	89	30698765	gb|AAO64014.1| putative homeodomain leucine zipper protein [Arabidopsis thaliana]
132	430	PHE0006424_15520	9	photosystem II reaction center 6.1KD protein	3.00E-37	75	15224531	gb|AAM64951.1| photosystem II reaction center 6.1KD protein [Arabidopsis thaliana]
133	431	PHE0006392_15480	9	Corn PsbP	9.00E-99	84	115489108	gb|ABA98969.2| Thylakoid lumenal 21.5 kDa protein [Oryza sativa (japonica cultivar-group)]
134	432	PHE0006589_16093	13	Arabidopsis thioredoxin-like protein	3.00E-84	100	42563272	dbj|BAE99426.1| thioredoxin-like protein [Arabidopsis thaliana]
135	433	PHE0009258_21487	16	Arabidopsis sodium proton exchanger1	0	87	15240448	ref|NP_198067.1|NHX1 (NA+/H+ EXCHANGER); sodium:hydrogen antiporter [Arabidopsis thaliana]
135	433	PHE0009258_20132	34	Arabidopsis sodium proton exchanger1	0	87	15240448	ref|NP_198067.1|NHX1 (NA+/H+ EXCHANGER); sodium:hydrogen antiporter [Arabidopsis thaliana]
136	434	PHE0006680_16226	13	Corn flavanone 3-hydroxylase	1.00E-161	81	18057095	gb|AAL58118.1| putative flavanone 3-hydroxylase [Oryza sativa (japonica cultivar-group)]
137	435	PHE0006481_16072	13	Corn Os09g0570300 homolog	1.00E-145	81	115480783	ref|NP_001063985.1|Os09g0570300 [Oryza sativa (japonica cultivar-group)]
138	436	PHE0006269_8820	23	Synechocystis conserved tetratricopeptide repeat protein	0	96	16329708	ref|NP_440436.1|hypothetical protein slr2048 [Synechocystis sp. PCC 6803]
138	436	PHE0006269_8821	9	Synechocystis conserved tetratricopeptide repeat protein	0	96	16329708	ref|NP_440436.1|hypothetical protein slr2048 [Synechocystis sp. PCC 6803]
139	437	PHE0006783_16365	11	Brassica napus amino acid permease 6	0	97	31455393	emb|CAD92450.1|amino acid permease 6 [Brassica napus]
140	438	PHE0003151_18392	28	Soy aintegumenta 2	1.00E-141	47	38492172	gb|AAR22388.1| ANT-like protein [Nicotiana tabacum]
141	439	PHE0008272_18723	30	Arabidopsis heat shock factor protein 5	0	88	15220611	sp|Q9S7U5| Heat shock transcription factor 5) (HSTF 5) [Arabidopsis thaliana]

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
142	440	PHE0008277_18706	30	Yeast chromatin structure remodeling complex protein SFH1	0	95	6323354	sp|Q06168| Chromatin structure remodeling complex protein SFH1 [Saccharomyces cerevisiae]
143	441	PHE0008109_18411	30	Yeast uncharacterized protein YOR154W precursor	0	93	6324728	sp|Q12232| Yeast uncharacterized protein YOR154W precursor
143	441	PHE0008109_18568	13	Yeast uncharacterized protein YOR154W precursor	0	93	6324728	sp|Q12232| Yeast uncharacterized protein YOR154W precursor
144	442	PHE0008280_18826	30	Sinorhizobium putative trehalose synthase	0	93	16263847	ref|NP_436639.1| putative trehalose synthase protein [Sinorhizobium meliloti 1021]
145	443	PHE0002424_15825	13	Corn NADPH:protochlorophyllide oxidoreductase A	1.00E-140	77	90398977	gb|EAY96061.1| hypothetical protein OsI_017294 [Oryza sativa (indica cultivar-group)]
146	444	PHE0006914_16804	13	Corn 26S protease regulatory subunit 7	0	100	115466876	dbj|BAD35266.1| 26S protease regulatory subunit 7 [Oryza sativa (japonica cultivar-group)]
147	445	PHE0006642_16172	14	Rice ent-kaurene oxidase	0	97	115468620	dbj|BAD54598.1| ent-kaurene oxidase [Oryza sativa (japonica cultivar-group)]
148	446	PHE0007578_17852	29	Arabidopsis unknown protein	2.00E-71	100	18395285	ref|NP_564202.1|unknown protein [Arabidopsis thaliana]
149	447	PHE0009926_21079	30	Arabidopsis putative dual-specificity protein phosphatase	1.00E-84	100	18397475	gb|AAM62982.1| putative dual-specificity protein phosphatase [Arabidopsis thaliana]
150	448	PHE0006206_19159	30	Arabidopsis ubiquitin ligase SINAT5	0	97	15234306	ref|NP_194517.1 |ubiquitin-protein ligase/zinc ion binding [Arabidopsis thaliana]
151	449	PHE0008274_18705	30	Arabidopsis putative BURP domain containing protein	1.00E-154	95	15222103	gb|AAD43166.1| Putative BURP domain containing protein [Arabidopsis thaliana]
152	450	PHE0009510_20526	30	Cucurbita spermidine synthase	1.00E-164	89	49425361	gb|AAT66041.1| spermidine synthase [Cucumis sativus]
153	451	PHE0009937_21097	34	Cucurbita spermidine synthase	1.00E-164	89	49425361	gb|AAT66041.1| spermidine synthase [Cucumis sativus]
154	452	PHE0009927_21080	30	Brassica putative dual-specificity protein phosphatase	4.00E-79	88	30679726	dbj|BAC42108.1| putative dual-specificity protein phosphatase [Arabidopsis thaliana]
155	453	PHE0006460_15962	13	Arabidopsis unknown protein	0	86	18394658	ref|NP_564064.1|unknown protein [Arabidopsis thaliana]
156	454	PHE0006445_15956	13	Arabidopsis allene oxide cyclase	1.00E-142	100	18404656	emb|CAC83762.1| allene oxide cyclase [Arabidopsis thaliana]
157	455	PHE0006987_16893	13	Arabidopsis calcineurin B-like protein 2	1.00E-125	99	15241101	dbj|BAB09281.1| calcineurin B-like protein 2 [Arabidopsis thaliana]
158	456	PHE0004230_15865	13	Corn E2F3 like	0	72	115446539	dbj|BAD73815.1| putative E2F homolog [Oryza sativa (japonica cultivar-group)]
159	457	PHE0006814_16395	13	Arabidopsis 2-on-2 hemoglobin	2.00E-99	100	18418064	dbj|BAD42999.1| 2-on-2 hemoglobin (GLB3) [Arabidopsis thaliana]
160	458	PHE0002773_15881	13	Rice glucose-1-phosphate adenylyltransferase large subunit 1	0	100	115465649	ref|NP_001056424.1|Os05g0580000 [Oryza sativa (japonica cultivar-group)]
161	459	PHE0006060_15842	13	Corn ubiquitin precursor	0	86	60677681	dbj|BAD90972.1|cytochrome P450 [Oryza sativa (japonica cultivar-group)]

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162	460	PHE0006850_16451	2	Corn kernel specific yabby	4.00E-73	74	115454365	dbj|BAF45803.1| YABBY2 protein [Oryza sativa (japonica cultivar-group)]
163	461	PHE0008043_18199	2	Corn homeobox-9	0	99	40950648	gb|AAR97952.1| rolled leaf1 [Zea mays]
164	462	PHE0000125_18852	13	Corn receiver domain (TOC1-like) 10	1.00E-171	73	51571875	dbj|BAD38854.1| pseudo-response regulator 1 [Oryza sativa (japonica cultivar-group)]
165	463	PHE0008269_18720	30	Arabidopsis protein kinase	0	96	15222311	ref|NP_172195.1|MAPKKK13; protein kinase/ protein serine/ threonine kinase/ protein-tyrosine kinase [Arabidopsis thaliana]
166	464	PHE0008264_18712	30	Arabidopsis ubiquitin-protein ligase	0	97	42567081	ref|NP_194117.3| ubiquitin-protein ligase/ zinc ion binding [Arabidopsis thaliana]
167	465	PHE0002782_20059	8	Corn phosphoglucose isomerase	0	89	115479643	dbj|BAD17509.1| putative glucose-6-phosphate isomerase [Oryza sativa (japonica cultivar-group)]
167	465	PHE0002782_22452	13	Corn phosphoglucose isomerase	0	89	115479643	dbj|BAD17509.1| putative glucose-6-phosphate isomerase [Oryza sativa (japonica cultivar-group)]
168	466	PHE0009438_20404	9	Corn GNC	8.00E-84	52	115445073	dbj|BAD17612.1| zinc finger protein-like [Oryza sativa (japonica cultivar-group)]
169	467	PHE0009429_20392	13	Rice sulphiredoxin	6.00E-68	85	71905635	gb|AAZ52795.1| sulfiredoxin-like protein [Oryza sativa (japonica cultivar-group)]
170	468	PHE0009440_20406	13	Yeast sulphiredoxin	3.00E-66	100	6322764	sp|P36077|SRX1_YEAST Sulfiredoxin
171	469	PHE0008543_18945	13	Agrobacterium siroheme synthase	0	97	17937602	ref|NP_534391.1| siroheme synthase [Agrobacterium tumefaciens str. C58]
171	469	PHE0008543_18946	20	Agrobacterium siroheme synthase	0	97	17937602	ref|NP_534391.1| siroheme synthase [Agrobacterium tumefaciens str. C58]
171	469	PHE0008543_18947	10	Agrobacterium siroheme synthase	0	97	17937602	ref|NP_534391.1| siroheme synthase [Agrobacterium tumefaciens str. C58]
172	470	PHE0006960_16857	8	Rice nitrate reductase	0	96	115448947	dbj|BAD16843.1| putative nitrate reductase [Oryza sativa (japonica cultivar-group)]
173	471	PHE0009427_20389	13	Arabidopsis imidazoleglycerol-phosphate dehydratase	1.00E-118	88	30686639	dbj|BAB01781.1| imidazoleglycerol-phosphate dehydratase [Arabidopsis thaliana]
174	472	PHE0006437_15906	13	Arabidopsis putative protein kinase	1.00E-170	95	22327062	ref|NP_197926.2| protein kinase/ protein serine/ threonine kinase/ protein-tyrosine kinase [Arabidopsis thaliana]
175	473	PHE0007409_17652	3	Rice mono- or diacylglycerol acyltransferase	0	99	115467862	dbj|BAD33251.1| putative mono- or diacylglycerol acyltransferase [Oryza sativa (japonica cultivar-group)]
176	474	PHE0009426_20387	13	Yeast histidinol phosphatase	0	100	14318548	dbj|BAA09264.1| histidinol phosphatase [Saccharomyces cerevisiae]
177	475	PHE0009430_20393	13	Soy sulphiredoxin	2.00E-45	74	85719364	gb|ABC75369.1|ParB-like nuclease [Medicago truncatula]

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178	476	PHE0007003_16906	13	Rice putative phosphotyrosyl phosphatase activator	1.00E-171	82	115467132	dbj|BAD37240.1| putative phosphotyrosyl phosphatase activator [Oryza sativa (japonica cultivar-group)]
179	477	PHE0008378_18741	13	Yeast general control protein	1.00E-144	94	6320828	sp|P03069| General control protein GCN4 (Amino acid biosynthesis regulatory protein) [Saccharomyces cerevisiae]
180	478	PHE0008273_18725	30	Arabidopsis nucleic acid binding protein	0	95	30683900	ref|NP_180412.2| nucleic acid binding [Arabidopsis thaliana]
181	479	PHE0008547_18953	20	Corn ferredoxin	1.00E-38	75	119961	sp|P27789| Maize ferredoxin-5 (Ferredoxin V)
182	480	PHE0006922_16812	13	Corn putative hexokinase I	0	87	115439869	gb|AAZ93623.1| hexokinase 6 [Oryza sativa (japonica cultivar-group)]
183	481	PHE0006920_16810	13	Corn putative CAD ATPase	0	88	115439215	gb|AAP92128.1| putative ATPase ATP1 [Oryza sativa (japonica cultivar-group)]
184	482	PHE0008396_18765	9	Corn altered glutamine synthetase2	0	97	121341	sp|P25462| Maize glutamine synthetase (Glutamate--ammonia ligase) (GS2)
185	483	PHE0006957_16854	13	Corn glutamine synthetase2 C299A/C364A double mutant	0	97	121341	sp|P25462| Maize glutamine synthetase (Glutamate--ammonia ligase) (GS2)
186	484	PHE0006404_15936	13	Arabidopsis RNA-binding protein	0	96	18423760	ref|NP_568826.1|RNA binding protein [Arabidopsis thaliana]
187	485	PHE0006820_16401	13	Nostoc punctiforme A-type flavoproteins	0	97	23129576	ref|ZP_00111402.1|Uncharacterized flavoproteins [Nostoc punctiforme PCC 73102]
188	486	PHE0006690_16236	13	Corn putative high affinity nitrate transporter	0	79	108794607	gb|ABG20828.1| high affinity nitrate transporter NRT2.5 [Hordeum vulgare subsp. vulgare]
189	487	PHE0006395_15932	13	Arabidopsis leucine-rich repeat protein	0	100	18391461	gb|AAW57412.1| plant intracellular Ras-group-related LRR protein 3 [Arabidopsis thaliana]
190	488	PHE0006759_16384	6	Arabidopsis glycine hydroxymethyltransferase/serine hydroxymethyltransferase	0	100	15235745	ref|NP_195506.1| Serine hydroxymethyltransferase 1); glycine hydroxymethyl-transferase [Arabidopsis thaliana]
191	489	PHE0006576_16028	13	Corn tuber-specific and sucrose-responsive element binding factor	1.00E-87	57	115469098	dbj|BAD37513.1| putative tuber-specific and sucrose-responsive element binding factor [Oryza sativa (japonica cultivar-group)]
192	490	PHE0006979_16885	13	Arabidopsis xylogen like protein 12	1.00E-103	100	18405294	dbj|BAE73268.1| xylogen like protein 12 [Arabidopsis thaliana]
193	491	PHE0009786_21754	9	Arabidopsis HFR1-delta N105 mutant	1.00E-97	93	18378953	sp|Q9FE22| Long hypocotyl in far-red 1 (bHLH-like protein HFR1); gb|AAK15282.1 basic helix-loop-helix FBI1 protein [Arabidopsis thaliana]
193	491	PHE0009786_20911	30	Arabidopsis HFR1-delta N105 mutant	1.00E-97	93	18378953	sp|Q9FE22| Long hypocotyl in far-red 1 (bHLH-like protein HFR1); gb|AAK15282.1 basic helix-loop-helix FBI1 protein [Arabidopsis thaliana]

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
194	492	PHE0006572_16007	13	Arabidopsis phosphoenolpyruvate carboxylase kinase 2	1.00E-160	100	18396914	ref|NP_566229.1|Phosphoenol pyruvate carboxylase kinase 2 [Arabidopsis thaliana]
195	493	PHE0006828_16408	13	Arabidopsis de-etiolated 1	0	94	42566387	ref|NP_192756.2|DET1 (De-etiolated 1) [Arabidopsis thaliana]
196	494	PHE0006588_16092	13	Corn electron transporter/ thiol-disulfide exchange intermediate	9.00E-90	100	30693659	ref|NP_175021.2 |electron transporter/ thiol-disulfide exchange intermediate [Arabidopsis thaliana]
197	495	PHE0006985_16891	13	Corn putative nuclear protein NAP	0	75	115450034	dbj|BAD23003.1| putative nuclear protein NAP [Oryza sativa (japonica cultivar-group)]
198	496	PHE0006875_16688	13	Rice putative Photosystem I reaction center subunit V	6.00E-61	85	115479799	dbj|BAD33396.1| putative photosystem I reaction center subunit V [Oryza sativa (japonica cultivar-group)]
199	497	PHE0006626_16155	13	Corn (1-3, 1-4)-beta-glucanase	1.00E-134	72	18984	emb|CAA36801.1| (1-3,1-4)-beta-D-glucanase; emb|CAB41401.1| lichenase [Hordeum vulgare subsp. vulgare]
200	498	PHE0006689_16235	13	Corn amino acid transporter family protein	0	84	115443611	dbj|BAD08181.1| putative amino acid transport protein [Oryza sativa (japonica cultivar-group)]
201	499	PHE0006799_16382	9	Rhodopseudomonas Mg-protoporphyrin IX monomethylester cyclase	0	97	39934738	ref|NP_947014.1 |Mg-protoporphyrin IX monomethyl ester oxidative cyclase 66kD subunit [Rhodopseudomonas palustris CGA009]
202	500	PHE0006959_16856	8	nitrate reductase	0	95	115476820	dbj|BAD09558.1| nitrate reductase apoenzyme [Oryza sativa (japonica cultivar-group)]
203	501	PHE0011615_23861	31	Arabidopsis activated YODA	0	78	38049268	gb|AAR10436.1| YDA [Arabidopsis thaliana]
204	502	PHE0006925_16814	13	Corn translocase inner membrane-like protein	2.00E-74	66	115473245	dbj|BAC83874.1| translocase inner membrane-like protein [Oryza sativa (japonica cultivar-group)]
205	503	PHE0006713_16274	25	Arabidopsis cell division cycle protein 23-like	0	96	15228343	emb|CAB51062.1| cell division cycle protein 23 homolog [Arabidopsis thaliana]
206	504	PHE0008447_18848	13	Corn light regulated protein	1.00E-23	63	77023866	gb|ABA61130.1| light-induced protein 1 [Lolium perenne]
207	505	PHE0006674_16221	13	Rice putative nitrate transporter NRT1-5	0	95	115441781	dbj|BAD82445.1| putative nitrate transporter NRT1-5 [Oryza sativa (japonica cultivar-group)]
208	506	PHE0010613_22398	3	Corn high oil DAG kinase-like	1.00E-165	80	115450433	gb|ABF93745.1| expressed protein [Oryza sativa (japonica cultivar-group)]
208	506	PHE0010613_22404	7	Corn high oil DAG kinase-like	1.00E-165	80	115450433	gb|ABF93745.1| expressed protein [Oryza sativa (japonica cultivar-group)]
209	507	PHE0002720_22558	18	Corn DNA J protein	0	83	115463255	gb|AAV43840.1| putative DnaJ protein [Oryza sativa (japonica cultivar-group)]

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210	508	PHE0011760_24005	31	Arabidopsis YODA	0	83	15222512	ref|NP_176557.1| YDA (YODA); protein kinase/ protein serine/threonine kinase/ protein-tyrosine kinase [Arabidopsis thaliana]
211	509	PHE0010612_22397	3	Corn high oil nucleic acid binding	1.00E-165	77	115450431	gb|ABF93744.1| D111/G-patch domain-containing protein [Oryza sativa (japonica cultivar-group)]
211	509	PHE0010612_22403	7	Corn high oil nucleic acid binding	1.00E-165	77	115450431	gb|ABF93744.1| D111/G-patch domain-containing protein [Oryza sativa (japonica cultivar-group)]
212	510	PHE0010615_22400	3	Corn high oil unknown protein	1.00E-32	28	125581548	gb|EAZ22479.1| hypothetical protein OsJ_005962 [Oryza sativa (japonica cultivar-group)]
213	511	PHE0007411_17654	3	Rice putative tafazzin isoform	0	97	57899656	dbj|BAD87325.1| putative tafazzin isoform [Oryza sativa (japonica cultivar-group)]
214	512	PHE0010617_22402	3	Corn high oil hydrolase-like protein	5.00E-98	69	115447203	dbj|BAD20134.1| isochorismatase hydrolase-like protein [Oryza sativa (japonica cultivar-group)]
215	513	PHE0010610_22395	3	Corn high oil RING-finger protein	9.00E-27	75	2894379	emb|CAA74911.1| ring finger protein [Hordeum vulgare subsp. vulgare]
216	514	PHE0006883_16702	7	Corn glucosyl transferase	0	93	136757	sp|P04713| Granule-bound starch synthase 1, (GBSS-I) emb|CAA27574.1| glucosyl transferase [Zea mays]
217	515	PHE0010636_22408	3	Corn glucose 6-phosphate translocator	0	89	2997589	gb|AAC08524.1| glucose-6-phosphate/phosphate-translocator precursor [Zea mays]
217	515	PHE0010636_22410	7	Corn glucose 6-phosphate translocator	0	89	2997589	gb|AAC08524.1|glucose-6-phosphate/phosphate-translocator precursor [Zea mays]
218	516	PHE0011082_23038	30	Soy SAG13 like	3.00E-98	66	15239327	gb|AAO22710.1| putative short chain alcohol dehydrogenase [Arabidopsis thaliana]
219	517	PHE0009475_20462	15	Arabidopsis gibberellin 20-oxidase	0	100	15235072	ref|NP_194272.1| gibberellin 20-oxidase/ gibberellin 3-beta-dioxygenase [Arabidopsis thaliana]
219	517	PHE0009475_20468	2	Arabidopsis gibberellin 20-oxidase	0	100	15235072	ref|NP_194272.1| gibberellin 20-oxidase/ gibberellin 3-beta-dioxygenase [Arabidopsis thaliana]
219	517	PHE0009475_20899	1	Arabidopsis gibberellin 20-oxidase	0	100	15235072	ref|NP_194272.1| gibberellin 20-oxidase/ gibberellin 3-beta-dioxygenase [Arabidopsis thaliana]
219	517	PHE0009475_21740	any	Arabidopsis gibberellin 20-oxidase	0	100	15235072	ref|NP_194272.1| gibberellin 20-oxidase/ gibberellin 3-beta-dioxygenase [Arabidopsis thaliana]
219	517	PHE0009475_20469	30	Arabidopsis gibberellin 20-oxidase	0	100	15235072	ref|NP_194272.1| gibberellin 20-oxidase/ gibberellin 3-beta-dioxygenase [Arabidopsis thaliana]
220	518	PHE0007415_17661	7	Corn EIL2	0	75	125543684	gb|EAY89823.1|hypothetical protein OsI_011056 [Oryza sativa (indica cultivar-group)]

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221	519	PHE0006614_16129	22	Soy STN7-LHCII protein kinase	0	72	15241093	emb|CAB82763.1| (1-4)-beta-mannan endohydrolase-like protein [Arabidopsis thaliana]
222	520	PHE0010611_22396	3	Corn high oil flavin containing monooxygenase	0	77	116317828	gb|EAY92887.1| hypothetical protein OsI_014120 [Oryza sativa (indica cultivar-group)]
223	521	PHE0011719_23946	na	Arabidopsis 1-acylglycerol-3-phosphate O-acyltransferase	0	99	18410774	ref|NP_567052.1| 1-acylglycerol-3-phosphate O-acyltransferase [Arabidopsis thaliana]
224	522	PHE0006861_16463	29	Arabidopsis unknown protein	0	87	5734789	gb|AAD50054.1| Hypothetical protein [Arabidopsis thaliana]
225	523	PHE0006568_16005	13	Corn receptor-like protein kinase	0	60	115472561	dbj|BAC84489.1| putative serine/threonine-specific protein kinase [Oryza sativa (japonica cultivar-group)]
226	524	PHE0010652_22429	3	Corn high oil tRNA methyltransferase	1.00E-172	94	115469828	dbj|BAD53799.1| putative FtsJ homolog 1 isoform b [Oryza sativa (japonica cultivar-group)]
227	525	PHE0008158_18448	7	ZMEN1	7.00E-65	56	115437056	dbj|BAD73580.1| zinc-binding protein-like [Oryza sativa (japonica cultivar-group)]
228	526	PHE0003316_20755	30	E. coli beta-glucuronidase with intron 2 from potato light-inducible tissue-specific gene	0	100	475169	emb|CAA83649.1| beta-glucuronidase [synthetic construct]
229	527	PHE0009478_20470	2	Corn gibberellin 20 oxidase	1.00E-167	82	37359180	gb|AAN73384.1| putative gibberellin 20 oxidase [Oryza rufipogon]
229	527	PHE0009478_20900	1	Corn gibberellin 20 oxidase	1.00E-167	82	37359180	gb|AAN73384.1| putative gibberellin 20 oxidase [Oryza rufipogon]
229	527	PHE0009478_20471	any	Corn gibberellin 20 oxidase	1.00E-167	82	37359180	gb|AAN73384.1| putative gibberellin 20 oxidase [Oryza rufipogon]
230	528	PHE0010395_21760	30	Soy DUF716	3.00E-84	55	124365521	gb|ABN09755.1| Proteinase inhibitor I4, serpin [Medicago truncatula]
231	529	PHE0010391_21753	30	Soy Unknown protein	7.00E-64	91	115463333	gb|AAV43818.1| unknown protein [Oryza sativa (japonica cultivar-group)]
232	530	PHE0010396_21761	30	Soy DUF716	2.00E-83	56	124365521	gb|ABN09755.1|Proteinase inhibitor I4, serpin [Medicago truncatula]
233	531	PHE0009511_22422	26	Cucurbita S-adenosylmethionine decarboxylase	1.00E-147	73	21239731	gb|AAM44307.1| S-adenosylmethionine decarboxylase [Citrofortunella mitis]
234	532	PHE0010614_22399	3	Corn high oil SOUL-like protein	2.00E-94	80	115435220	dbj|BAA96146.1| putative heme binding protein 2 [Oryza sativa (japonica cultivar-group)]
235	533	PHE0011420_23634	3	Corn unknown protein	0	91	115464535	ref|NP_001055867.1|Os05g0482600 [Oryza sativa (japonica cultivar-group)]
236	534	PHE0011454_23667	13	Corn H2B2	3.00E-36	87	122044864	sp|P05621| Wheat histone H2B.2
237	535	PHE0011443_24001	13	Corn H2B1	2.00E-39	77	1708107	sp|P54348| Maize histone H2B.5 (H2B)
238	536	PHE0011452_23665	13	Corn H3-2	3.00E-69	99	15232146	sp|P69246| Maize hHistone H3.2
239	537	PHE0010394_21758	30	Soy Sin3 associated ploypeptide	2.00E-52	82	115443887	dbj|BAD07609.1| putative P18 [Oryza sativa (japonica cultivar-group)]

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
240	538	PHE0010397_21762	30	Soy unknown protein	7.00E-17	57	82568700	dbj|BAE48663.1|Pm52 [Prunus mume]
241	539	PHE0010398_21763	30	Soy unknown protein	1.00E-17	56	82568700	dbj|BAE48663.1|Pm52 [Prunus mume]
242	540	PHE0010100_21467	20	Klebsiella nitrite reductase	0	90	585562	gb|AAA25099.1| nitrite reductase [Klebsiella oxytoca]
243	541	PHE0011503_23734	1	Corn ethylene receptor etr1 mutant	0	91	38607373	gb|AAR25566.1| ethylene receptor [Zea mays]
243	541	PHE0011503_23736	15	Corn ethylene receptor etr1 mutant	0	91	38607373	gb|AAR25566.1| ethylene receptor [Zea mays]
244	542	PHE0010099_21319	13	Klebsiella nitrate reductase large subunit	0	87	18314343	gb|AAA25100.2| nitrate reductase large subunit [Klebsiella oxytoca]
244	542	PHE0010099_21461	9	Klebsiella nitrate reductase large subunit	0	87	18314343	gb|AAA25100.2| nitrate reductase large subunit [Klebsiella oxytoca]
244	542	PHE0010099_21466	20	Klebsiella nitrate reductase large subunit	0	87	18314343	gb|AAA25100.2| nitrate reductase large subunit [Klebsiella oxytoca]
245	543	PHE0011269_23400	13	Arabidopsis HIO2019A	0	96	42562138	gb|AAF97271.1| meristem L1 layer homeobox protein like (ATML1) from Arabidopsis thaliana gb|U37589 and contains Transposase PF|01527, Homeobox PF|00046, and START PF|01852 domains.
246	544	PHE0008393_18762	20	Corn roothairless 1	0	95	34733383	gb|AAQ81632.1| roothairless 1 [Zea mays]
247	545	PHE0010223_21491	3	Corn sucrose transport protein 4	0	87	49066602	gb|AAT51689.1| sucrose transport protein [Zea mays]
248	546	PHE0011613_23858	3	Corn WRI1	1.00E-109	56	85815798	dbj|BAE78578.1|aintegumenta -like [Oryza sativa (japonica cultivar-group)]
248	546	PHE0011613_23860	7	Corn WRI1	1.00E-109	56	85815798	dbj|BAE78578.1|aintegumenta -like protein [Oryza sativa (japonica cultivar-group)]
249	547	PHE0001582_22064	1	Corn ethylene receptor ETR1 like	0	93	10241927	gb|AAR25568.1| ethylene receptor [Zea mays]
250	548	PHE0007444_22314	18	Corn aminopropyl transferase	0	94	74481421	gb|AAW57523.1| spermidine synthase [Zea mays]
251	549	PHE0010543_22298	16	Corn en-like protein FDR2	4.00E-92	94	115498267	gb|ABI98712.1| terminal flower 1 [Zea mays]
252	550	PHE0010543_22323	12	Cen-like protein FDR2	3.00E-83	94	115498267	gb|ABI98712.1| terminal flower 1 [Zea mays]
253	551	PHE0011081_23033	30	Arabidopsis SUC2	0	88	15239921	ref|NP_199174.1|carbohydrate /sugar transporter [Arabidopsis thaliana]
254	552	PHE0011075_23026	30	Wheat P-II	3.00E-62	77	125550734	gb|EAY96443.1| hypothetical protein Osl_017676 [Oryza sativa (indica cultivar-group)]
255	553	PHE0010100_21462	9	Klebsiella Nitrite reductase	0	90	585562	sp|Q06458| Nitrite reductase [NAD(P)H] large subunit [Klebsiella oxytoca]
256	554	PHE0009640_21775	30	Arabidopsis ME10609	3.00E-53	100	15236188	ref|NP_195203.1| unknown protein [Arabidopsis thaliana]
257	555	PHE0010092_21307	13	Corn translation initiation factor 2 alpha subunit	1.00E-158	84	115452457	gb|ABF95443.1| Eukaryotic translation initiation factor 2 alpha subunit [Oryza sativa (japonica cultivar-group)]
258	556	PHE0004611_24123	13	Arabidopsis putative peptide transporter	0	89	12325237	gb|AAG52567.1| putative peptide transporter [Arabidopsis thaliana]

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
259	557	PHE0010093_21308	13	Corn translation initiation factor 2 alpha subunit	1.00E-162	85	115452457	gb|ABF95443.1| Eukaryotic translation initiation factor 2 alpha subunit [Oryza sativa (japonica cultivar-group)]
260	558	PHE0008605_23084	13	Arabidopsis putative histone H2B	4.00E-40	98	15226943	gb|AAM62619.1| putative histone H2B [Arabidopsis thaliana]
261	559	PHE0009939_21100	16	Arabidopsis glycine dehydrogenase	0	98	15234036	ref|NP_195027.1| glycine dehydrogenase [Arabidopsis thaliana]
262	560	PHE0009941_21105	8	Corn Moc1-like	1.00E-124	80	125540188	gb|EAY86583.1| hypothetical protein OsI_007816 [Oryza sativa (indica cultivar-group)]
263	561	PHE0009951_21135	8	Yeast QSR1-like	1.00E-115	93	6323104	sp|P41805| Yeast 60S ribosomal protein L10 (L9) (Ubiquinol-cytochrome C reductase complex subunit VI-requiring protein)
264	562	PHE0009943_21127	8	ArabidopsisQSR1-like	1.00E-127	100	18408550	gb|AAM64974.1| 60S ribosomal protein L10 [Arabidopsis thaliana]
265	563	PHE0009948_21132	8	Soy QSR1-like	1.00E-120	93	92870109	gb|ABE79479.1 |Ribosomal protein L10E [Medicago truncatula]
266	564	PHE0011084_23040	30	Soy SNG1_sinapoylglucose:malate sinapoyltransferase	0	77	92896730	gb|ABE93328.1|Peptidase S10, serine carboxypeptidase [Medicago truncatula]
267	565	PHE0008233_23042	30	Soy Phi-1 protein	1.00E-122	71	3759184	dbj|BAA33810.1| phi-1 [Nicotiana tabacum]
268	566	PHE0010854_22730	13	Soy regulator of G-protein signaling	1.00E-160	58	22331342	ref|NP_189238.2| Regulator of G-protein signaling 1 [Arabidopsis thaliana]
269	567	PHE0003797_23051	10	Corn DNA-binding protein Dof2	1.00E-44	63	1061306	emb|CAA56287.1| Dof2 [Zea mays]
270	568	PHE0010194_21769	30	Arabidopsis putative endo-1, 4-beta glucanase	0	94	15235301	emb|CAB80722.1| putative endo-1, 4-beta glucanase [Arabidopsis thaliana]
271	569	PHE0010197_21429	13	Corn putative DNA binding protein	2.00E-25	72	115451027	gb|ABF94221.1| bHLH family protein [Oryza sativa (japonica cultivar-group)]
271	569	PHE0010197_21454	5	Corn putative DNA binding protein	2.00E-25	72	115451027	gb|ABF94221.1| bHLH family protein [Oryza sativa (japonica cultivar-group)]
271	569	PHE0010197_21455	16	Corn putative DNA binding protein	2.00E-25	72	115451027	gb|ABF94221.1| bHLH family protein[Oryza sativa (japonica cultivar-group)]
271	569	PHE0010197_21456	20	Corn putative DNA binding protein	2.00E-25	72	115451027	gb|ABF94221.1| bHLH family protein[Oryza sativa (japonica cultivar-group)]
271	569	PHE0010197_21770	30	Corn putative DNA binding protein	2.00E-25	72	115451027	gb|ABF94221.1| bHLH family protein [Oryza sativa (japonica cultivar-group)]
272	570	PHE0011665_23899	30	Arabidopsis chlorophyll a/b binding protein	1.00E-146	96	15225630	gb|AAM20369.1| putative chlorophyll a/b binding protein [Arabidopsis thaliana]
273	571	PHE0012178_24443	3	Corn putative chain C, structure of the plant transcriptional regulator Pbf-2	4.00E-99	78	115444353	dbj|BAD28032.1| putative chain C, structure of the plant transcriptional regulator Pbf-2 [Oryza sativa (japonica cultivar-group)]
274	572	PHE0012170_24424	3	Arabidopsis AGROS-like	7.00E-46	74	30689607	gb|ABC96792.1| ARGOS-like [Arabidopsis thaliana]

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
275	573	PHE0011064_22994	4	Corn LPA1	0	85	115436512	dbj|BAD52962.1| unknown protein [Oryza sativa (japonica cultivar-group)]
275	573	PHE0011064_23000	8	Corn LPA1	0	85	115436512	dbj|BAD52962.1| unknown protein [Oryza sativa (japonica cultivar-group)]
275	573	PHE0011064_23002	18	Corn LPA1	0	85	115436512	dbj|BAD52962.1| unknown protein [Oryza sativa (japonica cultivar-group)]
275	573	PHE0011064_23003	9	Corn LPA1	0	85	115436512	dbj|BAD52962.1| unknown protein [Oryza sativa (japonica cultivar-group)]
275	573	PHE0011064_23004	10	Corn LPA1	0	85	115436512	dbj|BAD52962.1| unknown protein [Oryza sativa (japonica cultivar-group)]
276	574	PHE0010201_21768	30	Soy DNA-binding protein	9.00E-30	75	30689711	ref|NP_849712.1 | transcription regulator [Arabidopsis thaliana]
277	575	PHE0010838_22702	30	Soy PIN1	3.00E-32	71	25140423	gb|AAN71616.1| PIN-like protein [Gossypium hirsutum]
278	576	PHE0010201_21433	13	Soy DNA-binding protein	1.00E-29	75	30689711	ref|NP_849712.1| transcription regulator [Arabidopsis thaliana]
279	577	PHE0011446_23659	13	Corn histone H3-1	7.00E-59	87	166384	gb|AAA32655.1| histone H3 (H3-1.1)
280	578	PHE0001424_22077	9	Corn seed storage protein, 35K isoform AmA1 -	1.00E-68	41	57233444	gb|AAW48295.1| pore-forming toxin-like protein Hfr-2 [Triticum aestivum]
281	579	PHE0011445_23658	13	Corn putative elicitor-responsive gene-3	3.00E-71	89	115447529	dbj|BAD25356.1| putative elicitor-responsive gene-3 [Oryza sativa (japonica cultivar-group)]
282	580	PHE0010090_21297	21	Corn receptor protein kinase CLAVATA1	0	79	115451705	gb|ABF94768.1| Receptor protein kinase CLAVATA1 precursor,[Oryza sativa (japonica cultivar-group)]
283	581	PHE0012180_24445	7	Corn regulatory protein viviparous-1	0	83	138603	sp|P26307| Maize regulatory protein viviparous-1
284	582	PHE0011083_23039	30	Soy SAG13 like homolog	8.00E-87	63	15239327	ref|NP_196225.1|oxidoreductase; dbj|BAA98195.1| short chain alcohol dehydrogenase-like [Arabidopsis thaliana]
285	583	PHE0011085_23041	30	Soy SNG1_sinapoylglucose:malate sinapoyltransferase	1.00E-157	62	92896727	gb|ABE93325.1|Peptidase S10, serine carboxypeptidase [Medicago truncatula]
286	584	PHE0012175_24440	3	Corn TSO1_like	0	62	115489476	gb|ABA99828.1| Tesmin/TSO1-like CXC domain containing protein [Oryza sativa (japonica cultivar-group)]
286	584	PHE0012175_24448	7	Corn TSO1_like	0	62	115489476	gb|ABA99828.1| Tesmin/TSO1-like CXC domain containing protein [Oryza sativa (japonica cultivar-group)]
287	585	PHE0012177_24442	3	Corn P24_like	1.00E-101	74	119638471	gb|ABL85062.1| expressed protein [Brachypodium sylvaticum]
288	586	PHE0011447_23660	13	Corn P0031D02.12 protein	1.00E-71	80	115440763	gb|EAY76322.1| hypothetical protein OsI_004169 [Oryza sativa (indica cultivar-group)]
289	587	PHE0010194_21426	13	Arabidopsis putative endo-1, 4-beta glucanase	0	95	15235301	emb|CAB80722.1| putative endo-1, 4-beta glucanase [Arabidopsis thaliana]

NUC SEQ ID NO	PEP SEQ ID NO	Gene ID	BV ID	Gene Name	e-value	% id	GenBank ID	Annotation
290	588	PHE0011666_23900	30	Soy SPDS	1.00E-165	87	99083515	gb|ABF66657.1| spermidine synthase [Ammopiptanthus mongolicus]
291	589	PHE0011448_24160	13	Corn histone H4-2	2.00E-30	80	115479301	ref|NP_001063244.1| Os09g0433500 [Oryza sativa (japonica cultivar-group)]
292	590	PHE0008324_18636	13	Arabidopsis T27I1.4 protein	0	86	15218372	ref|NP_172473.1| unknown protein [Arabidopsis thaliana]
293	591	PHE0006971_16875	13	Ceres82Rice unknown protein	1.00E-124	84	125583584	gb|EAZ24515.1| hypothetical protein OsJ_007998 [Oryza sativa (japonica cultivar-group)]
294	592	PHE0009939_21147	20	Arabidopsis glycine dehydrogenase	0	98	15234036	ref|NP_195027.1| glycine dehydrogenase [Arabidopsis thaliana]
295	593	PHE0009953_21137	9	Corn dicarboxylate translocator 1	0	89	40363459	dbj|BAD06219.1| plastidic 2-oxoglutarate/malate transporter [Zea mays]
296	594	PHE0006907_16797	13	Corn DUF150_MON_ZM41251	5.00E-94	60	115458524	ref|NP_001052862.1| Os04g0438300 [Oryza sativa (japonica cultivar-group)]
297	595	PHE0008557_18970	13	Corn FAD_binding_6[1]::NAD_binding_1[1] ferredoxin-NADP reductase	0	89	115455751	dbj|BAA07479.1| root ferredoxin-NADP+ reductase [Oryza sativa (japonica cultivar-group)]
298	596	PHE0006443_15955	13	Arabidopsis putative SET-domain protein	0	98	15226918	gb|AAL32813.1| putative SET-domain protein [Arabidopsis thaliana]

Selection methods for transgenic plants with enhanced agronomic trait

Within a population of transgenic plants regenerated from plant cells transformed with the recombinant DNA many plants that survive to fertile transgenic plants that produce seeds and progeny plants will not exhibit an enhanced agronomic trait. Selection from the population is necessary to identify one or more transgenic plant cells that can provide plants with the enhanced trait. Transgenic plants having enhanced traits are selected from populations of plants regenerated or derived from plant cells transformed as described herein by evaluating the plants in a variety of assays to detect an enhanced trait, e.g. enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil. These assays also may take many forms including, but not limited to, direct screening for the trait in a greenhouse or field trial or by screening for a surrogate trait. Such analyses can be directed to detecting changes in the chemical composition, biomass, physiological properties, morphology of the plant. Changes in chemical compositions such as nutritional composition of grain can be detected by analysis of the seed composition and content of protein, free amino acids, oil, free fatty acids, starch or tocopherols. Changes in biomass characteristics can be made on greenhouse or field

grown plants and can include plant height, stem diameter, root and shoot dry weights; and, for corn plants, ear length and diameter. Changes in physiological properties can be identified by evaluating responses to stress conditions, for example assays using imposed stress conditions such as water deficit, nitrogen deficiency, cold growing conditions, pathogen or insect attack or light deficiency, or increased plant density. Changes in morphology can be measured by visual observation of tendency of a transformed plant with an enhanced agronomic trait to also appear to be a normal plant as compared to changes toward bushy, taller, thicker, narrower leaves, striped leaves, knotted trait, chlorosis, albino, anthocyanin production, or altered tassels, ears or roots. Other selection properties include days to pollen shed, days to silking, leaf extension rate, chlorophyll content, leaf temperature, stand, seedling vigor, internode length, plant height, leaf number, leaf area, tillering, brace roots, stay green, stalk lodging, root lodging, plant health, barreness/prolificacy, green snap, and pest resistance. In addition, phenotypic characteristics of harvested grain may be evaluated, including number of kernels per row on the ear, number of rows of kernels on the ear, kernel abortion, kernel weight, kernel size, kernel density and physical grain quality. Although the plant cells and methods of this invention can be applied to any plant cell, plant, seed or pollen, e.g. any fruit, vegetable, grass, tree or ornamental plant, the various aspects of the invention are preferably applied to corn, soybean, cotton, canola, alfalfa, wheat and rice plants.

The following examples are included to demonstrate aspects of the invention, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific aspects which are disclosed and still obtain a like or similar results without departing from the spirit and scope of the invention.

Example 1. Plant Expression Constructs

This example illustrates the construction of plasmids for transferring recombinant DNA into plant cells which can be regenerated into transgenic plants of this invention

A. Plant expression constructs for corn transformation

A base corn transformation vector pMON93039, as set forth in SEQ ID NO: 30521, illustrated in Table 3 and Figure 2, was fabricated for use in preparing recombinant DNA for *Agrobacterium*-mediated transformation into corn tissue.

Table 3.

Function	Name	Annotation	Coordinates of SEQ ID NO: 30521

Function	Name	Annotation	Coordinates of SEQ ID NO: 30521
Agrobacterium T-DNA transfer	B-AGRtu.right border	Agro right border sequence, essential for transfer of T-DNA.	11364-11720
Gene of interest expression cassette	E-Os.Act1	Upstream promoter region of the rice actin 1 gene	19-775
	E-CaMV.35S.2xA1-B3	Duplicated35S A1-B3 domain without TATA box	788-1120
	P-Os.Act1	Promoter region of the rice actin 1 gene	1125-1204
	L-Ta.Lhcb1	5' untranslated leader of wheat major chlorophyll a/b binding protein	1210-1270
	I-Os.Act1	First intron and flanking UTR exon sequences from the rice actin 1 gene	1287-1766
	T-St.Pis4	3' non-translated region of the potato proteinase inhibitor II gene which functions to direct polyadenylation of the mRNA	1838-2780
Plant selectable marker expression cassette	P-Os.Act1	Promoter from the rice actin 1 gene	2830-3670
	L-Os.Act1	First exon of the rice actin 1 gene	3671-3750
	I-Os.Act1	First intron and flanking UTR exon sequences from the rice actin 1 gene	3751-4228
	TS-At.ShkG-CTP2	Transit peptide region of Arabidopsis EPSPS	4238-4465
	CR-AGRtu.aroA-CP4.nat	Coding region for bacterial strain CP4 native aroA gene.	4466-5833
	T-AGRtu.nos	A 3' non-translated region of the nopaline synthase gene of Agrobacterium tumefaciens Ti plasmid which functions to direct polyadenylation of the mRNA.	5849-6101
Agrobacterium T-DNA transfer	B-AGRtu.left border	Agro left border sequence, essential for transfer of T-DNA.	6168-6609
Maintenance in E. coli	OR-Ec.oriV-RK2	The vegetative origin of replication from plasmid RK2.	6696-7092
	CR-Ec.rop	Coding region for repressor of primer from the ColE1 plasmid. Expression of this gene product interferes with primer binding at the origin of replication, keeping plasmid copy number low.	8601-8792
	OR-Ec.ori-ColE1	The minimal origin of replication from the E. coli plasmid ColE1.	9220-9808
	P-Ec.aadA-SPC/STR	Promoter for Tn7 adenylyltransferase (AAD(3"))	10339-10380

Function	Name	Annotation	Coordinates of SEQ ID NO: 30521
	CR-Ec.aadA-SPC/STR	Coding region for Tn7 adenylyltransferase (AAD(3")) conferring spectinomycin and streptomycin resistance.	10381-11169
	T-Ec.aadA-SPC/STR	3' UTR from the Tn7 adenylyltransferase (AAD(3")) gene of E. coli.	11170-11227

Other base vectors similar to the one described above were also constructed as listed in Table 4. These base corn transformation vectors are also used for wheat and rice transformations. See Table 4 for a summary of base vectors and base vector ID's which are referenced in Table 2. Also see Table 5 for a summary of regulatory elements used in the gene expression cassettes for these base vectors and the SEQ ID NOs for these elements.

Primers for PCR amplification of protein coding nucleotides of recombinant DNA are designed at or near the start and stop codons of the coding sequence, in order to eliminate most of the 5' and 3' untranslated regions. Each recombinant DNA coding for a protein identified in Table 2 is amplified by PCR prior to insertion into the insertion site within the gene of interest expression cassette of one of the base vectors as referenced in Table 2.

Table 4

Base Vector ID	Corn Base Vector
1	pMON92709
2	pMON92713
3	pMON92715
4	pMON92716
5	pMON92718
6	pMON92719
7	pMON92721
8	pMON92722
9	pMON92724
10	pMON92729
11	pMON93025
12	pMON93026
13	pMON93039
14	pMON93043
15	pMON94781
16	pMON94830
17	pMON99009
18	pMON99048
19	pMON99051
20	pMON99052
21	pMON100406

Base Vector ID	Corn Base Vector
Base Vector ID	**Soy And Canola Base Vector**
22	pMON74537
23	pMON74552
24	pMON74553
25	pMON82053
26	pMON83145
27	pMON92671
28	pMON97027
29	pMON99006
30	pMON100407
31	pMON102815
32	pMON102819
Base Vector ID	**Cotton Base Vector**
33	pMON95549
34	pMON99053

Table 5

Base vector	Promoter	SEQ ID NO	Leader	SEQ ID NO	Intron	SEQ ID NO
pMON92709	P-Os.GT1	30469	L-Os.GT1	30493	I-Zm.DnaK	30516
pMON92713	P-Zm.P39486	30470	L-Zm.39486	30494	I-Zm.DnaK	30516
pMON92715	P-Hv.Per1	30471	L-Hv.Per1	30495	I-Zm.DnaK	30516
pMON92716	P-Zm.FDA	30472	L-Zm.FDA	30496	I-Zm.DnaK	30516
pMON92718	P-Zm.CLK1	30473	L-Zm.Cik1	30497	I-Zm.Cik1	30517
pMON92719	P-Zm.RAB17	30474	L-Zm.RAB17	30498	I-Zm.DnaK	30516
pMON92721	P-Zm.SzeinC1	30475	L-Zm.SzeinC1	30499	I-Zm.DnaK	30516
pMON92722	P-CaMV.35S-enh	30476	L-CaMV.35S	30500	I-Zm.DnaK	30516
pMON92724	P-Zm.-636aldolase-0:1:2 + P-Zm.PPDK	30477	L-Zm.PPDK	30501	I-Zm.DnaK	30516
pMON92729	P-Zm.PPDK	30478	L-Zm.PPDK	30501	I-Zm.DnaK	30516
pMON93025	P-At.SUC2	30479	L-At.SUC2	30502	I-Zm.DnaK	30516
pMON93026	P-Os.H1	30480	L-Os.H1	30503	I-Zm.DnaK	30516
pMON93039	E-Os.Act1 + E-CaMV.35S. 2xA1-B3 + P-Os.Act1	30481	L-Ta.Lhcb1	30504	I-Os.Act1	30518
pMON93043	P-Zm.EM	30482	L-Zm.EM	30505	I-Zm.DnaK	30516
pMON94781	P-Zm.Brittle-2	30483	L-Zm.Brittle-2	30506	I-Zm.DnaK	30516
pMON94830	P-Os.Act16	30484	L-Os.Act16	30507	I-Os.Act16	30519
pMON99009	P-CaMV.35S-enh	30476	L-Ph.DnaK	30508	None	/
pMON99048	P-Os.Act1+E-CaMV.35S.2xA1-B3	30485	L-Ta.Lhcb1	30504	I-Os.Act1	30518
pMON99051	P-Zm.Kn1	30486	L-Zm.Kn1	30509	None	/
pMON99052	P-Os.Rcc3	30487	L-Os.Rcc3	30510	I-Zm.DnaK	30516
pMON100406	P-Zm.Kn1	30486	L-Zm.Kn1	30509	I-Zm.DnaK	30516
pMON74537	P-At.RbcS4	30488	L-At.RbcS4	30511	None	/
pMON74552	P-CaMV.35S-enh	30476	None	/	None	/
pMON74553	P-CaMV.35S-enh	30476	None	/	None	/
pMON82053	P-CaMV.35S-enh	30476	None	/	None	/
pMON83145	P-Gm.Hsp20	30489	L-Gm.Hsp20	30512	None	/
pMON92671	P-At.SAMS3	30490	L-At.SAMS3	30513	I-At.SAMS3	30520
pMON97027	P-At.GRP7	30491	L-At.GRP7	30514	None	/
pMON99006	P-CaMV.35S-enh	30476	None	/	None	/
pMON100407	P-CaMV.35S-enh	30476	L-Ph.DnaK	30508	None	/
pMON102815	P-At.Erecta	30492	L-At.Erecta	30515	None	/
pMON102819	P-CaMV.35S-enh	30476	None	/	None	/
pMON95549	P-At.SAMS3	30490	L-At.SAMS3	30513	I-At.SMAS3	30520
pMON99053	P-CaMV.35S-enh	30476	L-Ph.DnaK	30508	None	/

For construct pMON94830, the L-Os.Act16 leader sequence (SEQ ID IN 30507) is interrupted by the I-Os.Act16 intron sequence (SEQ ID NO 30519) between nucleotide positions 3344 and 3861.

B. Plant expression constructs for soy and canola transformation

Vectors for use in transformation of soybean and canola were also prepared. Elements of an exemplary common expression vector pMON82053 are shown in Table 6 below and Figure 3. Another example of elements used in plant expression cassestte for gene of interest such as PEP SEQ ID NOs 366 and 521 were the P-Gm.Sphas1 promoter (SEQ ID NO: 30524) and the L-Gm.Sphas1 leader (SEQ ID NO: 30525) sequences.

Table 6.

Function	Name	Annotation	Coordinates of SEQ ID NO: 30522
Agrobacterium T-DNA transfer	B-AGRtu.left border	Agro left border sequence, essential for transfer of T-DNA.	6144-6585
Plant selectable marker expression cassette	P-At.Act7	Promoter from the Arabidopsis actin 7 gene	
	L-At.Act7	5'UTR of Arabidopsis Act7 gene	
	I-At.Act7	Intron from the Arabidopsis actin7 gene	6624-7861
	TS-At.ShkG-CTP2	Transit peptide region of Arabidopsis EPSPS	7864-8091
	CR-AGRtu.aroA-CP4.nno_At	Synthetic CP4 coding region with dicot preferred codon usage.	8092-9459
	T-AGRtu.nos	A 3' non-translated region of the nopaline synthase gene of Agrobacterium tumefaciens Ti plasmid which functions to direct polyadenylation of the mRNA.	9466-9718
Gene of interest expression cassette	P-CaMV.35S-enh	Promoter for 35S RNA from CaMV containing a duplication of the -90 to -350 region.	1-613
	T-Gb.E6-3b	3' untranslated region from the fiber protein E6 gene of sea-island cotton.	688-1002
Agrobacterium T-DNA transfer	B-AGRtu.right border	Agro right border sequence, essential for transfer of T-DNA.	1033-1389
Maintenance in E. coli	OR-Ec.oriV-RK2	The vegetative origin of replication from plasmid RK2.	5661-6057
	CR-Ec.rop	Coding region for repressor of primer from the ColE1 plasmid. Expression of this gene product interferes with primer binding at the origin of replication, keeping plasmid copy number low.	3961-4152
	OR-Ec.ori-ColE1	The minimal origin of replication from the E. coli plasmid ColE1.	2945-3533
	P-Ec.aadA-SPC/STR	Promoter for Tn7 adenylyltransferase (AAD(3"))	2373-2414

Function	Name	Annotation	Coordinates of SEQ ID NO: 30522
	CR-Ec.aadA-SPC/STR	Coding region for Tn7 adenylyltransferase (AAD(3")) conferring spectinomycin and streptomycin resistance.	1584-2372
	T-Ec.aadA-SPC/STR	3' UTR from the Tn7 adenylyltransferase (AAD(3")) gene of E. coli.	1526-1583

Primers for PCR amplification of protein coding nucleotides of recombinant DNA are designed at or near the start and stop codons of the coding sequence, in order to eliminate most of the 5' and 3' untranslated regions. Each recombinant DNA coding for a protein identified in Table 2 is amplified by PCR prior to insertion into the insertion site within the gene of interest expression cassette of one of the base vectors as referenced in Table 2.

C. Cotton transformation vector

Plasmids for use in transformation of cotton were also prepared. Elements of an exemplary common expression vector pMON99053 are shown in Table 7 below and Figure 4. Primers for PCR amplification of protein coding nucleotides of recombinant DNA are designed at or near the start and stop codons of the coding sequence, in order to eliminate most of the 5' and 3' untranslated regions. Each recombinant DNA coding for a protein identified in Table 2 is amplified by PCR prior to insertion into the insertion site within the gene of interest expression cassette of one of the base vectors as referenced in Table 2.

Table 7.

Function	Name	Annotation	Coordinates of SEQ ID NO: 30523
Agrobacterium T-DNA transfer	B-AGRtu.right border	Agro right border sequence, essential for transfer of T-DNA.	1-357
Gene of interest expression cassette	Exp-CaMV.35S-enh+Ph.DnaK	Enhanced version of the 35S RNA promoter from CaMV plus the petunia hsp70 5' untranslated region	388-1091
	T-Ps.RbcS2-E9	The 3' non-translated region of the pea RbcS2 gene which functions to direct polyadenylation of the mRNA.	1165- 1797
Plant selectable marker expression	Exp-CaMV.35S	Promoter and 5' untranslated region from the 35S RNA of CaMV	1828-2151

Function	Name	Annotation	Coordinates of SEQ ID NO: 30523
cassette	CR-Ec.nptII-Tn5	Coding region for neomycin phosphotransferase gene from transposon Tn5 which confers resistance to neomycin and kanamycin.	2185-2979
	T-AGRtu.nos	A 3' non-translated region of the nopaline synthase gene of Agrobacterium tumefaciens Ti plasmid which functions to direct polyadenylation of the mRNA.	3011-3263
Agrobacterium T-DNA transfer	B-AGRtu.left border	Agro left border sequence, essential for transfer of T-DNA.	3309-3750
Maintenance in E. coli	OR-Ec.oriV-RK2	The vegetative origin of replication from plasmid RK2.	3837-4233
	CR-Ec.rop	Coding region for repressor of primer from the ColE1 plasmid. Expression of this gene product interferes with primer binding at the origin of replication, keeping plasmid copy number low.	5742-5933
	OR-Ec.ori-ColE1	The minimal origin of replication from the E. coli plasmid ColE1.	6361-6949
	P-Ec.aadA-SPC/STR	Promoter for Tn7 adenylyltransferase (AAD(3"))	7480-7521
	CR-Ec.aadA-SPC/STR	Coding region for Tn7 adenylyltransferase (AAD(3")) conferring spectinomycin and streptomycin resistance.	7522-8310
	T-Ec.aadA-SPC/STR	3' UTR from the Tn7 adenylyltransferase (AAD(3")) gene of E. coli.	8311-8368

Example 2. Corn Transformation

This example illustrates plant cell transformation methods useful in producing transgenic corn plant cells, plants, seeds and pollen of this invention and the production and identification of transgenic corn plants and seed with an enhanced trait, i.e. enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil. Plasmid vectors were prepared by cloning DNA

identified in Table 2 in the identified base vectors for use in corn transformation of corn plant cells to produce transgenic corn plants and progeny plants, seed and pollen.

For *Agrobacterium*-mediated transformation of corn embryo cells corn plants of a readily transformable line are grown in the greenhouse and ears are harvested when the embryos are 1.5 to 2.0 mm in length. Ears are surface sterilized by spraying or soaking the ears in 80% ethanol, followed by air drying. Immature embryos are isolated from individual kernels on surface sterilized ears. Prior to inoculation of maize cells, *Agrobacterium* cells are grown overnight at room temperature. Immature maize embryo cells are inoculated with *Agrobacterium* shortly after excision, and incubated at room temperature with *Agrobacterium* for 5-20 minutes. Immature embryo plant cells are then co-cultured with *Agrobacterium* for 1 to 3 days at 23°C in the dark. Co-cultured embryos are transferred to selection media and cultured for approximately two weeks to allow embryogenic callus to develop. Embryogenic callus is transferred to culture medium containing 100 mg/L paromomycin and subcultured at about two week intervals. Transformed plant cells are recovered 6 to 8 weeks after initiation of selection.

For *Agrobacterium*-mediated transformation of maize callus immature embryos are cultured for approximately 8-21 days after excision to allow callus to develop. Callus is then incubated for about 30 minutes at room temperature with the *Agrobacterium* suspension, followed by removal of the liquid by aspiration. The callus and *Agrobacterium* are co-cultured without selection for 3-6 days followed by selection on paromomycin for approximately 6 weeks, with biweekly transfers to fresh media. Paromomycin resistant calli are identified about 6-8 weeks after initiation of selection.

For transformation by microprojectile bombardment maize immature embryos are isolated and cultured 3-4 days prior to bombardment. Prior to microprojectile bombardment, a suspension of gold particles is prepared onto which the desired recombinant DNA expression cassettes are precipitated. DNA is introduced into maize cells as described in U.S. Patents 5,550,318 and 6,399,861 using the electric discharge particle acceleration gene delivery device. Following microprojectile bombardment, tissue is cultured in the dark at 27°C. Additional transformation methods and materials for making transgenic plants of this invention, for example, various media and recipient target cells, transformation of immature embryos and subsequence regeneration of fertile transgenic plants are disclosed in U.S. Patents 6,194,636 and

6,232,526 and U.S. Patent application Serial No. 09/757,089, which are incorporated herein by reference.

To regenerate transgenic corn plants a callus of transgenic plant cells resulting from transformation and selection is placed on media to initiate shoot development into plantlets which are transferred to potting soil for initial growth in a growth chamber at 26°C followed by a mist bench before transplanting to 5 inch pots where plants are grown to maturity. The regenerated plants are self-fertilized and seed is harvested for use in one or more methods to select seeds, seedlings or progeny second generation transgenic plants (R2 plants) or hybrids, e.g. by selecting transgenic plants exhibiting an enhanced trait as compared to a control plant.

Transgenic corn plant cells are transformed with recombinant DNA from each of the genes identified in Table 2. Progeny transgenic plants and seed of the transformed plant cells are screened for enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil as reported in Example 7.

Example 3. Soybean transformation

This example illustrates plant transformation useful in producing the transgenic soybean plants of this invention and the production and identification of transgenic seed for transgenic soybean having enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil.

For *Agrobacterium* mediated transformation, soybean seeds are imbided overnight and the meristem explants excised. The explants are placed in a wounding vessel. Soybean explants and induced *Agrobacterium* cells from a strain containing plasmid DNA with the gene of interest cassette and a plant selectable marker cassette are mixed no later than 14 hours from the time of initiation of seed imbibition, and wounded using sonication. Following wounding, explants are placed in co-culture for 2-5 days at which point they are transferred to selection media for 6-8 weeks to allow selection and growth of transgenic shoots. Resistant shoots are harvested approximately 6-8 weeks and placed into selective rooting media for 2-3 weeks. Shoots producing roots are transferred to the greenhouse and potted in soil. Shoots that remain healthy on selection, but do not produce roots are transferred to non-selective rooting media for an additional two weeks. Roots from any shoots that produce roots off selection are tested for

expression of the plant selectable marker before they are transferred to the greenhouse and potted in soil. Additionally, a DNA construct can be transferred into the genome of a soybean cell by particle bombardment and the cell regenerated into a fertile soybean plant as described in U.S. Patent 5,015,580, herein incorporated by reference.

Transgenic soybean plant cells are transformed with recombinant DNA from each of the genes identified in Table 2. Transgenic progeny plants and seed of the transformed plant cells are screened for enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil as reported in Example 7.

Example 4. Cotton transgenic plants with enhanced agronomic traits

Cotton transformation is performed as generally described in WO0036911 and in U.S. Pat. No. 5,846,797. Transgenic cotton plants containing each of the recombinant DNA having a sequence of SEQ ID NO: 1 through SEQ ID NO: 298 are obtained by transforming with recombinant DNA from each of the genes identified in Table 1. Progeny transgenic plants are selected from a population of transgenic cotton events under specified growing conditions and are compared with control cotton plants. Control cotton plants are substantially the same cotton genotype but without the recombinant DNA, for example, either a parental cotton plant of the same genotype that was not transformed with the identical recombinant DNA or a negative isoline of the transformed plant. Additionally, a commercial cotton cultivar adapted to the geographical region and cultivation conditions, i.e. cotton variety ST474, cotton variety FM 958, and cotton variety Siokra L-23, are used to compare the relative performance of the transgenic cotton plants containing the recombinant DNA. The specified culture conditions are growing a first set of transgenic and control plants under "wet" conditions, i.e. irrigated in the range of 85 to 100 percent of evapotranspiration to provide leaf water potential of -14 to -18 bars, and growing a second set of transgenic and control plants under "dry" conditions, i.e. irrigated in the range of 40 to 60 percent of evapotranspiration to provide a leaf water potential of -21 to -25 bars. Pest control, such as weed and insect control is applied equally to both wet and dry treatments as needed. Data gathered during the trial includes weather records throughout the growing season including detailed records of rainfall; soil characterization information; any herbicide or insecticide applications; any gross agronomic differences

observed such as leaf morphology, branching habit, leaf color, time to flowering, and fruiting pattern; plant height at various points during the trial; stand density; node and fruit number including node above white flower and node above crack boll measurements; and visual wilt scoring. Cotton boll samples are taken and analyzed for lint fraction and fiber quality. The cotton is harvested at the normal harvest timeframe for the trial area. Enhanced water use efficiency is indicated by increased yield, improved relative water content, enhanced leaf water potential, increased biomass, enhanced leaf extension rates, and improved fiber parameters.

The transgenic cotton plants of this invention are identified from among the transgenic cotton plants by agronomic trait screening as having increased yield and enhanced water use efficiency.

Example 5. Canola transformation

This example illustrates plant transformation useful in producing the transgenic canola plants of this invention and the production and identification of transgenic seed for transgenic canola having enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil.

Tissues from *in vitro* grown canola seedlings are prepared and inoculated with overnight-grown *Agrobacterium* cells containing plasmid DNA with the gene of interest cassette and a plant selectable marker cassette. Following co-cultivation with *Agrobacterium*, the infected tissues are allowed to grow on selection to promote growth of transgenic shoots, followed by growth of roots from the transgenic shoots. The selected plantlets are then transferred to the greenhouse and potted in soil. Molecular characterization are performed to confirm the presence of the gene of interest, and its expression in transgenic plants and progenies. Progeny transgenic plants are selected from a population of transgenic canola events under specified growing conditions and are compared with control canola plants. Control canola plants are substantially the same canola genotype but without the recombinant DNA, for example, either a parental canola plant of the same genotype that is not transformed with the identical recombinant DNA or a negative isoline of the transformed plant

Transgenic canola plant cells are transformed with recombinant DNA from each of the genes identified in Table 2. Transgenic progeny plants and seed of the transformed plant cells are screened for enhanced water use efficiency, enhanced cold tolerance, increased yield,

enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil as reported in Example 7.

Example 6. Homolog Identification

This example illustrates the identification of homologs of proteins encoded by the DNA identified in Table 2 which is used to provide transgenic seed and plants having enhanced agronomic traits. From the sequence of the homologs, homologous DNA sequence can be identified for preparing additional transgenic seeds and plants of this invention with enhanced agronomic traits.

An "All Protein Database" was constructed of known protein sequences using a proprietary sequence database and the National Center for Biotechnology Information (NCBI) non-redundant amino acid database (nr.aa). For each organism from which a polynucleotide sequence provided herein was obtained, an "Organism Protein Database" was constructed of known protein sequences of the organism; it is a subset of the All Protein Database based on the NCBI taxonomy ID for the organism.

The All Protein Database was queried using amino acid sequences provided herein as SEQ ID NO: 299 through SEQ ID NO: 596 using NCBI "blastp" program with E-value cutoff of 1e-8. Up to 1000 top hits were kept, and separated by organism names. For each organism other than that of the query sequence, a list was kept for hits from the query organism itself with a more significant E-value than the best hit of the organism. The list contains likely duplicated genes of the polynucleotides provided herein, and is referred to as the Core List. Another list was kept for all the hits from each organism, sorted by E-value, and referred to as the Hit List.

The Organism Protein Database was queried using polypeptide sequences provided herein as SEQ ID NO: 299 through SEQ ID NO: 596 using NCBI "blastp" program with E-value cutoff of 1e-4. Up to 1000 top hits were kept. A BLAST searchable database was constructed based on these hits, and is referred to as "SubDB". SubDB was queried with each sequence in the Hit List using NCBI "blastp" program with E-value cutoff of 1e-8. The hit with the best E-value was compared with the Core List from the corresponding organism. The hit is deemed a likely ortholog if it belongs to the Core List, otherwise it is deemed not a likely ortholog and there is no further search of sequences in the Hit List for the same organism. Homologs from a large number of distinct organisms were identified and are reported by amino acid sequences of

SEQ ID NO: 597 through SEQ ID NO: 30468. These relationships of proteins of SEQ ID NO: 299 through 596 and homologs of SEQ ID NO: 597 through 30468 are identified in Table 8. The source organism for each homolog is found in the Sequence Listing.

Table 8.

PEP SEQ ID NO:homolog SEQ ID NOs

299:22505 17330 17332 3832 26661 24604 24601 26617 26553 26585 26591 26640
26582 26471 26635 26572 26576 26556 26609 26423 25650 26551 2373 2405
24690 25446 25450 26422 26473 25340 10489 21242 21238 21241 21279 24723
24725 25494 25690 25164 16529 7978 10037 16615 16621 11055 12923 6955
7874 7948 7839 8664 12144 14757 17096 14796 14828 26829 12171 21124
23048 20000 23191 22333 20933 15625 22212 19851 28192 27896 22998 22992
15629 28137 24133 22151 29931 985 2060 944 28955 28930 28990 29965
15658 21486 21255 20071 19073 19014 18158 18127 18109 15620 2239 2233
16398 14851 11657 12588 11744 14570 13748 13790 12143 12170 12173 12919
15622 15628 15656 15604 16393 16394 16392 12916 11037 22432 8701 24656
19135 19853 7817 18495 2869 8263 29437 29438 22440 22435 21711 22465
21146 17031 19010 21907 27742 22415 5429 1188 5421 18365 23435 12382
12090 12087 10571 18362 23640 15382 987 6445 6417 5413 8888 2538
24892 15423 1089 1127 5417 21701 24630 24633 21702 21705 25589 25558
21697 7438 22989 17339 1478 1506 1479 9199 25171 26307 1472 25424
25418 5394 23942 8884 8887 12914 23541
300:16906 20559 21806 5055 4905 9056 13559 14510 4167 13364 13441 11165
3862 1913 6572 9353 29885 5672 23667 20433 11405 26215 8142 13983
9317 1831 16167 26355 11049 12454 607 20163 21550 8941 3691 19336
8960 11635 2500 12784 9832 13853 14431 6891 25892 24022 26873 8074
10050 6314 15883 23231 829 4758 4754 28646 4717 20464 9858 7971
12622 20501 4271 12195 9291 19503 21548 4124 10854 26029 12272 1775
25590 22837 22812 22556 10678 16223 25625 21334 8456 22176 14327 14302
17822 17624 16859 16850 17780 17619 17629 16852 10588 10650 1001 29219
15690 20293 25766 12151 21485 14320 17504 15478 15477 15066 15835 30365
30421 2696 20311 3417 19744 18488 3839 8782 17965 3743 3775 17509
17514 7891 6457 12394 12411 4075 4077 17590 21909 28286 3388 16788
15982 7848 29135 20349 19157 14248 29979 17396 17358 17398 17407 17324
17410 17366 17403 17357 17368 17360 19996 19846 11904 23714 2173 22814
22809 20197 29687 3983 24318 21492 20319 26103 28858 6802 20161 3448
4055 12112 7750 12606 13406 12501 6292 9527 13807 3896 12598 5152
5149 5155 368 10357 26816
301:23796 2040 5965 5087 6920 4213 4210 3372 5323 16802 27336 6010
8202 26922 2187 12208 19871 11517 22010 9197
302:23710 23757 13694 19592 23650 22298 8204 25706 24745 15742 30254 26637
15339 21922 20145 25220 16474 13767 24177 23168 13704
303: 3118 4379 29540 19057 23492 24494 29662 25823 22886 22040 8636
304:26286 15828 29381 9463 1447 21905 14853 28599 20781 27729 18418 27603
25716 26765 16175 23282 13862 1876 2531 5158 2533 1911 11707 6224
26059 22774 10047 19527 24245 14187 10461 29433 11856 11359 8254 1796
13315 5545 10252 6191 13314 17203 23729 19203 6192 6228 29464 11849
11846 6202 6193 27557 1797 10397 18417 9442 9470 9471 9441 2568
1789 6233 6227 6222 2751 26484 19634 2753 2526 10548 2523 1794
6151 4931 4935 5392 10581 10578 2167 28003 16045 2532 18805 25644
25642 9834 12912 12907 15640 633 25781 4120 9293 11181 8741 3780
18808 26630 26485 24195 4726 377 14947 2501 18997 20773 19301 16345
18906 7189 15422 18210 21903 25270 24910 2503 10083 10113 10084
305:18758 19672 29878 23393 24854 10271 27282 5416 23140 27083 2820 18194

12159 9628 23204 13486 8110 12511 29407 1503 1698 28122 17599 5085
4965 5960 13895 14534 22724 18476 18472 18083 6734 4997 23491 17752
26978 29291 24877 24879 27070 7649 1949 21111 19488 21730 23945 21686
27651 27650 25269 26607 2212 12263 12554 13918 14019 13994 24823

306: 4073 22555 4100 27267 26975 4996 15934 19811 15228 25434 15706 9550
6935 23481 1480 1482 22482 23641 21374 10611 11281 1517 1518 20953
27704 18146 5286 3202 20016 12556 8797 12122 11246 15875 7987 28966
24975 15587 1524 1520 1525 22082 16220 12190 17779 13034 6068 14309
1994 8624 24174 25476 7577 15375 9142 6515

307:16517 13655 16944 13657 7250 7253 12523 12555 12496 12558 12553 12525
12447 12531 12529 11716 12499 12463 12453 11714 12577 12494 11711 21462
12491 12601 12582 12580 21464 21465 21507 21503 16115 29908 3927 21531
11721 12449 12456 12564 12574 12579 12597 4697 27420 29700 887 888
29909 10124 10158 3563 3567 2220 19295 11183 6149 23835 18693 5946
11374 7886 10617 22967 2516 25498 16862 19210 19213 20295 12609 20321
19214 21959 21952 9615 9870 2134 24575 19069 12843 24557 21955 9616
5341 17200 24499 19179 16766 27442 27444 27475 11328 16431 22721 27181
5216 30264 17131 27106 28708 27417 27447 877 873 875 871 8082
6820 25860 22904 13900 14638 15177 21498 16830 27002 26518 10906

308:21299 5872 17817 22477 17074 5710 14819 10604 17874 1813 10959 10605
15093 26425 26689 15978 7997 20445 27568 22238 24487 14436 8017 11460
11177 23024 7463 17377 12864 16004 7043 10148 5131 17879 2644 4412
30069 7754 23829 27906 18649 18440 12984 10731 1103 9112 16047 10098
7912 16569 16562 6731 17991 27109 737 4745 1628 1918 8843 10143
23669 15513 26808 624 8628 24803 29563 22820 5123 10502 15852 29463
22220 10856 18502 23546 13041 11144 7740 22616 15720 20242 23531 5722
20633 5177 20273 19174 27912 17072 2243 16163 25856 5049 6431 1557
7243 920 10764 22406 29189 5927 25074 30314 11963 2240 3307 17229
1852 25569 23447 28980 12872 29015 25605 11835 16360 5509 30059 20235
4232 777 18344 30237 16693 10540 9369 8759 9835 6007 24894 10066
18130 29536 17856 26868 3903 16811 18196 21620 26548 7365 15998 3435
27303 23365 7429 11162 26510 12980 9574 23826 21579 5242 18786 14691
24298 12247 3422 15077 5617 2735 26140 997 29968 12410 12430 10035
14455 7492 23858 24253 17897 14804 22708 1951 3376 2209 19943 4307
28095 17187 11974 15174 24900 18475 20119 16286 18268 17385 22117 27898
8793 15872 15906 4708 21261 26796 17926 27187 29242 3511 15315 28909
14450 15500 18402 15627 29217 28317 25837 1144 10601 28448 696 19865
19308 28313 24999 21607 2359 23902 7170 26313 18751 16086 24401 23719
26186 20994 6420 27239 24526 30413 5823 15225 19802 25521 16776 22806
5296 9126 19695 20534 28027 27517 28414 17210 6379 15657 5267 30048
12519 10872 7999 3409 15362 18419 9898 24104 13253 13534 12783 16649
21079 7630 26772 22421 1477 28203 24992 8063 2672 9815 13717 30388
28256 22915 19937 8726 10192 5739 848 22943 28147 3316 5705 29741
13175 9919 22404 1054 27138 29191 24754 9604 14403 23630 10191 14456
29106 21213 2964 12239 28568 17977 6904 14167 29917 25066 6760 11818
10338 11895 9584 13160 28158 4901 13340 8682 19451 2848 22433 2436
25490 10991 20029 19992 12490 12785 26327

309:20952 3236 24334 17181 27703 2623 29914 6200 7062 3530 3554 23659
25523 881 10869 12110 6449 24143 20219 18629 16735 28415 10036 20347
20380 24855 4163 9382 18086 13740 12955 3770 21914 17308 26890 5621
4316 22499 13629 7216 14424 1693 15401 20539 26693 4545 6048 10842
24512 4305 18585 2506 19115 14873 12308 18961 24529 9057 20738 7334
29837 22519 24768 3969 15041 24322 23592 29505 3601 11341 994 29145
11268 22722 19591 4525 2445 29611 3707 4339 23545 23449 26341 20094
26889 7940 9580 26304 16101 5572 635 10465 4954 10916 15869 8855
6601 3351 23384 4094 29250 21718 17876 28965 5077 21429 17490 6375
25086 13446 13636 23483 22483 7618 2598 29424 27960 18996 2440 7224

13322 7785 27104 8134 6761 3390 3119 13435 20599 17077 24289 23988
18684 20468 22443 27060 22953 27019 17163 27831 3678 18733 1346 17225
3681 5554 26543 26495 19458 25397 1456 21781 24859 9246 9688 8767
27583 7360 22732 20947 14430 3682 8790 14166 5271 28857 4601 1785
6220 8856 8812 14515 5548 17409 11560 19742 5005 1435 30386 18204
11765 23599 25429 24305 3499 7990 5931 13351 3632 7908 14951 7389
24053 1667 1034 21846 12920 10712 17253 14508 17438 17283 5056 15270
6588 24518 25812 8928 19389 21797 1648 15618 22165

310:27320 24987 3155 1338 1958 13881 9392 5380 13563 19750 8872 25913
27110 26243 7854 7289 14448 28326 27162 12993 29870 16402 16956 13426
7595 22122 24090 19304 11864 17988 24435 18484 5785 18625 19889 18382
5708 21735 18955 9538 23623 5991 16978 9399 20682 16184 15467 13163
12299 17857 17272 10117 9126 1908 20035 18868 1615 22344 21292 5575
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