**Title:** METHODS AND COMPOSITIONS FOR PRODUCTION OF MULTIMERIC PROTEINS IN TRANSGENIC PLANTS

This invention is related to the field of methods and compositions for production of multimeric proteins in transgenic plants. The invention provides a method for producing a heterologous multimeric protein in a transgenic plant cell by transforming a plant cell with a plurality of naked plasmids. Each plasmid encodes less than all of the polypeptide components of the multimeric protein, and the plurality of transformed plasmids encodes all of the polypeptide components of the multimeric protein. The invention also provides a transgenic plant or plant cell expressing a multimeric protein that is heterologous to the plant cell.
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METHODS AND COMPOSITIONS FOR PRODUCTION OF MULTIMERIC PROTEINS IN TRANSGENIC PLANTS

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the priority benefit of provisional U.S. Patent Application 60/079,249, filed March 25, 1998, pending, which is hereby incorporated herein by reference in its entirety.

TECHNICAL FIELD

This invention is related to the field of methods and compositions for production of multimeric proteins in transgenic plants.

BACKGROUND

Transgenic plants have been studied over the past several years for potential use in low cost production of high quality, biologically active mammalian proteins. Of the various mammalian proteins studied to date, monoclonal antibodies have received the most attention because of their potential value as therapeutic and clinical reagents (reviewed in Ma and Hein, 1995; Ma and Hein, 1996). Initially, individual transgenic tobacco plants each expressing either the heavy or light immunoglobulin chains were crossed, generating a hybrid plant that co-expressed both chains. More recently, through successive crosses, it has been possible to generate tobacco plants expressing functional secretory IgA (SIgA) consisting of heavy and light chains plus a J chain and secretory component. However, such cross-breeding is a time-consuming process and in some plants, like alfalfa, that do not readily self-fertilize, it is impractical. There thus remains a need for methods and compositions for simultaneous introduction of genes into plants encoding multiple protein chains capable of associating
together or assembling together, for example antibody chains, and rapid scale-up to commercial production levels without laborious crosses to commercial varieties.

SUMMARY OF THE INVENTION

The present invention provides compositions and methods for the transformation of plant cells with multiple genes, and proper association or assembly of multimeric proteins that are heterologous to the plant cells. At least two separate plasmid constructs encoding the individual polypeptide components of the multimeric protein are used. Preferably, the plasmids are introduced into the target cells as naked DNA. Typically, these plasmids used in the invention are made as "expression cassettes" which include the required regulatory sequences. For example, such DNA constructs (expression cassettes) may include: a promoter that is functional in a given host plant cell; nucleic acid encoding a signal peptide fused to nucleic acid molecule encoding a subunit protein; nucleic acid encoding the amino acid sequence KDEL for retention in the endoplasmic reticulum; and a polyadenylation signal sequence. The transformed plant cells are cultured under conditions to produce the assembled protein which can then be isolated.

In one aspect, the multimeric protein comprises an immunoglobulin molecule, a receptor ligand complex, a homodimer or heterodimer. Typically, the multimeric protein is biologically active. The present invention encompasses use of plant cells from a dicot genus, for example Nicotiana, or from a monocot genus, for example, Lemna.

In another aspect, additional sequences are introduced into the plant cells. These additional sequences include, but are not limited to, sequences encoding the amino acid sequence KDEL (which aids in accumulating the multimer in the endoplasmic reticulum); a signal sequence (which facilitates the targeting of the multimer to the endoplasmic reticulum); a selectable marker (which allows for identification of transformed cells) and a leader sequence. Thus, also
encompassed by the present inventions are DNA constructs for high level expression and secretion of heterologous proteins in plant cells. These additional sequences may be included on the plasmids carrying one or more structural components of the multimer. Alternatively, these additional sequences can be introduced on additional plasmids.

In yet another aspect, the present invention includes microparticles coated with multiple plasmids, where at least two of these multiple plasmids encode structural components made of a multimeric protein. Microparticles are preferably of inert metals such as tungsten or gold and are preferably between about 0.5 and 1.5 microns in size.

The present invention also includes transgenic plants and cells that produce multimeric proteins. These plants and plant cells are characterized by adjacent integration of multiple expression cassettes, wherein each expression cassette encodes at least one but less than all of the polypeptide components of a multimeric protein.

As will become apparent, preferred features and characteristics of one aspect of the invention are applicable to any other aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic depicting the plasmid SSpHuK (pSSpHuK).
Figure 2 is a schematic depicting the plasmid SSpHuA2 (pSSpHuA2).
Figure 3 is a schematic depicting the plasmid SHuJ (pSSpSHuJ).
Figure 4 is a schematic depicting the plasmid SSpHuSC (pSSpHuSC).
Figure 5 (SEQ ID NOS: 1 and 2) depicts the nucleotide and amino acid sequence of the coding region of pSSpGuyHuK.
Figure 6 (SEQ ID NOS: 3 and 4) depicts the nucleotide and amino acid sequence of the coding region of pSSpGuyHuA2.
Figure 7 (SEQ ID NOS: 5 and 6) depicts the nucleotide and amino acid sequence of the coding region of pSHuJ.
Figure 8 (SEQ ID NOS: 7 and 8) depicts the nucleotide and amino acid sequence of the coding region of pSHuSC.
MODES FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

Definitions


As used in the specification and claims, the singular form “a”, “an” and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a cell” includes a plurality of cells, including mixtures thereof.

A “variable region” of an antibody refers to the variable region of the antibody’s light chain or the variable region of the heavy chain, either alone or in combination.

As used herein, a “polynucleotide” is a polymeric form of nucleotides of any length, which contain deoxyribonucleotides, ribonucleotides, and/or their analogs. The terms “polynucleotide" and “nucleotide” as used herein are used interchangeably. Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term “polynucleotide”
includes double-, single-stranded, and triple-helical molecules. Unless otherwise specified or required, any embodiment of the invention described herein that is a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double stranded form.

The term “polypeptide” is used in its broadest sense to refer to a compound of two or more subunit amino acids, amino acid analogs, or peptidomimetics. The subunits may be linked by peptide bonds. In another embodiment, the subunit may be linked by other bonds, e.g. ester, ether, etc. As used herein the term “amino acid” refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of three or more amino acids is commonly called an oligopeptide if the peptide chain is short. If the peptide chain is long, the peptide is commonly called a polypeptide or a protein.

A "multimeric protein" as used herein refers to a globular protein containing more than one separate polypeptide or protein chain associated with each other to form a single globular protein in vitro or in vivo. The multimeric protein may consist of more than one polypeptide of the same kind to form a homodimeric or homotrimeric protein; the multimeric protein may also be composed of more than one polypeptide having distinct sequences to form, e.g., a heterodimer or a heterotrimer. Non-limiting examples of multimeric proteins include immunoglobulin molecules, receptor dimer complexes, trimeric G-proteins, and any enzyme complexes.

An "immunoglobulin molecule" or "antibody" is a multimeric protein containing the immunologically active portions of an immunoglobulin heavy chain and immunoglobulin light chain covalently coupled together and capable of specifically combining with antigen. An "immunoglobulin combining site" or "binding domain" is formed from the folding of variable domains of an antibody molecule(s) to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an epitope of an antigen, which allows an immunological reaction with the antigen. An antibody
combining site may be formed from a heavy and/or a light chain domain (VH and VL, respectively), which form hypervariable loops which contribute to antigen binding. The immunoglobulins, or antibody molecules, are a large family of molecules that include several types of molecules, such as IgD, IgG, IgA, IgM and IgE. The term "immunoglobulin molecule" includes, for example, hybrid antibodies, or altered antibodies, and fragments thereof, including but not limited to Fab fragment(s), and Fv fragment.

An Fab fragment of an immunoglobulin molecule is a multimeric protein consisting of the portion of an immunoglobulin molecule containing the immunologically active portions of an immunoglobulin heavy chain and an immunoglobulin light chain covalently coupled together and capable of specifically combining with an antigen. Fab fragments can be prepared by proteolytic digestion of substantially intact immunoglobulin molecules with papain using methods that are well known in the art. However, a Fab fragment may also be prepared by expressing in a suitable host cell the desired portions of immunoglobulin heavy chain and immunoglobulin light chain using methods disclosed herein or any other methods known in the art.

An Fv fragment of an immunoglobulin molecule is a multimeric protein consisting of the immunologically active portions of an immunoglobulin heavy chain variable region and an immunoglobulin light chain variable region covalently coupled together and capable of specifically combining with an antigen. Fv fragments are typically prepared by expressing in suitable host cell the desired portions of immunoglobulin heavy chain variable region and immunoglobulin light chain variable region using methods described herein and/or other methods known to artisans in the field.

"Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared. For example, a polynucleotide introduced by genetic engineering techniques into a different cell is a heterologous polynucleotide (and, when expressed, can encode a heterologous polypeptide). In particular, the term "heterologous" as applied to a multimeric protein means that the multimer is expressed in a host cell that is genotypically
distinct from the host cell in which the multimer is normally expressed. For example, the exemplified human IgA multimeric protein is heterologous to a plant cell.

The term "biologically active", as used herein, refers to a multimer having structural, regulatory, or biochemical functions of a naturally occurring molecule expressed in its native host cell. For instance, a biologically active immunoglobulin produced in a plant cell by the methods of this invention has the structural characteristics of the naturally occurring molecule, and/or exhibits antigen binding specificity of the naturally occurring antibody that is present in the host cell in which the molecule is normally expressed.

A “gene” refers to a polynucleotide containing at least one open reading frame that is capable of encoding a particular protein after being transcribed and translated.

As used herein, “expression” refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently being translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell.

The term “construct” refers to an artificially assembled DNA segment to be transferred into a target plant tissue or cell. Typically, the construct will include the gene of a particular interest, a marker gene and appropriate control sequences. The term “plasmid” refers to an autonomous, self-replicating extrachromosomal DNA molecule. In a preferred embodiment, the plasmid constructs of the present invention contain sequences coding for heavy or light chain constant regions of an antibody. The plasmids also preferably contain sequences encoding a signal peptide, a leader sequence, a sequence for retention in the endoplasmic reticulum (ER), such as KDEL. Plasmid constructs containing suitable regulatory elements are also referred to as “expression cassettes.” In a preferred embodiment, a plasmid construct can also contain a screening or selectable marker, for example an antibiotic resistance gene. The term “naked
DNA” or “naked plasmid” refers to plasmids which are not packaged in a gene delivery vehicle, for example agrobacterium.

The terms “screening marker”, “selectable marker” and “reporter gene” are used to refer to a gene that encodes a product that can readily be assayed. For example, reporter genes can be used to determine whether a particular DNA construct has been successfully introduced into a cell, organ or tissue. Non-limiting examples of selectable markers include genes encoding for antibiotic resistance, e.g., ampicillin, kanamycin or the like. Other selection markers will be known to those of skill in the art.

A "signal sequence" is a nucleic acid sequence encoding the "signal peptide" located typically at the N-terminus of a polypeptide, which direct the polypeptide to a specific cell compartment, e.g. the endoplasmic reticulum, wherein the assembly of multimer takes place.

A “primer” is a short polynucleotide, generally with a free 3’-OH group, that binds to a target or “template” potentially present in a sample of interest by hybridizing with the target, and thereafter promoting polymerization of a polynucleotide complementary to the target. A “polymerase chain reaction” ("PCR") is a reaction in which replicate copies are made of a target polynucleotide using a “pair of primers” or a “set of primers” consisting of an “upstream” and a “downstream” primer, and a catalyst of polymerization, such as a DNA polymerase, and typically a thermally-stable polymerase enzyme.

Methods for PCR are well known in the art, and taught, for example in MacPherson, et al., PCR: A Practical Approach (IRL Press at Oxford University Press (1991)). All processes of producing replicate copies of a polynucleotide, such as PCR or gene cloning, are collectively referred to herein as “replication.” A primer can also be used as a probe in hybridization reactions, such as Southern or Northern blot analyses.

“Hybridization” refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The
complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

When hybridization occurs in an antiparallel configuration between two single-stranded polynucleotides, the reaction is called "annealing" and those polynucleotides are described as "complementary". A double-stranded polynucleotide can be "complementary" or "homologous" to another polynucleotide, if hybridization can occur between one of the strands of the first polynucleotide and the second. "Complementarity" or "homology" (the degree that one polynucleotide is complementary with another) is quantifiable in terms of the proportion of bases in opposing strands that are expected to form hydrogen bonding with each other, according to generally accepted base-pairing rules.

A "transgenic plant" refers to a genetically engineered plant or progeny of genetically engineered plants. The transgenic plant usually contains material from at least one unrelated organism, such as from a virus, another plant, or animal.

As used herein, the term "isolated" means separated from constituents, cellular and otherwise, in which the polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, are normally associated with in nature. As is apparent to those of skill in the art, a non-naturally occurring polynucleotide, peptide, polypeptide, protein, antibody, or antibody fragments, does not require "isolation" to distinguish it from its naturally occurring counterpart. In addition, a "concentrated", "separated" or "diluted" polynucleotide, peptide, polypeptide, protein, antibody, or antibody fragments, is distinguishable from its naturally occurring counterpart in that the concentration or number of molecules per volume is greater than "concentrated" or less than "separated" than that of its naturally occurring counterpart. A polynucleotide, peptide, polypeptide, protein, antibody, or fragments thereof, which differs from the naturally occurring counterpart in its primary sequence or for example, by its glycosylation pattern, need not be present in its isolated form since it is distinguishable from its naturally
occurring counterpart by its primary sequence, or alternatively, by another
characteristic such as glycosylation pattern. A protein produced in a plant cell is
provided as a separate embodiment from the naturally occurring protein isolated
from another type of cell in which it is produced in nature.

A "control" is an alternative subject or sample used in an experiment for
comparison purpose. A control can be "positive" or "negative". For example,
where the purpose of the experiment is to determine the presence of an
exogenously introduced plasmid or the expression of a polypeptide encoded by
such plasmid in a plant transformant or its progenies, it is generally preferable to
use a positive control (a plant or a sample from a plant, carrying such plasmid
and/or expressing the encoded protein), and a negative control (a plant or a sample
from a plant lacking the plasmid of interest and/or expression of the polypeptide
encoded by the plasmid).

The present invention provides methods for producing multimeric proteins
by introducing multiple plasmids into a plant cell. The inventors have shown that
multimeric proteins are assembled and accumulated when the individual
polypeptide components are encoded by separate plasmids. In addition,
constructs carrying sequences encoding signal peptides, leader sequences,
selectable marker sequences and accumulation sequences have also been shown to
transform plant cells and aid in the production of functional multimeric proteins.
The invention, therefore, provides a fast and efficient method of making
heterologous multimeric proteins in plant cells. The plants transformed by the
methods described herein are also stably integrated and their progeny also
produce the multimeric protein(s).

The methods described herein also result in a transgenic plant that is
genetically distinguishable from plants which have been cross-bred to produce
multimers. Similarly, the plants and plant cells described herein are also
distinguishable from plants into which a single plasmid carrying sequences
encoding components of a multimeric protein has been introduced. In particular,
the transgenic plants described herein are characterized in that the separate
expression cassettes of the multiple plasmids are integrated adjacent to one another in the host genome. In contrast, single plasmid transformants will carry a single integrated expression cassette, while cross-bred plants will carry randomly integrated (non-adjacent) expression cassettes. These differences can be determined by methods known in the art, for example, by Southern blotting.

**Transformation of Plant Cells**

The present invention provides for methods of producing multimeric proteins by transforming plant cells with a plurality of plasmids. Typically, the plasmids are introduced as naked DNA, for example by particle bombardment. Sequence coding for individual components of the multimer is found on at least two plasmids. In one embodiment, each component is encoded for by a different plasmid. In another embodiment, for example where the multimer is made up of more than two structural components, it is possible to use a plasmid encoding all but one of the structural components in conjunction with a plasmid encoding the missing component. Non-limiting examples of multimeric proteins and their respective structural components include immunoglobulins made up of heavy and light chains and optional J-chains and secretory components; receptor molecules made up of two or more components and the like. In addition, using the methods described herein, it is possible to assemble a functional multimeric protein using plasmids carrying fragments of the components of the multimer, e.g., Fab or Fv fragments of immunoglobulins.

Methods of making plasmid constructs are well known in the art and described for example, in Sambrook, *supra*. Plasmids will typically contain at least one structural component and appropriate regulatory sequences, such as promoters suitable for use in plants. Non-limiting examples of promoters include constitutive (or nearly constitutive) promoters from agrobacterium such as a superpromoter; nopaline synthase; octopine synthase; mannopine synthase and 1’2’; the rbcS (small subunit of ribulose bis-phosphate carboxylase promoter from plants; chlorella virus adenine methyl transferase (AMT) and ubiquitin. Examples of inducible promoters include, chitinase (from bean), chalcone synthase
phenylalanine ammonia lyase and HRGP. Tissue-specific promoters may also be used, for example, legumin (or other seed storage protein promoters), patatin and the like. For a discussion of the 35S promoter, see, for example, Odell JT et al. (1985) Nature 313: 810-812 and U.S. Patent No. 5,352,605, issued October 1994).

At least two of the plasmids will each encode at least one of the structural components of the multimer. In a preferred embodiment, the structural components are linked to signal peptides necessary for the assembly of a multimeric protein. Suitable signal peptides can be readily determined by those of skill in the art and include, by way of example, the signal peptide of 2S2 storage protein of A. thaliana, Vicia faba legumin B4, alpha amylase or patatin. One of skill in the art could readily construct a plasmid carrying sequence encoding a polypeptide in proper frame with a signal peptide coding sequence.

In another preferred embodiment, at least one of the plasmids used in transformation will encode a peptide, for example the amino acid sequence “KDEL,” which results in accumulation of the multimer in the endoplasmic reticulum. In yet another preferred embodiment, at least one of the plasmids used in transformation encodes a selectable marker. Suitable selectable markers for plants will be known to those in the art and will be appropriate for use in transgenic plants, which are regenerated via direct shoot organogenesis or somatic embryogenesis. Cloning and expression vectors typically contain a selectable marker (for example, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the vector). Only those host cells into which a selectable gene has been introduced will grow under selective conditions. Typical selection genes either: (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, kanamycin, methotrexate; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from complex media. The choice of the proper marker gene will depend on the host cell, and appropriate genes for different hosts are known in the art.

In one embodiment, described in the Examples below, generic expression vectors to fuse any mouse variable region to human heavy and light chain constant regions for expression in plants were used. The heavy chain vector,
pSSpHuA2 (Figure 2; Figure 6; SEQ ID NOS: 3 and 4), contains sequence encoding a bean legumin signal peptide and the human IgA2m(2) constant region with Spe I and Sac I sites in between for cloning variable regions. The light chain vector, pSSpHuK (Figure 1; Figure 5: SEQ ID NOS: 1 and 2), contains sequence encoding a bean legumin signal peptide and the human kappa constant region with Spe I and Hind III sites in between for cloning variable regions. Expression of these genes is under the control of the same promoter and terminator as in the heavy chain vector. Heavy and light chain constructs containing the variable regions of an anti-S. mutans SA I/II antibody (Guy's 13; see Figs. 1 and 2), along with constructs for the expression of human J chain, pSHuJ (Figure 3; Figure 7; SEQ ID NOS: 5 and 6) and secretory component, pSHuSC (Figure 4; Figure 8; SEQ ID NOS: 7 and 8), are used for transformation (USSN 08/430,000 incorporated by reference).

The target plant cells may be in the form of whole plants, isolated cells or protoplasts. Preferably, the cells are "intact" in that the cell comprises an outer layer of cell wall, typically composed of cellulose for protection and maintaining the rigidity of the plant cell. These plant cells may be transformed with the plurality of plasmids using any method known in the art. In a particularly preferred embodiment, the plant cells are bombarded with naked DNA using microparticles coated with the plurality of plasmids. Bombardment with DNA-coated microparticles has been successfully used to produce stable transformants in both plants and animals (see, for example, Sanford et al., 1993, infra). As many as 12 different plasmids, introduced simultaneously, have been stably integrated into soybean cells via particle bombardment (Hadi et al., 1996, infra). However, these plasmids did not encode polypeptides and expression was not shown. In rice, as many as 13 unrelated genes were inserted into the genome, expressed, and stably inherited (Chen et al., 1998, infra). However, assembly of polypeptides expressed from multiple plasmids into functional multimeric protein complexes in regenerated plants has not previously been demonstrated.

Suitable microparticles for bombardment are available for example, from BioRad (e.g., Bio-Rad's PDS-1000/He). Typically, particles suitable for use in
the methods described herein are made of metal, preferably tungsten or gold. Their average diameter is generally in the range of about 0.5 microns to about 1.5 microns.

As noted above, the methods described herein involve the use of at least two plasmids each encoding less than all of the structural components of a multimer. In addition, these at least two plasmids, or additional plasmids, may encode signal peptides, leader sequences, selectable marker genes or the like. Although not necessarily required, it is believed that the microparticles should be coated with all the plasmids to be used. Without being bound by one theory, it is believed to be unlikely that more than one microparticle will enter and stably transform a plant cell. Accordingly, it is preferable to ensure that each microparticle carries the plasmids of interest.

As will be evident to those of skill in the art, the particle bombardment protocol can be optimized for any plant by varying parameters such as He pressure, quantity of coated particles, distance between the macrocarrier and the stopping screen and flying distance from the stopping screen to the target. An optimal protocol is described in the Examples.

In one embodiment, secretory IgA molecules can be assembled by using plasmid containing heavy chain, light chain, J chain and secretory component, are used for particle bombardment of leaf disks. A fifth vector containing an antibiotic resistance gene, such as pSZeo (containing the Phe\(^{\prime}\) gene encoding resistance to the antibiotic Zeocin; Perez et al., 1989; Drocourt et al, 1990) or pBMSP-1 (containing the Kan\(^{\prime}\) gene encoding resistance to kanamycin; S. Gelvin, Purdue University) is also included. The transgenic plants obtained may be screened for production of assembled SlgA using the methods described below.

**Selection of Plant Transformants**

Transformation of a host plant with a plurality of plasmids, each plasmid encoding less than all of the components of the multimeric protein, yields a population of transformants that may carry some or all of the plasmids of interest. To select those transformants containing multiple plasmids encoding each and
every component of the multimeric protein, one generally proceeds by detecting
the presence of the plasmids, and/or the expression of each component of the
multimer in the transformants or the transgenic plant produced therefrom.

The presence of all plasmids encoding each component of the multimeric
protein can be confirmed by hybridization assays, amplification reactions using a
probe or a primer pair derived from the plasmids used for transformation.
Preferably, the probe or primer pair comprises the sequences complementary to
the nucleic acid encoding the full-length or a fragment of each polypeptide chain
that constitutes the multimer. In an alternative, the success of transformation can
also be determined by restriction enzyme digestion, in which the appearance of
restricted fragments representative of the plasmids is indicative of the presence of
the exogenously introduced plasmids.

The test nucleic acid can be obtained from the cultured plant
transformants, such as the plasmid-bombarded leaf disks, or the regenerated plant
therefrom. The nucleic acid to be tested can be extracted from plant according to
standard methods in the art. For instance, nucleic acid can be isolated using
various lytic enzymes or chemical solutions according to the procedures set forth
Edition, 1989), or extracted by nucleic-acid-binding resins following the
accompanying instructions provided by manufactures.

Hybridization can be performed under conditions of different
“stringency”. Conditions that vary levels of stringency are well known in the art
(see, for example, Sambrook, et al. supra). Briefly, relevant conditions include
temperature, ionic strength, time of incubation, the presence of additional solutes
in the reaction mixture such as formamide, and the washing procedure. Higher
stringency conditions are those conditions, such as higher temperature and lower
sodium ion concentration, which require higher minimum complementarity
between hybridizing elements for a stable hybridization complex to form. In
general, a low stringency hybridization reaction is carried out at about 40 °C in 10
x SSC or a solution of equivalent ionic strength/temperature. A moderate
stringency hybridization is typically performed at about 50 °C in 6 X SSC, and a
high stringency hybridization reaction is generally performed at about 60 °C in 1 X SSC. In determining the presence of an exogenously introduced plasmid carrying a gene of interest in the plant transformants, stringent hybridization is preferred.

For the purpose of this invention, amplification means any method employing a primer-dependent polymerase capable of replicating a target sequence with reasonable fidelity. Amplification may be carried out by natural or recombinant DNA-polymerases such as T7 DNA polymerase, Klenow fragment of E. coli DNA polymerase, and reverse transcriptase.

A preferred amplification method is PCR. General procedures for PCR are taught in “PCR: a practical approach” (M. MacPherson et al., IRL Press at Oxford University Press 1991). After amplification, the resulting DNA fragments can be detected by agarose gel electrophoresis followed by visualization with ethidium bromide staining and ultraviolet illumination. A specific amplification of the structural gene can be verified by demonstrating that the amplified DNA fragment has the predicted size, exhibits the predicted restriction digestion pattern, and/or hybridizes to the correct cloned DNA sequence.

The probe or primers employed in the hybridization and amplification reactions can be conjugated to a detectable marker, e.g., an enzymatic label or a radioisotope for detection of nucleic acid and/or expression of the gene in a cell. A wide variety of appropriate detectable markers are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known which can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

To ascertain the expression of each polypeptide contained in the multimeric protein, plant transformants or progeny made therefrom can be tested
in an immunoassay using antibodies specific for individual polypeptides that constitute the multimer. Procedures for carrying out immunoassays which include but are not limited to immunoblotting and immunoprecipitation which are well established and commonly practiced by an ordinary skill in the art. Briefly, the reaction is performed by contacting the detecting antibody with a sample containing the test proteins of the plant transformants or their progenies under conditions that will allow a complex to form between the antibody and the target polypeptide. The sample of test proteins can be prepared by homogenizing the plant transformants or their progenies made therefrom, and optionally solubilizing the test protein using detergents, preferably non-reducing detergents such as triton and digitonin. The binding reaction in which the test proteins are allowed to interact with the detecting antibodies may be performed in solution, or on a solid tissue sample, for example, using tissue sections or solid support that has been immobilized with the test proteins. The formation of the complex can be detected by a number of techniques known in the art. For example, the antibodies may be supplied with a label and unreacted antibodies may be removed from the complex; the amount of remaining label thereby indicating the amount of complex formed.

The amount of the target polypeptides that are immunologically reactive with the detecting antibodies can also be quantified by standard quantitative immunoassays. For instance, the target protein may be solubilized or extracted from a solid tissue sample, and mixed with a pre-determined non-limiting amount of the reagent antibody specific for the protein. The detecting antibody may contain a directly attached label, such as an enzyme or a radioisotope, or a second labeled reagent may be added, such as protein A. For a solid-phase assay, unreacted reagents are removed by washing. For a liquid-phase assay, unreacted reagents are removed by some other separation technique, such as filtration or chromatography. The amount of label captured in the complex is positively related to the amount of target protein present in the test sample. Alternatively, a competitive assay in which the target protein is tested for its ability to compete with a labeled analog for binding sites on the specific antibody. In this case, the amount of label captured is negatively related to the amount of target protein.
present in a test sample. Results obtained using any such assay on a sample from a plant transformant or a progeny thereof is compared with those from a non-transformed source as a control.

Detection of the Assembled, Biologically Active Multimeric Proteins

Production of biologically active multimer in a plant transformant or its progeny generally requires assembly of individual polypeptide components to assume a proper conformation that confers a biological activity. Selected plant transformants and/or the progenies made therefrom can be assayed for the presence of the assembled, biologically active multimeric protein using a variety of methods well known in the art. Such methods include ELISA, Western blotting, immunoprecipitation, and any assay designed to detect a functional multimeric protein by measuring, e.g. its enzymatic activity or immunological activity. The enzymatic activity of a multimer include but are not limited to phosphorylation, glycosylation, peptidase activity, GTPase activity, voltage-dependent ATPase activity, and any combinations thereof.

Preferred screening assays are those where the biological activity of an assembled multimeric protein is detected in such a way as to produce a detectable signal. This signal may be produced directly or indirectly and such signals include, for example, the production of a complex via protein-protein interaction, induction of a conformational change of the multimer, formation of a catalytic reaction product, the release or uptake of energy, and the like. Where the multimer is an immunoglobulin molecule, its biological activity can be assessed by its ability to bind specifically to its cognate antigen. As used herein, the specificity of an antibody refers to the ability of the antibody to distinguish cognate (or native) antigens from any other unrelated antigens. Where the multimer is a receptor-ligand complex or a complex of receptor molecules, its biological activity can be confirmed by the ability of the complex to interact with downstream signaling molecules and/or its ability to phosphorylate substrates on the tyrosine, serine and/or histidine residues. The biological activity of a trimeric G protein can be ascertained by the presence of GTPase activity. Assaying
procedures for phosphorylation and GTPase activity, as well as protocols for detecting protein-protein interactions are well established in the art, and thus are not detailed herein.

Isolation of Biologically Active Multimeric Proteins

A transgenic plant of the present invention expressing the desired multimeric protein is cultivated to isolate the desired multimeric protein they contain. After cultivation, the transgenic plant is harvested to recover the produced multimeric protein. This harvesting step may consist of harvesting the entire plant, or only the leaves, or roots of the plant. This step may either kill the plant, or if only the portion of the transgenic plant is harvested, may allow the remainder of the plant to continue to grow. In a specific embodiment, harvesting the transgenic plant may further comprise (a) homogenizing at least a portion of said transgenic plant to produce a plant pulp; (b) extracting the multimeric protein from the plant pulp to produce a multimeric protein containing solution; and (c) isolating said multimeric protein from said solution. More specifically, at least a portion of the transgenic plant is homogenized to produce a plant pulp using methods well known to one skilled in the art. This homogenization may be done manually, by a machine, or by a chemical means as long as the transgenic plant portions are broken up into small pieces to produce a plant pulp. This plant pulp consists of a mixture of varying sizes of transgenic plant particles. The size of the plant particles and the amount of variation in size that can be tolerated will depend on the exact method used to extract the multimeric protein from the plant pulp and these parameters are well known to one skilled in the art. The multimeric protein is extracted from the plant pulp produced above to form a multimeric protein containing solution. Such extraction processes are common and well known to articians in this art. For example, the extracting step may consist of soaking or immersing the plant pulp in a suitable solvent. A suitable solvent dissolves the multimeric protein present in the plant pulp to produce a multimeric protein containing solution. Solvents useful for such an extraction process include but are not limited to aqueous solvents, organic solvents and combinations of both. A
preferred solvent is non-reducing detergent such as digitonin or Triton-X100. The multimeric protein can then be isolated from the solution produced above by methods such as immunoprecipitation with antibody, and standard techniques such as gel filtration, ion-exchange, reversed-phase, and affinity chromatography. For such methodology, see for example Deutscher et al. (1999) GUIDE TO PROTEIN PURIFICATION: METHODS IN ENZYMOLOGY (Vol. 182, Academic Press).

REFERENCES


EXCEPTIONS

Example 1 Transformation of Tobacco with SIgA

Construction of Secretory IgA Expression Vectors

We have constructed generic expression vectors to fuse any mouse variable region to human heavy and light chain constant regions for expression in plants. The heavy chain vector is called pSSpHuA2 (Figure 2), and contains sequence encoding a bean legumin signal peptide and the human IgA2m(2) constant region with Spe I and Sac I sites in between for cloning variable regions. The light chain vector is called pSSpHuK(Figure 1), and contains sequence encoding a bean legumin signal peptide and the human kappa constant region with Spe I and Hind III sites in between for cloning variable regions. Expression is under the control of the same promoter and terminator as in the heavy chain vector. Heavy and light chain constructs containing the variable regions of an anti-S. mutans SA I/II antibody (Guy’s 13; see Figs. 1 and 2), along with constructs for the expression of human J chain (pSHuJ, Fig. 3) and secretory component (pSHuSC, Fig. 4), were used for transformation.

The expression vectors constructed for plant transformation were:

1. The plasmid pSSpGuyHuK is 4163 base pairs in length. Nucleotides 16-1139 represent the Superpromoter (Ni et al., 1995), which drives expression in plant cells. Nucleotides 1140-1864 encode the light chain (Figure 5) and comprise a sequence encoding a mouse light chain variable/human kappa constant hybrid with linker sequences. A consensus Kozak sequence (Kozak, 1986) is included (nt 1147-1154) to enhance translation initiation, and the native mouse signal peptide has been replaced with the signal peptide from bean legumin (nt 1150-1218; Bäumlein et al., 1986). The variable region (nt 1219-1533) is from the murine monoclonal Guy’s 13 (Smith and Lehner, 1989, US Patent No. 5,518,721 and 5,352,446). The sequence of the human kappa constant region (nt 1534-1864) has been previously published (Hieter et al., 1980). Nucleotides 1865-2118 derive from the nopaline synthase 3’ end (transcription termination
and polyadenylation signal; Depicker et al., 1982). The remainder of the plasmid derives from the vector pSP72 (Promega Corporation).

2. The plasmid pSSPGuyHuA2 is 5296 bp in length. Nucleotides 49-1165 represent the Superpromoter (Ni et al., 1995). Nucleotides 1166-2645 (Figure 6) comprise a sequence encoding a mouse heavy chain variable/human IgA2m(2) constant hybrid with linker sequences. A consensus Kozak sequence (Kozak, 1986) is included (nt 1186-1192) to enhance translation initiation, and the native mouse signal peptide has been replaced with the signal peptide from bean legumin (nt 1189-1257; Bäumlein et al., 1986). The variable region (nt 1258-1595) is from the murine monoclonal Guy’s 13 (Smith and Lehner, 1989). The sequence of the human IgA2m(2) constant region (nt 1596-2645) has been previously published (Chintalacharuvu, et al., 1994). Nucleotides 2646-2371 derive from the nopaline synthase 3’ end (transcription termination and polyadenylation signal; Depicker et al., 1982). The remainder of the plasmid derives from the vector pSP72 (Promega).

3. The plasmid pSHuJ is 4283 bp in length. Nucleotides 14-1136 represent the Superpromoter (Ni et al., 1995) and nucleotides 1137-1648 are shown in Figure 7 and comprise a sequence encoding the human J chain including the native signal peptide (Max et al, 1985) along with linker sequences. A consensus Kozak sequence (Kozak, 1986) is included (nt 1162-1168) to enhance translation initiation. Nucleotides 1649-1902 derive from the nopaline synthase 3’ end (transcription termination and polyadenylation signal; Depicker et al., 1982). The remainder of the plasmid derives from the vector pSP72 (Promega).

4. The plasmid pSHuSC is 5650 bp in length. Nucleotides 13-1136 are derived from the Superpromoter (Ni et al., 1995) and nucleotides 1137-2981 are shown in Figure 8 and comprise a sequence encoding the human Secretory Component including the native signal peptide (Krajci et al., 1989) along with linker sequences. A consensus Kozak sequence (Kozak, 1986) is included (nt 1151-1157) to enhance translation initiation. Nucleotides 2982-3236 derive from the nopaline synthase 3’ end (transcription termination and polyadenylation

24
signal; Depicker et al., 1982). The remainder of the plasmid derives from the
vector pSP72 (Promega).

**Particle Bombardment and Regeneration of Plant**

The four vectors described above, containing heavy chain, light chain, J
chain and secretory component, were used for particle bombardment of tobacco
leaf disks using Biolistic® PDS-1000 HE instrument (Bio-Rad). A fifth vector
containing an antibiotic resistance gene, such as pSZeo (containing the Phe' gene
encoding resistance to the antibiotic Zeocin; Perez et al., 1989; Drocourt et al.,
1990) or pBMSP-1 (containing the Kan' gene encoding resistance to kanamycin;
S. Gelvin, Purdue University) was also used. Transgenic plants were screened
using chain-specific antibodies by Western blot to identify individual
transformants expressing assembled human SIgA.

A stock suspension of microprojectiles was prepared by mixing 60 mg of
0.7 micron tungsten or gold particles in 1 mL of 70% ethanol in a sterile
microcentrifuge tube. This suspension was vortexed 5 minutes and incubated at
room temperature for 5 minutes. After microcentrifuging for 5 seconds the
ethanol was removed and the pellet was resuspended in 1ml sterile water and
centrifuged for 5 minutes. Particles were washed 3 times with sterile water,
removing wash each time after a brief centrifugation. Sterile 50% glycerol was
then added to particles to bring the concentration to 60 mg/mL. This suspension
was dispensed in 100 microliter aliquots in sterile 1.5 mL microcentrifuge tubes.

An aliquot of 60 mg of particles (in 50% glycerol) was vortexed for 5
minutes. While vortexing, 50 microliters of this suspension was removed to a
sterile 0.5 mL microfuge tube. While vortexing this tube, 1 microgram of each
plasmid DNA was added. While vortexing, 50 ml of CaCl2 (2.5M), and 20 ml of
spermidine (0.1 M) were added (in that order). This mixture was vortexed for
another 3 minutes, allowed to settle for 2 min, and then centrifuged for 5 seconds.
The supernatant was removed and 140 ml of absolute ethanol was added to the
DNA coated particles. The particles were allowed to settle for 5 minutes, and the
supernatant was removed. The particles were resuspended in 140 ml of absolute
ethanol and allowed to settle a second time. The supernatant was removed, and the particles were resuspended in 50 ml of absolute ethanol.

Ten ml of the suspension was applied as evenly as possible onto the center of macrocarrier sheet made of Kapton (DuPont) and the ethanol was evaporated. The macrocarrier sheet and a rupture disk were placed in the unit (Bio-Rad). A petri dish containing pieces of a surface-sterilized tobacco leaf was placed below the stopping screen. The chamber was evacuated to 28-29mm Hg and the target was bombarded once.

Tobacco leaf disks were bombarded with tungsten particles (1 μm) coated with the plasmids pSSpGuyHuK, pSSpGuyHuA2, pSHuJ, pSHuSC and pBMSP-1. Leaves from plants grown axenically were used, with the optimal transformation efficiency resulting from leaves taken 10 days after transplanting a node to new media. Regeneration of bombarded leaf disks was performed essentially according to standard protocols, for example Horsch et al., 1985. Leaf disks were immediately placed on MS media supplemented with NAA and BAP, along with 50 mg/L kanamycin sulfate. After 4 days, leaves were cut into small segments, dipped in water and placed on fresh plates. Leaves were dipped in water every week. Small calli appeared between 1 and 2 months after bombardment, and these were maintained under selection until shoots appeared about 2 weeks later. Shoots were transferred to fresh media, and when leaves were at least 3 cm in length, cuttings were taken and extracted for ELISA and immunoblot analysis. At least 1 of 10 putative transgenic plants produced fully assembled SIGA.

**Screening of transgenic plants - Western blot analysis of immunoglobulin**

We have found that immunoblotting is a sensitive and reliable method to detect expression of antibody chains in plants. Two 4 mm diameter leaf punches were homogenized with 75 mM Tris-HCl (pH 6.8), 2% SDS, under reducing and non-reducing conditions (± DTT). Homogenates were centrifuged to pellet debris and supernatants were loaded onto SDS poly-acrylamide gels. Electrophoresis
was performed and the gels were blotted onto nitrocellulose. Blots were rinsed 2 times for 10 min in TBST (TBS with 0.05% Tween 20), then incubated for 1 hr in TBST + 5% non-fat dry milk. Blots were rinsed twice again with TBST, then incubated for 1 hr at room temperature with titered goat anti-human chain-specific HRP-conjugated antisera in TBST + 2% goat serum. After washing twice with TBST and once with TBS, antibody binding was detected by incubation with chemiluminescent reagents (Pierce), and exposure of the blot to x-ray film.

**Example 2 Transformation of Duckweed with SIgA**

In a transformation system for *Lemma gibba*, bombardment of surface-sterilized leaf tissue with DNA-coated particles is much the same as with tobacco. Whole thalloid fronds and excised portions of the frond having meristematic areas of the plus and minus pockets are used. Surface sterilization is accomplished by treating with a solution of sodium dichloroisocyanurate for 20-25 min followed by three rinses in sterile water. Subsequent to bombardment, the explants are placed on modified medium of Hillman (1961), having the following constituents:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>680</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1515</td>
</tr>
<tr>
<td>Ca(NO₃)₂•4H₂O</td>
<td>1180</td>
</tr>
<tr>
<td>MgSO₄•7H₂O</td>
<td>500</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>2.86</td>
</tr>
<tr>
<td>ZnSO₄•7H₂O</td>
<td>0.22</td>
</tr>
<tr>
<td>Na₂MoO₄•2H₂O</td>
<td>0.12</td>
</tr>
<tr>
<td>CuSO₄•5H₂O</td>
<td>0.08</td>
</tr>
<tr>
<td>MnCl₂•4H₂O</td>
<td>3.62</td>
</tr>
<tr>
<td>FeCl₃•6H₂O</td>
<td>5.4</td>
</tr>
<tr>
<td>Tartaric acid</td>
<td>3.00</td>
</tr>
<tr>
<td>EDTA</td>
<td>9.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>4,000-10,000</td>
</tr>
<tr>
<td>Coconut milk</td>
<td>10% (v/v)</td>
</tr>
</tbody>
</table>

Also included in the medium are a kinetin, zeatin riboside, thidiazuron and 2,4-D, separately or in combinations (concentration ranging from 0.1 to 10 mg/L). Culture conditions are 28±1°C at a relative humidity of 65-75% and a day length
of 10 hours. Light is provided by day light fluorescent tubes. After 5 days the leaf discs are transferred to regeneration medium containing Zeocin to prevent regeneration from untransformed plant cells. After three weeks, the callus that forms from these leaf disks are transferred to medium without hormones to stimulate the development of somatic embryos. After 3-4 months, regenerated plants are then screened for production of immunoglobulin chains and assembled SIgA.

Explants are incubated in the light under these conditions until whole new fronds and roots form, after which the new plants are transferred to liquid medium and allowed to propagate clonally. Conditions for maintaining *Lemna* in aquaculture are described by Porath (U.S.Patent # 5,269,819) which is herein incorporated by reference. When sufficient material is available, it is harvested and extracted for ELISA and immunoblot analysis. We expect to find fully assembled SIgA.
CLAIMS

WHAT IS CLAIMED IS:

1. A method for producing a multimeric protein in a plant cell wherein the multimeric protein is heterologous to the plant cell, the method comprising the steps of:

   (a) transforming a plant cell with a plurality of naked plasmids, each plasmid encoding less than all of the polypeptide components of the multimeric protein, and said plurality encoding all of the polypeptide components of the multimeric protein; and

   (b) culturing the plant cell under conditions suitable for protein expression, thereby producing the multimeric protein.

2. The method of claim 1, further comprising the step of isolating the produced multimeric protein from the cell.

3. The method of claim 1, wherein the plant cell is intact.

4. The method of claim 1, wherein the multimeric protein is biologically active.

5. The method of claim 1, wherein each plasmid encodes a single polypeptide component of the multimeric protein.

6. The method of claim 1, wherein at least one plasmid encodes multiple polypeptide components of the multimeric protein.

7. The method of claim 1, wherein at least one plasmid comprises a sequence encoding a signal peptide.
8. The method of claim 1, wherein at least one plasmid comprises a sequence encoding the amino acid sequence KDEL.

9. The method of claim 1, wherein at least one plasmid comprises a sequence encoding a selectable marker.

10. The method of claim 1, wherein the plant cell is from a dicotyledonous plant.

11. The method of claim 1, wherein the plant cell is from a monocotyledonous plant.

12. The method of claim 10, wherein said dicotyledonous plant is tobacco.

13. The method of claim 11, wherein said monocotyledonous plant is *Lemma gibba* (L.)

14. The method of claim 1, wherein the multimeric protein is selected from the group consisting of an immunoglobulin molecule, a receptor-ligand complex, a receptor homodimer, a receptor heterodimer, and a trimeric G-protein.

15. The method of claim 14, wherein the immunoglobulin molecule is selected from the group consisting of IgA, IgM, IgG, IgD, and IgE.

16. The method of claim 14, wherein the immunoglobulin molecule is IgA.

17. Microparticles coated with a plurality of plasmids, each plasmid encoding less than all of the polypeptide components of a multimeric protein, and
said plurality encoding all of the polypeptide components of the multimeric protein.

18. The microparticles of claim 17, wherein the microparticles are tungsten or gold.

19. A transgenic plant or plant cell expressing a multimeric protein that is heterologous to the plant cell, wherein said plants or plant cells are characterized by adjacent integration of multiple expression cassettes, each expression cassette encoding less than all of the polypeptide components of the multimeric protein, and said multiple expression cassettes encoding all of the polypeptide components of the multimeric protein.

20. The method of claim 16, wherein the IgA molecule is secretory.
Figure 2

pSSpHuA2
4960 bp

SupersPromoter

Ampr

IgA2 heavy chain constant region

Bean Legumin Signal Peptide

Variable Region Cloning Site

KDEL sequence
Figure 4

pSSpHuSC
5650 bp

SuperPromoter

human Secretory Component

Ampr

EcoRV
BglII
ClaI

Xhol

XbaI
SmaI
KpnI
NcoI

Nos 3'

HindIII
SalI
EcoRI

Smal
Figure 5

1140  
GGATCTAACC ATG GGA TCT AAA CCT TTT TTG TCT CTT TCA TTG CCA TTT TTT  
met gly ser lys pro phe leu ser leu leu ser leu leu leu leu phe  
1201/18 Spe I  
ACA TCT ACT AGT TTG GCA GAC ATT GTG ATG ACC CAG TCT CCA GCA ATC AGT TCT GCA TCT  
thr ser thr ser leu ala asp ile val met thr gln ser pro ala ile met ser ala ser  
1261/38  
CCA GGG GAG GTC ACC ATA ACC TGC AGT GCC AGC TCA AGT GTA AGT TAC ATG CAC TGG  
pro gly glu lys val thr ile thr cys ser ala ser ser ser val tyr met his trp  
1321/58  
TTC CAG CAG AAG CCA GGC ACT TCT CCC AAA CTC TGG CTT TAT AGC ACA TCC AAC CTG GCT  
phe glu glu lns pro gly thr ser pro lys leu trp leu tyr ser thr ser asn leu ala  
1381/78  
TCT GGA GTC CCT GCT GCC TTC AGT GGC AGT GGA TCT GGG ACC TCT TAC TCT CTC ACA ATC  
ser gly val pro ala arg phe ser gly ser gly thr ser tyr ser leu thr ile  
1441/98  
AGC CGA ATG GAG GCT GAA GAT GCT GCC ACT TAT TAC TGC CAT CAA AGG ACT AGC TAC CCA  
ser arg met glu ala glu asp ala ala thr tyr tyr cys his gln arg thr ser tyr pro  
1501/118  
Hind III  
TAC ACA TTC GGA GGG GGC ACC AAG CTT GAG ATC AAA CGA ACT GTG GCT GCA CCA TCT GTC  
tyr thr phe gly gly thr lys leu glu ile lys thr val ala ala pro ser val  
1561/138  
TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG TGC CTG  
phe ile phe pro pro ser asp glu gln leu lys ser gly thr ala ser val lys cys leu  
1621/158  
CTG AAG AAC TTC TAT CCC AGA GAG GCC AAA GAA GAG GAT AAG GCC CTC CAA  
leu asn asn phe tyr pro arg glu ala lys val gln trp lys val asp asn ala leu gln  
1681/178  
TGC GGT AAG TCC CAG GAG GTG AAG TCA CAG GAC AAC AAG GAC AGC ACC TAC AGC TCT  
ser gly asn ser gln glu ser val thr glu gln asp ser lys asp thr tyr ser leu  
1741/198  
AGC AGC ACC CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA CAC AAG GTC TAC GCC TGC GAA  
ser ser thr leu thr leu ser lys ala asp tyr glu lys his lys val tyr ala cys glu  
1801/218  
GTC ACC CAT CAG GCC CTG ACG TCG CCC GTC ACA AAG ACG TTC AAC AGG GGA GAG TGT TGA  
val thr his glu lys leu ser ser pro val thr lys ser phe asn arg gly glu cys  
1861/237  
ATTC
Figure 6

GGATCTATCGATTCCCGGGTACCTG GGA TCT AAA
met gly ser lys

1201/5
CCT TTT TG TCT CCT TTT TCA TTG TCA TTG CTT TTG TT TAA ACT AAT TTG GCA GGG pro phe leu ser leu leu ser leu ser leu leu leu leu thr ser thr ser leu ala gly
1261/25
GTC CAG CTT CAG CCA GGA CCT GAC CTG ATG AAA CCT GGG GCC TCA GTG AAG ATA TCC val glu leu glu gln ser gly pro asp leu val yis pro gly ala ser val lys ile ser
1321/45
TGC AAG GCT TCT GGA TAC ACA TTC ACT GAC TAC AAT CAC TGG GTG AAG CAG ACC CTT cys lys ala ser gly tyr thr phe thr asp tyr asn ile his his trp val yis gln ser arg
1381/65
GGA AAG AGC CTT GAG TGG ATT GGA TAT ATT TAT CCT TAC AAT GGT AAT ACT TAC TAC AAG gly lys ser leu glu trp ile gly tyr ile tyr pro tyr asn gly thr thr thr tyr asn
1441/85
CAG AAG TTC AAG AAC AAG GCC ACA TTG ACT GGA GAC AAT TCC TCC ACC TCA GCC TAC ATG gln lys phe lys asn lys ala thr leu thr val asp ser ser thr ser ala tyr met
1501/105
GAG CTC CGC AGC AGC ACA TCT GAG GAC TCT GCA GTC TAT TAC TTG AAG TAC TTG GAC glu leu arg ser leu thr ser leu val ser thr tyr asp ser ala thr ser thr thr asp
1561/125
Sac I
TAC TGG GCC CAA GGC ACC ACT CTC ACA GTG AGC TCA GCA TCC CGG ACC AGC CCC AAG GTC tyr trp gly gln gly thr thr leu thr val ser ser ala ser pro thr ser pro lys val
1621/145
TTC CGG CTG AGC CTC AGC ACC ACC CCC CAA GAT GGG AAC GTG GTC GCA TGC CTG GTG phe pro leu ser asp ser thr pro gln asp gly asn val val lys leu cyu ser
1681/165
GAG GCC TTC TTC CCC CAG GAG CCA CTC AGT GTG ACC TGG AGC GAA AGC GAA CAG ACC GTG gln gly phe phe pro glu pro leu ser val thr thr ser gly glu asn ser
1741/185
ACC GCC AGA AAC TTC CCA CCT AGC CAG GAT GCC TCC GGG GCC CTG TAC ACC AGC AGC thr ala arg asn phe pro pro ser gln asp ala ser gly asp leu tyr thr thr ser ser pro pro glu thr his leu leu arg met pro pro pro gly thr thr thr pro arg ala ala arg gly lys leu pro thr AMB pro gly cyu asp arg gly pro val his gly gln pro
1801/205
GCG CTG ACC CTG CGG GCC ACA CAG TCC CCA GAC GGC AAG TCC GTG ACA TGC CAC GTG AAG gln leu thr leu pro ala thr gln cys pro asp gly lys ser val thr cyu thr his val lys
1861/225
CAC TAC AGC AAT TCC AGC CAG GAT GTG ACT GTG CCC TGC GAA GTT CCC CCA CTT CCT CCA his tyr thr asp leu ser glu thr val thr thr val pro cys arg pro pro pro pro pro
1921/245
TGC TCC CAC CCC CGA CTG TGG CTC CAC CGA CCC GCC CTC GAG GAG GTC TCT TTA GTT CTA cys his pro arg leu ser leu his arg pro ala leu leu lys leu ser
1981/265
GAA GGC AAC CAG CTC ACC GCT ACC GGC CTC GAG GAG GTC TCT TTA GTT CTA CYS HIS pro arg leu ser leu his arg pro ala leu leu gly ser
2041/285
TGG AGC CCC TCA AGT GGG AAG ACC GTG CTT CAA GGA CCA CCT CGT GAC CTC TGG GCC trp thr pro ser ser gly lys ser ala val gln glu pro pro glu arg asp leu cys gly
TGC TAC AGC GTG TCC AGA GTA CTT CCT GGC TGT GCC CAG CCA TGG AAC CAT GGG GAG ACC
cys tyr ser val ser arg val gly cys ala gln pro trp asn gln gln thr

TTC ACC TGC ACT GCT GCC CAC CCC GAG TTG AAG ACC CCA TTA ACC GCC AAC ATC ACA AAA
phe thr cys thr ala ala his pro glu leu lys thr pro leu thr ala asn ile thr lys

TCC GGA AAC ACA TTC CGG CCC CAG GTC CAC CTG CCG CCG TCG GAG GAC CTG GCC
ser gly asn thr phe arg pro glu val his leu leu pro pro pro ser glu glu leu ala

TCT AAC GAG CTG GTG ACG ATG ACC TGC CTC CTA ACC ACC TGG AAG GAT GTG CTG
leu asn glu leu val thr leu thr cys leu ala arg gly phe ser pro lys asp val leu

GTT CCC TGG CTG CAG GGG TCA CAG GAG CTG CCC CCG CAG GAG AGG TAC CTG ACT TGG GCA TCC
val arg trp leu gln gly ser glu glu leu pro arg glu lys tyr leu thr trp ala ser

GGG CAG GAG CCC AGC CAG GCC ACC ACC TAT GCT GTG ACC AGC ATA CTG CGC GTG GCA
arg gln glu pro ser glu gly thr thr thr thr thr leu leu ser ile val arg val ala

GCC GAG GAC TGG AAG GAG GCC TCC TCC TGC ATG GTG GCC CAC GAG GCC TCG CCG
ala glu asp trp lys lys gly glu thr phe ser cys met val gly his glu ala leu pro

CTG GCC TTC ACA CAG AAC ACC ATC GAC CGG TGG GCC CAG GCC TCG CCG
leu ala phe thr gln lys thr ile asp arg leu ala glylys pro thr his ile asn val

TCT GTT GTC ATG GCG GAG GCC GCC ACC TGC TAC AGA TCT GAA AAG GAT GAA CTT TAG
ser val val met ala glu ala asp gly thr cys tyr arg ser glu lys asp glu leu

AAT TC
Figure 7

AGGATCCTATGATCCTGGGTCACC ATG GAG AAC CAT TTG CTT TTC TGG GGA GTC CTG GCG
met glu asn his leu leu phe trp gly val leu ala

1201/13
GTT TTT ATT AAG GCT GTT CAT GTG AAA GCC CAA GAA GAT GAA AGG ATT GTT CTT GTT GAC
val phe ile lys ala val his val lys ala gln glu asp glu arg ile val leu val asp

AAA AAG TGG AAG TCT GCC CGG ATT ACT TCC AAG ATC ATC CCT GAT CCT AAT
asn lys lys cys ala arg ile thr ser arg ile ile arg ser ser glu asp pro asn

1321/53
GAG GAC ATT GTG GAG AGA AAG ATC CGA ATT ATT GTT CCT CTG AAC AAC AAG GAG AAT ATC
glu asp ile val glu arg asn ile arg ile ile val pro leu asn asn arg glu asn ile

1381/73
TCT GAT CCC ACC TCA CCA TTG AGA ACC AGA AAT ATT GTT CCT CTG AAC AAC AAG GAG AAT
er ser pro thr ser pro leu arg thr arg phe val tyr his leu ser asp leu cys lys

1441/93
AAA TGT GAT CCT ACA GAA GTG GAG CTG GAT AAT CAG ATA GTT ACT GCT ACC CAG AGC AAT
lys cys asp pro thr glu val glu leu asp asn gln ile val thr ala thr gln ser asn

1501/113
ATC TGT GAT GAA GAC AGT GCT ACA GAG ACC TGC TAC ACT TAT GAC AGA AAC AAG TGC TAC
ile cys asp glu asp ser ala thr glu thr cys tyr thr thr asp arg asn lys cys tyr

1561/153
ACA GCT GTG GTC CCA CTC GTA TAT GTT GGT GAG ACC AAA ATG GTG GAA ACA GCC TTA ACC
thr ala val val pro leu val tyr gly gly glu thr lys met val glu thr ala leu thr

1621/153
CCA GAT GCC TGC TAT CCT GAC TGA ATTC
pro asp ala cys tyr pro asp
2078/309
AGT GTG GTG ATC ACA GCC GTG AGG AAG GAG GAT GCA GGG CGC TAC CTG TGT GGA GCC CAT
ser val val ile thr gly leu arg lys glu asp ala gly arg tyr leu cys gly ala his
2138/329
TGG GAT GGT CAG CGT CAG GAA GCC TGG CCT ATC CAG GCC TGG CAA CTC TGC GTG AAT GAG
ser asp gly gln leu gln gly gly ser pro ile gln ala trp gln leu phe val asn glu
2198/349
GAG TCC ACG ATT CCC CCG AGC CCC ACT GTG GTG AAG GGG GTG GCA GGA AGC TCT GTG GCC
glu ser thr ile pro arg pro ile pro val lys gly gly ser ser val ala
2258/369
GTT CTC TGC CCC TAC AAC CGT AAG GAA AGC AAA AGC ATC AAG TAC TGG TGT CTC TGC TGG GAA
val leu cys pro tyr asn arg lys glu ser lys ser ile lys tyr trp cys leu thr glu
2318/389
GGG GCC CAG AAT GCC CCC TCC CTG CTG GAC AGC GAG GGG TGG GTT AAG GCC CAG
gly ala gln asp ala gly cys pro ile leu val asp ser glu gly thr val lys ala gln
2378/409
TAC GAG GCC CCG TCC TCC CTG CTG GAG GCA GCC AAG GCC ACC TTC ACT GTC ATC CTC
tyr glu gly arg leu ser leu leu glu glu pro gly asn gly thr phe thr val ile leu
2438/429
AAC CAG CTC ACC AGC CGG GAC GCC GCC TTC TAC TGG TGT CTC ACC AAG GCC GAT ACT CTC
asn gln leu thr ser arg asp ala gly phe tyr thr cys leu thr asn gly asp thr leu
2498/449
TGG AGG ACC ACC GTG GAG ATC AAG ATT ATC GAA GGA GAA CCA AAC CTC AAG GTT CCC GSG
trp arg thr thr val glu ile lys ile ile glu gly glu pro asn leu lys val pro gly
2558/469
AAT GTC ACG GCT GTG CTG GGA GAG ACT CTC AAG GTC CCC TGT CAC TTT CCA TGC AAA TAC
asn val thr ala pro ser lys tyr thr leu lys thr leu lys pro cys his phe pro cys lys phe
2618/489
TCC TGT TAC GAG AAA TAC TGG TGC AAG TGG AAT AAC ACG GCC TGC CAG GCC CTG CCC AGC
ser tyr glu lys tyr thr cys lys thr asn thr asp gly cys gln ala leu pro ser
2678/509
CAA GAC GAA GCC CCC AGC AAG GCC TTC GTG AAC TGT GAG GAG AAG AGC CGG CTT GTC TCC
gln asp gly gly pro ser lys ala phe val asc gly glu asp ser arg leu val ser
2738/529
CTG ACC CTC AAC CTG GTC ACC AGG GCT GAT GAG GCC TGG TAC TGG TGT GGA GTG AAG CAG
leu thr leu asn leu val thr arg ala asp glu gly thr trp tyr trp cys gly val lys gln
2798/549
GCC CAC TTC TAT GGA GAG ACT GCA GCC GTC TAT GTC GTG GCA GTT GAA GAG AAG GCA GCG
gly his phe tyr gly glu thr ala ala val tyr val ala val glu arg lys ala ala
2858/569
GGG TCC CCG GAT GTC AGC CTA GCG AAG GCA GAC GCT GCT CTT GAT GAG AAG GTG CTA GAC
gly ser arg asp val ser leu ala lys ala asp ala ala pro asp glu lys val leu asp
2918/589
TCT GTT TTT CGG GAG ATT GAG AAC AAA GCC ATT CAG CCC AGG CTT TTT GCA GAG TGA
ser gly phe arg glu ile glu asn lys ala ile gln asp pro arg leu phe ala glu
2978
ATTC