Title: CO-EXPRESSSION OF ZEIN PROTEINS

Bgl II

β zein (no stop) SP δ zein (including signal peptide)

Abstract: Methods and constructs for transformed plants and plant tissues that are capable of expressing high levels of stable proteins localized as protein bodies within the plant cell. Transformed plants co-expressing high levels of both the 15 kD and 10 kD zein proteins are disclosed which accumulate to high levels as protein bodies in the vegetative tissue of the plant. Transformed plants co-expressing the 15 kD and 10 kD zein proteins are useful for providing forage crops containing increased levels of sulfur containing amino acids, such as methionine, in the diet of animals that normally feed on such crops. In one embodiment, a stable protein body is expressed in a plant or plant tissue as a fusion protein comprising both the 15 kD and 10 kD zein proteins operably linked by a polypeptide or peptide linker. The protein bodies provided in the present invention are resistant to rumen digestion or environmental degradation.
CO-EXPRESSSION OF ZEIN PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the filing of U.S. Provisional Patent Application Serial No. 60/284,732, entitled "Co-Expression of 15 kD and 10 kD Zein Proteins", filed on April 18, 2001, and the specification thereof is incorporated herein by reference.

This application is a continuation-in-part application of U.S. Patent Application Serial No. 09/479,724, entitled "Co-Expression of Proteins", to Suman Bagga, Champa Sengupta-Gopalan and John D. Kemp, filed on January 7, 2000, which is a division of application Serial No. 09/224,655, filed December 31, 1998, now abandoned, which is a continuation-in-part application of application Serial No. 08/866,879, now United States Patent No. 5,990,384, issued November 23, 1999, and provisional application Serial No. 60/020,424, filed May 31, 1996, now abandoned; and the specification thereof of each is incorporated herein by reference.

GOVERNMENT RIGHTS

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. 99-34331-7524 and Grant No. 99-34250-7420, both awarded by the United States Department of Agriculture.

BACKGROUND OF THE INVENTION

Field of the Invention (Technical Field):

The present invention relates to methods and constructs for co-expression of zein proteins in plants, including forage plants, including co-expression of a 15 kD and 10 kD zein protein by means of a gene construct coding for such proteins and for a linker peptide sequence joining such proteins.
Background Art:

Note that the following discussion refers to a number of publications by author(s) and year of publication, and that due to recent publication dates certain publications are not to be considered as prior art vis-à-vis the present invention. Discussion of such publications herein is given for more complete background and is not to be construed as an admission that such publications are prior art for patentability determination purposes.

Alfalfa (Medicago sativa L.) is considered to be the most important cultivated forage crop in the world. It is widely grown, has a superb balance of vitamins and minerals, is high yielding, is an excellent source of biological nitrogen fixation, and it serves as an attractive nectar source for honeybees. Alfalfa has been bred for years for both forage quality and plant performance. Although alfalfa and other leguminous forage crops are high in protein, these plants are deficient in the sulfur amino acids (S-amino acids), methionine and cysteine. It has been shown that wool growth in sheep is limited by the availability of S-amino acids. Similarly, milk production by dairy animals is affected by the deficiency of S-amino acids in plants. Efforts to use conventional plant breeding and cell selection techniques to increase the S-amino acid content of alfalfa have met with little or no success.

A genetic engineering approach to improve the amino acid balance of alfalfa and other forage crops would be to introduce into these plants genes encoding proteins high in methionine driven by a strong constitutive promoter or a leaf promoter. In order to significantly alter the amino acid balance of legume forage, the foreign proteins should contain about 15 to 25% of S-amino acids and constitute 5 to 10% of the total leaf protein. To achieve these levels of protein accumulation, one has to ensure not only maximum levels of transcription and translation of the gene but also the stability of the protein. In regard to forage crops for ruminant animals, the digestibility of S-amino acid containing proteins by the rumen bacteria and the stomach enzymes is also an extremely critical issue in regard to providing a suitable forage crop for ruminant animals, but is often overlooked. Thus, the S-amino acid rich protein should be relatively resistant to degradation in the rumen (first stomach) of the ruminant animals and should be assimilated in the lower gastrointestinal tract.
Most of the concerted efforts in regard to nutritional improvement in plants have focused on seed proteins. Since corn and other cereal crops are not easily transformable, most work directed to seed protein modification has involved testing stability of modified prolamine proteins in transgenic tobacco and *Xenopus oocytes*. The synthesis of lysine containing α zeins was also analyzed in transgenic tobacco and petunia seeds. Both the normal and modified proteins were found to have a very short half-life.

Efforts to improve the S-amino acid content of legume seed proteins have included introducing a 45 bp oligonucleotide containing six methionine codons into the third exon of a β-phaseolin gene. Transformants containing this modified gene showed that the high methionine phaseolin was synthesized at the same level as the normal protein, but was very unstable and was rapidly turned over. Introduction of the extra amino acids in the β-phaseolin protein probably caused a distortion in its secondary structure making it more susceptible to proteolytic degradation.

DeClercq et al. (DeClercq, A., M. Vandewele, R. De Rycke, J. Van Damme, M. Van aMontagu, E. Krebbers, J. Vandekerckhove (1990) "Expression and processing of an Arabidopsis 2S albumin in transgenic tobacco" *Plant Physiol* 94:970-979) replaced a 23 amino acid coding segment between the sixth and seventh cysteine residues of Arabidopsis 2S albumin, with three different high methionine coding fragments. These modified Arabidopsis 2S genes were transformed into *A. thaliana*, *B. napus* and tobacco. There was some accumulation of the protein in the seeds but not as much as predicted. The gene of the 2S albumin of Brazil nut, which contains up to 19% methionine, and driven by the β-phaseolin gene promoter, has been introduced into tobacco, rape and soybean. Saalbach et al. (Saalbach, I., et al. (1994) "A chimeric gene encoding the methionine-rich 2S albumin of the Brazil nut (*Bertholletia excelsa* H.B.K.) is stably expressed and inherited in transgenic grain legumes" *Mol Gen Genet* 242:226-236) synthesized the 2S albumin gene and engineered it behind the CaMV 35S promoter. The gene, when introduced into tobacco and some grain legumes, showed the highest level of expression in the plant leaves with the protein localized in vacuoles. However, Brazil nut albumin protein is extremely allergenic and may not be acceptable for consumption. U.S. Patent No. 5,939,599, to Chui et al., issued August 17, 1999, discloses a nucleic acid fragment for
overexpression of a high methionine seed storage protein in plants, which nucleic acid fragment is derived from and related to the 10 kD δ zein.

Another approach to increase the pools of particular amino acids in plants has been to introduce bacterial genes encoding for key regulatory enzymes in amino acid biosynthetic pathways in plants. A bacterial gene encoding for aspartate kinase which is desensitized to feedback inhibition by lysine and threonine was fused to the β-phaseolin gene promoter and introduced into tobacco. The seeds of the transgenic tobacco showed increased levels of free threonine and methionine.

Very little effort has been made with regards to improving forage crop protein quality. Schoeder et al. (Schoeder, H.E., et al. (1991) "Expression of a chicken ovalbumin gene in three lucerne cultivars" Aust J Plant Physiol 18: 495-505) introduced the chicken ovalbumin gene (cDNA), driven by a CaMV 35S promoter, into alfalfa. The transgenic alfalfa plants, however, showed very low level accumulation of the protein in the leaves (0.005%). The basis for such a low abundance of this protein in the transgenic alfalfa leaves was not determined.

Some efforts to obtain alfalfa mutants that have larger free methionine levels have also been attempted at the University of Wisconsin. Cell lines with resistance to growth inhibition by an amino acid analogs reportedly produce higher than normal amounts of the corresponding natural amino acid. Hence, growth on specific amino acid analogs has been used as a selection tool to select for plants accumulating high levels of a particular amino acid. Amino acid over-production is usually due to relaxed feedback control of an enzyme involved in its production. In an attempt to improve the methionine content of alfalfa, mutagenized suspension culture cells of alfalfa were selected for resistance to growth inhibition by a methionine analog. A few cell lines containing high methionine pools were obtained, however, regeneration of these cell lines did not produce plants with high methionine content.

Zeins are a group of alcohol soluble proteins that are synthesized during endosperm development in corn and constitute 50% of the total protein in mature seeds. The zeins can be
divided into four groups, the α, β, γ and δ, based on their solubility (Larkins, B. A., C. R. Lending, J.C. Wallace, G. Galli, E. E. Kawata, K. B. Geetha, A. L. Kirz, D. M. Martin, and C. E. Bracker (1989) "Zein gene expression during maize endosperm development" In: Goldberg R. B. (Ed) The Molecular Basis Of Plant Development, Alan R. Liss, NY pp. 109-120). The zeins can also be separated by size into groups. The α zeins, which are the most abundant class, are made up of the 22 kD and 19 kD zeins; the central region of these proteins consists of repetitive peptides of about 20 amino acid residues. The β zeins comprise the 15 kD zein which contains less proline and glutamine than the α zeins. The γ zeins include the 27 kD and 16 kD class and are very rich in proline (25%). The δ zeins are a relatively minor class consisting of the 10 kD zein. All the zein classes are structurally unique.

The repeat regions in the α and γ zeins probably have a major role in the packing of protein bodies. Zeins, in general, contain extremely low levels of the essential amino acids lysine, tryptophan and to a lesser extent methionine. The 15 kD and 10 kD zeins, however, are distinguished by their extremely high methionine content (10% and 22.5%, respectively).

The zeins are synthesized on the endoplasmic reticulum (ER) and they aggregate into protein bodies directly in the ER. Based on the analysis of the zein composition of developing protein bodies in corn endosperm, Lending and Larkins (Lending, C. R. and B. A. Larkins (1989) "Changes in the zein composition of protein bodies during maize endosperm development" Plant Cell 1:1011-1023), have proposed a descriptive model for the pattern of zein deposition during protein body formation in corn endosperm: the β and γ zeins are the first to start accumulating within the ER. Subsequently, α zeins begin to accumulate as locules within the β and γ zeins. With time, the α zein locules fuse and form a central core while the β and γ zeins form a continuous layer around the periphery of the protein body. In a separate study, Esen and Stetter (Esen, A. and D. A. Stetter (1992) "Immunochemical location of γ-zein in the protein bodies of maize endosperm" Am J Bot 79:243-248), demonstrated that the δ zein occurs throughout the core region of the protein body.

Mutations in maize affect the expression of the different zein genes. Changes in zein gene expression in turn have direct impact on the amino acid composition of the seeds. Seeds of plants homozygous for the recessive mutation opaque-2, have increased levels of lysine compared to the
wild-type seeds. The increase in lysine is due to the reduced expression of the 22 kD α zeins. The inbred line BSSS-53 has 30% higher level of seed methionine compared to other inbred lines. This increase in methionine content is because of a two-fold increase in the level of the 10 kD zein.

Proteins that accumulate in the endoplasmic reticulum are known to have the amino acid sequence Lys(His)-Asp-Glu-Leu (K(H)DEL) (SEQ ID NO:1) near their carboxy terminal end which prevents them from exiting into the Golgi. The zeins and other prolamines, however, lack this sequence. A cognate of the 70-kD heat shock protein, BIP, which functions as a molecular chaperone, has been shown to be involved in the formation of prolamine protein body formation in rice endosperm. The determination of involvement of BIP in the formation of zein protein bodies is based on the fact that BIP accumulates to high levels in the ER and on the abnormal protein bodies of some of the zein regulatory mutants of corn. Overall, however, the mechanisms of zein targeting and assembly in protein bodies are poorly understood and it is not known whether inter- and intramolecular interactions play a key role in protein body formation.

U.S. Patent No. 5,990,384 discloses and claims a method to modify plants such that the resulting transgenic plant expresses both a 15 kD zein, such as a β zein, and a 10 kD zein, such as a δ zein. In one embodiment, this is accomplished by introducing 15 kD or 10 kD zein coding sequences under the control of a promoter, such that the resulting transgenic plant expressed either the 15 kD or the 10 kD zein, and thereafter breeding sexual crosses. The resulting seeds produce seedlings expressing both genes. Genetic constructs can further be made expressing both the 15 kD and the 10 kD zein (S. Bagga et al. (1997) "Co-expression of the maize δ-zein and β-zein genes results in stable accumulation of δ-zein in endoplasmic reticulum-derived protein bodies formed by β-zein" Plant Cell 9:1683-1696). U.S. Patent Application Serial No. 09/479,724 discloses a specific chimeric gene, consisting of a 10 kD zein gene fused in frame to the front of an oryzacystatin I protease inhibitor gene, resulting in a construct producing proteins that both provide S-amino acids and control plant pests. The chimeric gene includes both the coding and signal peptide regions of the 10 kD zein. The 10 kD zein protein, which contains an N-terminal signal peptide that directs the protein into the ER, is folded into a tertiary conformation depending, in part, on the signal peptide.
Modification of the signal peptide can result in varying accumulation patterns, such that a morphologically distinct protein body results. (J. Randal et al. (2000) "A modified 10 kD zein protein produces two morphologically distinct protein bodies in transgenic tobacco" Plant Science 150:21-28).

As can be understood from the above, there remains a need in the art for plants and forage crops that contain stable protein bodies that are high in S-amino acid content. The subject invention provides a novel and advantageous means for improving the forage quality of plants.

SUMMARY OF THE INVENTION (DISCLOSURE OF THE INVENTION)

The invention provides an isolated and purified nucleic acid fragment, which fragment includes a nucleotide sequence encoding a 15 kD zein and a nucleotide sequence encoding a 10 kD zein, joined, directly or through one or more additional sequences, by a nucleotide sequence encoding a polypeptide linker sequence. In one embodiment, the polypeptide linker sequence is a signal peptide for one of the encoded zeins, and is more specifically a signal peptide for a 10 kD zein. In the nucleic acid fragment, the codon coding the polypeptide linker sequence is preferably from about ten to about thirty amino acid residues, and is more preferably about twenty amino acid residues. The nucleic acid fragment may optionally include the nucleotide sequence of either SEQ ID NO:4 or SEQ ID NO:6. In one embodiment, the nucleic acid fragment is operably linked to a promoter, a plant signal sequence, or a regulatory sequence directing expression in one or more organs of a plant, or any combination of the foregoing.

In another embodiment, the invention provides a transgenic plant transformed with a gene sequence encoding a 15 kD zein and a nucleotide sequence encoding a 10 kD zein, joined, directly or through one or more additional sequences, by a nucleotide sequence encoding a polypeptide linker sequence, and operably linked to a regulatory sequence directing expression in one or more organs of a plant. The invention further provides a polypeptide product including a 15 kD zein linked to a 10 kD zein by a polypeptide linker, wherein the polypeptide linker includes from about ten to
about thirty amino acid residues. The polypeptide product may result from the expression in a prokaryotic or eukaryotic host cell of a nucleic acid fragment as described above.

In yet another embodiment, the invention provides a method for expression of stable protein bodies in a plant, the method including transforming a plant or plant tissue with a polynucleotide molecule that encodes a storage protein comprising a first protein and a second protein joined by a polypeptide linker, wherein the storage protein is expressed and accumulated as a protein body in a vegetative tissue of the plant or plant tissue. In this method, the first protein and second protein may be zein proteins, and in any event, the first protein and second protein may be different. If the first protein and second protein are zein proteins, they may be a 10 kD zein protein and a 15 kD zein protein. The storage protein made by the method may, in a preferred embodiment, be rumin stable.

The invention further provides a composition that includes a rumin stable protein body, wherein said protein body comprises a first protein and a second protein joined by a polypeptide linker, and wherein the protein body is expressed and accumulated in a plant. In this composition, the first protein and second protein can be zein proteins.

A primary object of the present invention is to provide a fusion protein, composed of two zein proteins joined by a linker, to increase availability of S-amino acids in plants.

Another object of the present invention is to provide a gene fusion construct, which construct includes one or more promoters, and further includes sequences coding for two zein proteins and a linker.

Another object of the invention is to provide transgenic plants wherein leaves and/or seeds contain stable protein bodies, the protein bodies including a protein consisting of two zein protein sequences joined by a linker polypeptide.
Yet another object of the invention is to provide stably transformed plants, such that seeds derived from such plants contain nucleic acid sequences coding for two zein proteins and a linker. Preferred are plants with utility as forage plants, such as alfalfa, but other plants are included, such as corn, soybeans, rapeseed, tobacco and rice.

A primary advantage of the present invention is that it provides a single linked protein, consisting of two zein proteins joined by a polypeptide linker, which protein can be expressed in commercially useful quantities in plants.

Another advantage of the present invention is that, by means of the polypeptide linker, the two linked zein proteins may each assume a desired tertiary structure.

Another advantage of the present invention is that the expression of S-amino acid proteins and protein bodies is enhanced by means of two zein proteins joined by a polypeptide linker, as compared to two zein proteins directly joined without a polypeptide linker.

Other objects, advantages and novel features, and further scope of applicability of the present invention will be set forth in part in the detailed description to follow, taken in conjunction with the accompanying drawings and sequence listings, and in part will become apparent to those skilled in the art upon examination of the following, or may be learned by practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated into and form a part of the specification, illustrate one or more embodiments of the present invention and, together with the description, serve to explain the principles of the invention. The drawings are only for the purpose of illustrating one or more preferred embodiments of the invention and are not to be construed as limiting the invention. In the drawings:
FIG. 1 shows a diagrammatic representation of a fusion gene construct of a 15 kD β zein and a 10 kD δ zein without a linker.

FIG. 2 shows a diagrammatic representation of a fusion gene construct of a 15 kD β zein and a 10 kD δ zein with a linker, the linker depicted as the signal peptide (SP) gene sequence for the 10 kD δ zein.

FIG. 3 shows the western analysis of independently transformed tobacco plants accumulating the β/δ zein fusion proteins. Fifty micrograms of ethanol soluble protein was separated on a 16% SDS-PAGE gel, transferred to nitrocellulose, and immunodetected with β and δ zein antisera. The top blot was immunodetected with a δ zein antibody and the bottom blot with a β zein antibody. Lanes 1, 2, 3, and 4 are protein from transgenic plants containing the β/δ zein fusion construct without a linker. Lanes A, B, and C are protein from transgenic plants containing the β/δ zein fusion construct with a linker of the construct of FIG. 2, including the linker sequence of SEQ ID NO:5. Lane NT is the non-transformed negative control. The positive control on the top blot is a high accumulating altered 10 kD zein transgenic tobacco plant, and the positive control on the bottom blot is a high accumulating 15 kD zein transgenic tobacco plant. As depicted, protein levels in lanes A and C are higher than lanes 1, 2, 3, and 4. β/δ zein fusion protein accumulates to higher levels in plants A and C than in plants 1, 2, 3, and 4.

FIG. 4 shows an electron micrograph of leaves from independently transformed tobacco plants accumulating the β/δ zein fusion proteins with a linker of the construct of FIG. 2, including the linker sequence of SEQ ID NO:5, with localization using anti-β-zein polyclonal rabbit antisera bound to gold. Leaves from transformed plants were sectioned and fixed as described by Bagga et al., 1995. The grids were observed using a Hitachi H700 transmission electron microscope. In the micrograph, "RB" depicts ribosomes, "PB" depicts protein body, "CW" depicts cell wall and "MT" depicts mitochondria, and the arrows indicate the gold-conjugated antibody.
FIG. 5 shows an electron micrograph as in FIG. 4, but with localization using anti-δ-zein polyclonal rabbit antisera. In the micrograph, “PB” depicts protein body, “RB” depicts ribosomes and “CP” depicts chloroplasts, and the arrows indicate the gold-conjugated antibody.

FIG. 6 shows the fusion gene construct and coded proteins for the fusion portion of a 15 kD β zein and a 10 kD δ zein without a linker. The 5′ end through position 6 is the β zein; the 3′ end from position 13 to 30 is the δ zein; and the position 7 to 12 are two amino acids, Arg and Ser, introduced with the restriction site.

FIG. 7 shows the fusion gene construct and coded proteins for the fusion portion of a 15 kD β zein and a 10 kD δ zein with a linker, wherein the linker includes the signal peptide gene sequence for the 10 kD δ zein. The 5′ end through position 6 includes a portion of the β zein; the 3′ end from position 73 to 87 includes a portion of the δ zein; position 7 to 12 code for two amino acids, Arg and Ser, introduced with the restriction site; and position 13 to 72 denotes the linker, here the signal peptide sequence for the 10 kD δ zein.

FIG. 8 shows the fusion gene construct and coded proteins for the fusion portion of a 15 kD β zein and a 10 kD δ zein with a linker. The 5′ end through position 6 includes a portion of the β zein; the 3′ end from position 73 to 87 includes a portion of the δ zein; position 7 to 12 code for two amino acids, Arg and Ser, introduced with the restriction site; and position 13 to 72 denotes the linker.

**BRIEF DESCRIPTION OF THE SEQUENCES**

The accompanying sequence listing, which is incorporated into and forms a part of the specification, illustrate one or more embodiments of the present invention and, together with the description, serve to explain the principles of the invention. The sequences are only for the purpose of illustrating one or more preferred embodiments of the invention and are not to be construed as limiting the invention. In the sequence listing:
SEQ ID NO: 1 represents a plant protein sequence found in proteins that accumulate in the endoplasmic reticulum, which sequence is near the carboxy terminal end of the protein and serves to prevent the protein from exiting into the Golgi.

SEQ ID NO: 2 represents the fused gene construct of FIGS. 1 and 6.

SEQ ID NO: 3 represents the amino acid sequence coded by SEQ ID NO: 2.

SEQ ID NO: 4 represents the fused gene construct of FIGS. 2 and 7.

SEQ ID NO: 5 represents the amino acid sequence coded by SEQ ID NO: 4.

SEQ ID NO: 6 represents the fused gene construct of FIG. 8.

SEQ ID NO: 7 represents the amino acid sequence coded by SEQ ID NO: 6.

DESCRIPTION OF THE PREFERRED EMBODIMENTS
(BEST MODES FOR CARRYING OUT THE INVENTION)

The subject invention concerns plants and plant tissues that are capable of expressing high levels of stable storage proteins that are localized as protein bodies within the plant cell. Plants contemplated within the scope of the invention include forage crop plants, including, for example, alfalfa, clover, corn silage, sorghum and other leguminous crops, transformed to express the proteins of the invention. Also contemplated within the scope of the present invention are plants for human consumption, which have been transformed to express proteins that enhance the protein quality of the plant for improved nutrition. Specifically exemplified are plants expressing proteins containing high levels of S-amino acids, such as methionine and cysteine. In a preferred embodiment, a zein protein is expressed in the plant or plant tissue. More preferably, the zein protein expressed is the 15 kD and 10 kD zein proteins co-expressed in the plant or plant tissue, preferably joined by a polypeptide linker. The zein proteins expressed in plants are preferably resistant to rumin
degradation and, therefore, are useful for providing nutritionally important amino acids that can be 
digested in the stomach and absorbed by the ruminant animal because of the protein's capacity to 
"by-pass" the rumin.

Also contemplated by the subject invention are plants or plant tissue comprising rumin stable 
protein bodies which contain other proteinaceous material, for example, an antigenic determinant 
capable of eliciting an immune response, a proteinaceous drug, pesticide or antimicrobial peptide. 
Heterologous and endogenous proteins and synthetic peptides having essential amino acids can be 
expressed in plants transformed with the storage proteins of the subject invention which can act as a 
"carrier protein," whereby the proteins coalesce and accumulate in the cell as a protein body. In an 
alternative embodiment, a rumin stable protein body is expressed in a plant or plant tissue as a 
fusion protein comprising a zein protein and a heterologous protein or peptide. The fusion protein 
can be designed to yield the heterologous protein portion by cleavage with a selected enzyme or 
under certain physiological conditions. Preferably, both the 15 kD and 10 kD zein proteins are co-
expressed in the plant or plant tissue comprising the fusion protein, joined by a linker, such as a 
polypeptide linker.

The subject invention also pertains to a rumin stable protein body. Rumin stable protein 
bodies of the invention are not subject to digestion by rumin bacteria in the rumin of an animal but 
can be digested proteolytic enzymes of an animal's stomach. A rumin stable protein body of the 
present invention can be prepared which contains heterologous proteinaceous material in addition to 
the rumin stable protein, for example, an antigenic determinant capable of eliciting an immune 
response, a proteinaceous drug, pesticide or antimicrobial peptide. Rumin stable protein bodies can 
be isolated from plants that have been transformed with polynucleotide molecules encoding the 
desired rumin stable proteins. Plant cells expressing the polynucleotide molecules encoding the 
desired rumin stable proteins can be readily selected and regenerated into plants or plant tissue 
using standard techniques known in the art.
In one embodiment of the present invention, a first storage protein gene is co-expressed in a cell with a second storage protein gene whereby the first gene and second gene are joined by a linker, such that the first protein and second protein are joined by a polypeptide linker. Thus in one embodiment a gene fusion construct is provided, wherein the β zein coding region is fused to the δ zein coding region with a linker coding region between the genes. The gene fusion construct can be introduced into a plant to be transformed behind a suitable promoter, such as the 35S constitutive promoter. The resulting fusion protein construct with a linker between the two zein proteins accumulates to higher levels than a protein construct without a linker. Further, plants with the fusion protein containing the linker produce protein bodies, which protein bodies are different morphologically than the rosette, spherical or aggregate-shaped protein bodies previously noted with zein proteins.

The linker in the gene fusion construct is selected from any suitable sequence coding for a polypeptide that may be employed as a linker. In one embodiment, the linker is a signal peptide gene sequence for a zein protein, such as the signal peptide for the δ zein. In this embodiment, the expressed signal peptide sequence does not necessarily serve as a functional signal peptide, but rather serves to link the β zein and the δ zein. The linker may alternatively be any sequence coding a utilizable fusion polypeptide. Thus, for example, the linker polypeptide, and gene coding therefore, disclosed by Prescott et al. may be employed (Prescott, M. et al. (1999) "The length of polypeptide linker affects the stability of green fluorescent protein fusion proteins" Anal Biochem 273:305-307). It is hypothesized that the resulting linker polypeptide should be of such length as to permit the two fusion zein proteins to each assume a desired and typical tertiary structure. It is further hypothesized that two fusion zein proteins linked without a linker sequence do not assume a desired and typical tertiary structure, because of steric and related considerations, thereby adversely affecting the stability of the protein and the ability of the protein to form protein bodies. In general, the linker polypeptide may be of any desired length, such as from about ten to about thirty amino acids, and preferably about twenty amino acids.
Regulatory sequences employed with the protein genes (promoters, initiation sequences, termination sequences, polyadenylation sequences, enhancers, etc.) are readily chosen by one of ordinary skill in the art based on a variety of factors, such as, for example, i) the specific protein genes employed, ii) the target cell to be transformed, iii) the plant tissue where expression/accumulation is desired, iv) the particular plant (monocot, dicot, etc) species to be transformed, etc. For example, when a plant cell is the target cell then a constitutive promoter may be chosen (e.g., CaMV 35S, ubiquitin, etc.) or a tissue specific promoter may be employed that will express at high levels in specific tissues (seeds, green tissues, etc.). In general, promoters that can be employed with zeins include, in addition to the foregoing, the CvMV promoter, a constitutive promoter from cassava, and the phaseolin promoter.

In a preferred embodiment of the present invention, alfalfa, tobacco or other plant cells are transformed with a fusion gene construct composed of a 15 kD zein protein gene and a 10 kD zein protein gene, joined by a sequence coding a linker polypeptide, wherein both zein genes are driven by a constitutive promoter. Fertile, transgenic plants containing the fusion gene construct are regenerated. Progeny plants are grown and the 10 kD zein protein is accumulated in green tissue at levels significantly more than the accumulation level of the 10 kD protein when expressed alone, the 15 kD protein when expressed alone, or a construct of the 10 kD and 15 kD protein not joined by a polypeptide linker.

Additionally, the present invention encompasses novel protein bodies formed as a result of expressing a fusion protein gene construct in green plant tissues. In one embodiment, the novel protein body includes 10 kD zein protein segment joined, by means of a polypeptide linker, to a 15 kD zein protein segment. The protein body is typically located in leaf tissue. In a preferred embodiment, the novel protein body is located in leaf tissue and comprises a 15 kD zein protein segment and a 10 kD zein protein segment linked by means of a polypeptide linker.

The subject invention also concerns a method for increasing the forage quality of a plant comprising transforming a plant or plant tissue with a polynucleotide molecule that encodes a storage
protein of the present invention. Methods for transforming plants and selecting for expression of the transformed genotype are known in the art. In a preferred embodiment of the method, the polynucleotide encodes a zein protein which is expressed in the plant or plant tissue. More preferably, the zein protein expressed is the 15 kD and the 10 kD zein protein. Most preferably, the zein protein expressed is the 15 kD and 10 kD zein proteins joined by a polypeptide linker, and co-expressed in the transformed plant or plant tissue. Transgenic plants can be readily prepared from the transformed plant or plant tissue using standard techniques known in the art.

It is known that β and δ zeins produce ER derived protein bodies in the leaves and seeds of transgenic tobacco plants when under the control of the 35S constitutive promoter. Both proteins are stable and accumulate to high levels in the leaves of transgenic tobacco plants. ER-derived protein bodies have been observed in the leaves of these transgenic tobacco plants containing the δ (10 kD) and β (15 kD) genes. Morphology differences of the ER derived protein bodies in leaves have been observed between the δ and β zein transgenic plants. The β zein protein immunolocalizes in rosette-shaped ER protein bodies and the δ zein protein immunolocalized in spherical-shaped ER protein bodies. When plants containing the β zein protein are crossed with plants containing the δ zein protein, co-localization of both proteins is found in the β zein rosette bodies (Randall et al., 2000). The protein bodies produced by a fusion protein consisting of β and δ zeins joined by a polypeptide linker appear to have a slightly different morphology than the rosette or spherical bodies previously observed.

The zein proteins of the present invention include not only those proteins having the same amino acid sequence as found in nature, including allelic variants, but also includes those variant zein proteins having conservative amino acid substitutions, additions and deletions in the protein sequence, as long as the variant protein retains substantially the same relevant biological activity as the native zein protein. The skilled artisan, having the benefit of the teachings disclosed herein, can readily determine whether a variant protein retains the substantially the same biological activity as the non-modified protein.
Standard procedures can be employed for recombinant DNA manipulations. Plasmid pMZEl10k containing the 10 kD zein cDNA isolated from a corn endosperm cDNA library (Kiriha, J.A., et al. (1988) "Differential expression of a gene for a methionine-rich storage protein in maize" Mol Gen Genet 211: 477-484), was a gift from Dr. J. Messing. A 470 bp EcoR1/Xba1 fragment containing the entire coding region was removed from pUC 119 and cloned into the EcoR1 and Xba1 sites of pSP73. The stop codon for the 10 kD zein is contained within the Xba1 site. The 10 kD zein gene was then recovered as a BglI/Xhol fragment and inserted into the BglI and Xhol sites in the polylinker of pMON316 (Rogers et al., 1987). The translation terminator following the stop codon of the 10 kD zein is the NOS terminator. The resulting plasmid was called pM10Z. Plasmid pMEZ, containing the 15 kD zein cDNA, is as described by Bagga et al. (Bagga, S., Adams Hanke, J. D. Kemp, and C. Sengupta-Gopalan (1995) "Accumulation of 15 kD zein in novel protein bodies in transgenic tobacco" Plant Physiol 107:13-23).

The invention is further illustrated by the following non-limiting examples.

Example 1

The stop and 3' UTR were removed from the β (15 kD) zein gene and a Bgl II site was added to the 3' end for cloning purposes. The δ (10 kD) mature gene consists of the site from the mature coding sequence to the natural stop with the addition of a Bgl II at the 5' end of the gene for cloning purposes. The construct is graphically illustrated at FIG. 1, and the sequence is shown at FIG. 6, with the sequence of the gene at SEQ ID NO:2 and the resulting amino acid sequence at SEQ ID NO:3.

Example 2

The full length δ (10 kD) zein gene including its signal peptide was placed behind the β (15 kD) zein with no stop at the Bgl II site. The signal peptide is used as a 20 amino acid linker between the two zein proteins to maintain their tertiary configuration. Constructs are placed in pGGG prior to plant transformation. The construct is graphically illustrated at FIG. 2, and the sequence is shown at FIG. 7, with the sequence of the gene at SEQ ID NO:4 and the resulting amino acid sequence at SEQ ID NO:5.
Example 3

Transfer of the constructs of Examples 1 and 2 into *Agrobacterium tumefaciens* (strain pTIT37ASE) was accomplished through tri-parental matings. *Nicotiana tabacum* plants were transformed using the leaf disc transformation system. Transformants were selected on MS media containing 100 μg/mL Kanamycin and the presence of the genes in transgenic tobacco was confirmed by PCR or Nopaline analysis.

Example 4

Protein was isolated from leaf tissue of transformed plants of Example 3 as described by Bagga et al. (1995). Zein proteins were detected after separating 50 μg of ethanol soluble protein on a 16% SDS-PAGE gel and challenging the blot with anti-β-zein polyclonal rabbit antisera or anti-δ-zein polyclonal rabbit antisera.

Example 5

Leaves from transformed plants of Example 3, using the construct of Example 2 including a signal peptide linker sequence, were sectioned and fixed. Detection was using gold-conjugated anti-β-zein polyclonal rabbit antisera or anti-δ-zein polyclonal rabbit antisera. The grids were observed using a Hitachi H700 transmission electron microscope. As shown in FIGS. 4 and 5, protein bodies specific for both β zein and δ zein were detected.

Example 6

A synthetic linker containing a Bgl II site is synthesized to the 5' end of the 10 kD zein mature coding sequence. This synthetic linker with the 10 kD mature coding sequence is then placed behind the 15 kD zein, with no stop, at the Bgl II site. The resulting construct is then placed in pGG prior to plant transformation, resulting in a sequence as shown in SEQ ID NO:6.
The preceding examples can be repeated with similar success by substituting the generically or specifically described reactants and/or operating conditions of this invention for those used in the preceding examples.

Although the invention has been described in detail with particular reference to these preferred embodiments, other embodiments can achieve the same results. Variations and modifications of the present invention will be obvious to those skilled in the art and it is intended to cover in the appended claims all such modifications and equivalents. The entire disclosures of all references, applications, patents, and publications cited above are hereby incorporated by reference.
What is claimed is:

1. An isolated and purified nucleic acid fragment comprising a nucleotide sequence encoding a 15 kD zein and a nucleotide sequence encoding a 10 kD zein, joined by a nucleotide sequence encoding a polypeptide linker sequence.

2. The nucleic acid fragment of claim 1, wherein the polypeptide linker sequence is a signal peptide for one of the encoded zeins.

3. The nucleic acid fragment of claim 2, wherein the polypeptide linker sequence is a signal peptide for a 10 kD zein.

4. The nucleic acid fragment of claim 1, wherein the polypeptide linker sequence comprises codons coding from about ten to about thirty amino acid residues.

5. The nucleic acid fragment of claim 4, wherein the polypeptide linker sequence comprises codons coding about twenty amino acid residues.

6. The nucleic acid fragment of claim 1, comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:6.

7. The nucleic acid fragment of claim 1 operably linked to a promoter.

8. The nucleic acid fragment of claim 1 operably linked to a plant signal sequence.

9. A gene sequence comprising the nucleic acid fragment of claim 1 operably linked to a regulatory sequence directing expression in one or more organs of a plant.
10. A transgenic plant transformed with the gene sequence of claim 9.

11. A polypeptide product comprising a 15 kD zein linked to a 10 kD zein by a polypeptide linker, wherein the polypeptide linker comprises from about ten to about thirty amino acid residues.

12. A polypeptide product of the expression in a prokaryotic or eukaryotic host cell of a nucleic acid fragment of claim 1.

13. A method for expression of stable protein bodies in a plant, comprising transforming a plant or plant tissue with a polynucleotide molecule that encodes a storage protein comprising a first protein and a second protein joined by a polypeptide linker, wherein the storage protein is expressed and accumulated as a protein body in a vegetative tissue of the plant or plant tissue.

14. The method of claim 13 wherein the first protein and second protein are zein proteins.

15. The method of claim 13 wherein the first protein and second protein are different.

16. The method of claim 14 wherein the zein proteins are a 10 kD zein protein and a 15 kD zein protein.

17. The method of claim 13 wherein the storage protein is rumin stable.

18. A composition comprising a rumin stable protein body, wherein said protein body comprises a first protein and a second protein joined by a polypeptide linker, and wherein the protein body is expressed and accumulated in a plant.

19. The composition of claim 18 wherein the first protein and second protein are zein proteins.
20. The composition of claim 18 wherein the first protein and second protein are different.

21. The composition of claim 19 wherein the zein proteins are a 10 kD zein protein and a 15 kD zein protein.
FIG. 1

Bgl II

β zein (no stop)  δ zein

FIG. 2

Bgl II

β zein (no stop)  SP  δ zein (including signal peptide)
FIG. 5

"TACTACAGATCTATGGCGACCCATATTCCA"
Y Y R S M A T H I P

FIG. 6
<110> Kemp, John
    Randall, Jennifer

<120> Co-Expression of Zein Proteins

<130> 37000-0106

<150> US 60/284,732
<151> 2001-04-18

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30

<tactacagat ctttgctgca ccatattc>

10

PRT

artificial

protein sequence for beta and delta zein fusion without linker

Tyr Tyr Arg Ser Met Ala Thr His Ile Pro

1 5 10

beta and delta zein fusion with delta zein signal peptide linker

<tactacagat ctttgctgca ccatattc>

gcatgtttgc cttcctagc tctttgtgca

60

agcgca tattc

87

Arg Ser Met Ala Ala Lys Met Leu Ala Leu Phe Ala Leu Leu Ala Leu

2
Cys Ala Ser Ala Thr Ser Ala Thr His Ile Pro

6 87 DNA artificial

beta and delta zein fusion with linker

tactacagat ctatgtcccc gggatctaac atgtctggag ctcaagcttc gaattctgca

gtgcacggta ccgcgccca tatcaca

protein sequence for beta and delta zein fusion with linker

Tyr Tyr Arg Ser Met Ser Pro Gly Ser Asn Met Ser Arg Ala Gln Ala

Ser Asn Ser Ala Val Asp Gly Thr Ala Thr His Ile Pro