Biotechnology of Seed Crops: Genetic Engineering of Seed Storage Proteins

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During their development, seeds accumulate large amounts of storage, or reserve, products that are metabolized during germination and the early stages of seedling growth. Energy reserves include complex carbohydrates (starch) and lipids, while reserves of N, C, and S are accumulated in specific storage proteins. The uses of seed products by man are innumerable, ranging from fermentation of maize starch for alcohol production to refinement of soybean protein for use in infant formula. Agronomic crops, such as maize, wheat, oats, and soybeans, are grown because they generate large amounts of seed reserves per unit of cultivated land. A major concern of the agricultural industry is increasing yields of seed components to enhance the value of these crops. During the past century, plant breeders have been quite successful in developing new cultivars of seed crop plants that give higher yields of seed components than older ones. Advances have been made not only with respect to increasing the content of starch, oil, and protein in the seed, but also, in some cases, in altering the quality of these components. For example, naturally occurring genetic variation has been exploited in the development of maize cultivars with modified starch content (Nelson, 1979; Shannon and Garwood, 1984) and increased or altered oil content (Alexander 1988, 1989; Weber, 1987). In a similar manner, the composition of soybean oil has been altered through selection to decrease the content of linolenic acid, resulting in a product with a more desirable flavor (Fehr, 1989; Hymowitz, 1987).

The synthesis of carbohydrates and lipids involve complex metabolic processes, and although a great deal is known about these pathways, it remains to be determined how recent advances in genetic engineering technology will aid in the modification of these processes to enhance seed quality. For example, alteration of the activities of one or two enzymes in the starch biosynthetic pathway might allow for the accumulation of desirable intermediates of starch biosynthesis. Such a situation may, however, result in a serious imbalance in the overall carbon flow in the seed, which could affect factors such as grain yield and seed viability. Conventional selection and breeding strategies may, therefore, be more efficient than the use of genetic engineering in the development of crop cultivars possessing seed carbohydrates and lipids of desirable composition (Alexander, 1988).

An important aspect of seed quality that is directly amenable to manipulation through genetic engineering involves the nutritional characteristics of seed proteins. In most cases, seed proteins of any one crop do not contain sufficient quantities of certain amino acids that are essential in the diet of humans and other monogastric animals (Nelson, 1969). Generally, cereals are most limiting in lysine and tryptophan, and legumes are deficient in the sulfur amino acids methionine and cysteine (Table 1). The economic advantages for increasing the nutritional quality of seed proteins are enormous: estimates of total protein production for each of two major seed crops in the United States, maize and soybeans, exceed 16 million tons annually (Wilson, 1987). Development of nutritionally balanced seed proteins in high-production crops like maize and soybean would benefit all areas of the agricultural industry concerned with grain production for human and livestock consumption.

Plant breeders have had only limited success in improving the nutritional quality of seed proteins, primarily because genes encod-

ing seed storage proteins with high levels of essential amino acids do not normally exist in any given species. Modification of genes encoding seed proteins by using genetic engineering techniques may, therefore, be an ideal solution to this problem. However straightforward the genetic engineering approach might be, certain problems must be overcome for successful application of this technology to the particular problem of improving seed protein quality. It is a relatively simple matter to isolate recombinant DNA clones corresponding to genes that encode specific proteins and to modify these clones by *in vitro* mutagenesis so that they contain codons for desired amino acids (Kramer et al., 1984; Wallace et al., 1988a). In addition, recent progress in gene transfer technology will soon allow for the routine production of transgenic maize and soybean plants (Hinchee et al., 1988; Klein et al., 1988; Rhodes et al., 1988). It must be recognized, however, that seed storage proteins assume specific conformational structures within the seed and that any modifications to these proteins must allow for their proper deposition in the cell. As discussed below, most of the work in this area has been conducted with respect to the extent to which storage proteins of maize and soybean seeds can be modified without perturbing their structural organization.

CHARACTERISTICS AND CLASSIFICATION OF SEED STORAGE PROTEINS

The major proteins of most seeds are referred to as storage proteins. These proteins accumulate to high levels during seed devel-

Table 1. Amino acid composition of total maize and soybean seed protein and purified storage protein fractions.^a

Amino acid	Maize		Soybean	
	Total protein ^y	Alpha zein ^x	Total protein ^w	Glycinin ^v
Glycine	6.7	2.2	7.4	7.8
Alanine	11.1	13.3	6.0	6.7
Valine	5.6	3.6	5.5	5.6
Leucine	12.1	18.7	8.0	7.2
Isoleucine	3.6	3.8	4.7	4.6
Serine	5.8	6.3	6.3	6.6
Threonine	4.0	3.0	4.3	4.2
Tyrosine	3.0	3.5	2.6	2.5
Phenylalanine	3.8	5.2	3.7	3.9
Tryptophane	0.3	nd	0.7	nd
Proline	9.3	10.7	6.5	6.3
Methionine	1.4	0.9	1.2	1.0
Cysteine	2.1	1.0	1.6	1.1
Lysine	2.4	0.1	5.5	4.1
Histidine	2.1	1.0	2.1	1.8
Arginine	3.3	1.2	5.9	5.9
Asx ^u	7.0	5.1	11.3	11.8
Glx ^t	16.4	21.4	16.5	18.8

^aExpressed as mole percent; nd, not determined.

^yAverage of values in Nelson (1969) and Mosse and Baudet (1983).

^xFrom Wilson (1987).

^wAverage of values in Smith and Circle (1972) and Mosse and Baudet (1983).

^vFrom Kitamura and Shibasaki (1975).

^uDetermined as aspartic acid + asparagine.

^tDetermined as glutamic acid + glutamine.

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opment, usually in membrane-bound aggregates called protein bodies, and are degraded during germination to provide a source of N, C, and S for the early stages of seedling growth (reviewed by Higgins, 1984; Shotwell and Larkins, 1989). Seed storage proteins are generally distinguished on the basis of solubility as water-soluble albumins, saline-soluble, water-insoluble globulins, alcohol-soluble prolamins, and dilute acid- or alkali-soluble glutelins (Osborne, 1924). There is a general distinction among plant types with respect to the type of storage proteins found in the seed. Dicotyledonous plants, such as legumes, contain globulin types, while monocots, such as cereals, usually contain predominantly the prolamins types. Globulins and prolamins are quite different in their structures and in the mechanisms by which they are synthesized and deposited in the seed, and the two types are considered separately in the following sections.

MODIFICATION OF MAIZE ZEINS

Zeins, the prolamins of maize grain, account for 40% of the protein in maize kernels. These proteins can be fractionated into three distinct groups based on differential solubility in aqueous alcohol solutions (Esen, 1986). Recombinant DNA clones that correspond to genes encoding each of the different zein types have been characterized at the nucleotide sequence level (Kirihaara et al., 1988; Marks and Larkins, 1982; Pedersen et al., 1986; Prat et al., 1985), and comparative sequence analysis allows for the separation of these genes into four groups designated alpha, beta, gamma, and delta. The alpha zeins, *Mr* 22,000 and 19,000, are the most abundant group and account for 70% of the total zein fraction in mature grain. Extensive charge heterogeneity in alpha zeins is observed by isoelectric-focusing gel electrophoresis (Righetti et al., 1977; Wilson, 1984), and molecular analyses have demonstrated that these proteins are encoded by a large, multigene family (Hagen and Rubenstein, 1981; Wilson and Larkins, 1984). In contrast, the beta (*Mr* 14,000), gamma (*Mr* 27,000 and 16,000), and delta (*Mr* 10,000) zeins are each encoded by only one or two genes (Kirihaara et al., 1988; Prat et al., 1985; Wilson and Larkins, 1984). None of the zein genes analyzed to date have been found to encode even a single lysine residue, and in only one case has a tryptophan codon been identified (reviewed by Rubenstein and Geraghty, 1986).

All four of the zein types are synthesized as preproteins in the endosperm between 10 and 40 days after pollination (Hurkman et al., 1981; Rubenstein and Geraghty, 1986; Soave and Salamini, 1984). Zein mRNAs are translated on rough endoplasmic reticulum (RER) and, following cleavage of the signal peptide, zein proteins accumulate within the lumen of the RER as dense, membrane-bound protein bodies (Larkins and Hurkman, 1978). As viewed through the electron microscope, protein bodies are roughly spherical structures $\approx 1 \mu\text{m}$ in diameter (Fig. 1A). Immunocytochemical analyses have revealed that maize endosperm protein bodies are complex structures with unequal distribution of zeins (Lending et al., 1988). Immunolocalization of alpha zeins within protein bodies is shown in Fig. 1B. Note that these proteins are generally distributed within the protein body matrix. In contrast, the beta (Fig. 1C, panels 1-4) and gamma (Fig. 1D, panels 1-3) zeins generally occur on the surface of the protein bodies, or in dark-staining inclusions.

The poor nutritional quality of maize grain protein is primarily due to the near absence of lysine and tryptophan, both essential for humans and other monogastric animals, in the zein fraction (Table 1). In maize mutants such as *opaque-2*, *opaque-7*, and *floury-2*, the zein content of the endosperm is decreased and the relative lysine content is increased, resulting in grain of a more desirable nutritional quality (Nelson, 1969). These mutants primarily reduce synthesis of the alpha zeins (Soave and Salamini, 1984), which affect both the size and morphology of protein bodies (Christianson et al., 1974). The endosperm of these mutants has a soft, floury texture. The mutant grains are lower yielding than normal grains, and the soft kernel phenotype results in decreased kernel density, increased susceptibility to insect damage, and an increased frequency of kernel breakage (Mertz, 1986; Ortega and Bates, 1983). These agronomically undesirable features have limited the use of high-lysine maize mutants as a food source.

Since the technology required for gene transfer into maize will probably be available in the near future (Klein et al., 1988; Rhodes et al., 1988), in vitro genetic engineering of genes encoding zeins may provide an alternative to traditional breeding methods in the development of nutritionally desirable maize grain. This approach involves the use of recombinant DNA technology to modify zein genes so that lysine and tryptophan residues are incorporated into zein polypeptides. Genes encoding alpha zeins, the most abundant zein type, are logical candidates for modification through genetic engineering techniques. These genes have been extensively characterized with respect to nucleotide sequence, revealing the presence of a 20-amino-acid repeat within the corresponding polypeptides (Marks and Larkins, 1982; Spena et al., 1982). It has been proposed that these repeated sequences exist as antiparallel alpha helices separated by glutamine-rich turn regions (Argos et al., 1982). This structural model accounts for the tight packing of alpha zeins within protein bodies by way of hydrophobic and weak polar interactions between polypeptides. Such interactions must be taken into account when designing strategies for modification of alpha zeins: incorporation of highly charged lysine residues into these polypeptides may interfere with these associations and disrupt protein body formation. Clearly, any modifications must be such that they have no adverse effect on protein body structure and organization within the endosperm.

Even if the gene transfer technology for maize were presently available, a rapid, reliable, and sensitive assay for protein body formation would be necessary for the evaluation of modified gene constructions. Such an assay is provided by translation of zein mRNAs

Fig. 1. Analysis of purified maize protein bodies by transmission electron microscopy (TEM). Protein bodies were isolated from maize endosperm at 18 days after pollination by sucrose density gradient ultracentrifugation and processed for TEM analysis as described in Lending et al. (1988). (A) Protein bodies stained with lead citrate and uranyl acetate. $\times 9230$. (B) Protein bodies reacted with antibodies specific for alpha zeins followed by immunogold staining. The gold particles appear as discrete dots and serve to localize alpha zeins within the protein body matrix. $\times 28,400$. (C) Selected protein bodies showing immunogold localization of beta zeins. $\times 39,050$. (D) Staining patterns obtained with antibodies to gamma zein (panels 1-3) and with preimmune serum as a negative control. $\times 39,050$. Reproduced with permission from Larkins et al. (1989).

To determine whether the altered zeins were capable of aggregating into protein bodies, artificial mRNAs were synthesized from the modified cloned sequences in an SP6 polymerase in vitro transcription system. Each of these mRNAs, in addition to native zein mRNA, was injected into *Xenopus* oocytes, and after an 18-h incubation period, ^3H -labeled leucine was injected to radiolabel the modified proteins. Oocyte homogenates were then subjected to density ultracentrifugation in metrizamide gradients to separate the membrane components (Fig. 3, top). Fractions were collected from

Protein bodies containing alpha zein alone are slightly less dense than those containing the full zein complement, suggesting that the

other zein types influence protein body density. Protein bodies containing the single alpha zein are smaller than those containing all of the zein types, and their lower density may be due to a higher lipid : protein ratio in the smaller structures (Wallace et al., 1988b). Zeins modified by lysine substitution (Fig. 3 D and E) or oligopeptide insertion (Fig. 3 F and G) exhibit sedimentation patterns similar to that of the unmodified zein. Only the large peptide insertion (Fig. 3H) interfered with the aggregation of the polypeptide into protein bodies. Thus, introduction of lysine residues into an alpha zein polypeptide, either through substitution or insertion, does not appear to impair aggregation of zeins within protein bodies.

Although the environment within *Xenopus* oocytes is quite different from that of maize endosperm cells, the oocyte compartmentalization assay provides valuable information about the process of zein deposition in maize. The ability of individual alpha zeins to associate into protein bodies in oocytes demonstrates that such aggregation is largely a function of interactions between the polypeptides after they are translocated into the lumen of the RER. The addition of lysine residues to an alpha zein, either by amino acid substitution or insertion, does not interfere with the formation of protein bodies. It is therefore likely that lysine-containing zeins would be packaged into protein bodies in maize endosperm; this could be tested once the appropriate gene transfer technology is available for maize.

POTENTIAL FOR MODIFICATION OF SOYBEAN GLYCININ

Seed globulins are generally classified on the basis of their sedimentation properties. In most dicots, these proteins fall into two classes with sedimentation coefficients of 11-12S and 7-8S (Derbyshire et al., 1976). In soybean, the 12S globulin predominates and usually constitutes $\approx 62\%$ of the total globulin fraction. This 12S globulin, termed glycinin, accounts for $\approx 42\%$ of the total seed protein. Glycinin exists in seeds as a hexameric complex of *Mr* 360,000 and is composed of six subunits, each of which consists of an *Mr* 40,000 acidic polypeptide linked by a disulfide bond to an *Mr* 20,000 basic polypeptide (Nielsen, 1985a). A limited amount of heterogeneity has been observed for glycinin polypeptides (Nielsen, 1984), but all of these proteins are characterized by their low (2% or less) content of sulfur amino acids (Nielsen, 1985b). Five different subunits have been identified which fall into two classes: Group I consists of three members, each of which contains five to eight methionine residues; Group II consists of two members, each containing three methionine residues (Nielsen, 1985a). DNA clones corresponding to genes encoding each of the glycinin subunits have been characterized at the nucleotide sequence level (Nielsen et al., 1989).

Soybean storage proteins are localized in protein bodies, but the mechanism for their deposition is quite different than that of zeins in maize endosperm. The synthesis, processing, and transport of 12S globulins have recently been reviewed in detail (Chrispeels, 1985; Shotwell and Larkins, 1989), and only a brief overview is presented here. Glycinin subunits are synthesized throughout most of cotyledon development on RER as precursor polypeptides termed proglycinins (Barton et al., 1982; Turner et al., 1982). These precursors contain amino-terminal leader sequences that are cotranslationally removed during translocation into the lumen of the RER. In the RER, an intramolecular disulfide bond forms in proglycinin, after which these polypeptides assemble into 9S trimers (Barton et al., 1982). These trimers are transported via the golgi apparatus to the vacuole, where they associate to form protein bodies. Before protein body formation, each proglycinin molecule of the trimer is cleaved into the *Mr* 40,000 acidic and *Mr* 20,000 basic polypeptides, which remain associated via the disulfide bond (Nielsen, 1984; Staswick et al., 1984). Two trimers then associate to form the hexameric 12S holoprotein.

Since sulfur amino acids are limiting in soybean seed proteins (Table I), modification of glycinin polypeptides by addition of methionine residues would enhance the nutritional quality of soybean meal. As discussed above for maize zein polypeptides, any modifications to storage proteins must allow for proper deposition within

the seed. The structural domains of 12S globulins and the mechanisms by which these proteins are transported and processed within the seed are similar in all plants that have been examined (Shotwell and Larkins, 1989). Strong structural constraints may, therefore, exist in these proteins, and any modification of glycinin subunits by genetic engineering must allow for proper assembly of glycinin monomers into the hexameric 12S protein.

Recognizing the need for a rapid and sensitive assay for glycinin assembly, Nielsen and coworkers at Purdue Univ., West Lafayette, Ind., investigated self-assembly of proglycinin in vitro (Dickinson et al., 1987). This assay involved the in vitro transcription of cDNA clones corresponding to individual glycinin genes to produce synthetic mRNAs, which were then translated in a cell-free rabbit reticulocyte lysate system. Synthesis of proglycinin in the translation reactions was monitored by the incorporation of ^3H -labeled leucine into the polypeptide. Following termination of the translation reaction, the samples were incubated for various times to allow for self-assembly of the monomers into higher-order structures. Assembly was evaluated by subjecting the reaction mixtures to sucrose density gradient ultracentrifugation. After a 1-h incubation, all of the radiolabeled proglycinin polypeptides existed as 3S monomers, but after 30 h, all of the monomers were assembled into 9S trimers. Using this assay, Dickinson et al. (1987) demonstrated that removal of the signal peptide from proglycinin is required for proper assembly and that deletion of an internal hydrophobic domain of 27 amino acids in the basic subunit, which is conserved among all 12S seed globulins, prevented self-assembly. Since the presence of the hydrophobic signal sequence or absence of the conserved domain of the basic subunit would alter the structure of proglycinin monomers, these results indicate that assembly into 9S trimers depends on the proper conformation of the monomers.

Although the above experiments demonstrated that proglycinin monomers could self-assemble in vitro to form trimers, no evidence was obtained for hexamer formation. To investigate assembly of proglycinin into 12S hexamers, Dickinson et al. (1989) developed an assay to evaluate reassembly of proglycinin trimers and mature glycinin subunits (Fig. 4). In this system, native, mature glycinin was dissociated in a low-ionic-strength buffer to yield 3S monomers and 9S trimers. These mature subunits were mixed with either proglycinin monomers or trimers produced by the self-assembly process under conditions of high ionic strengths that promote reassembly of trimers into hexamers. The reassembly process was evaluated by subjecting the assay reaction to sucrose density ultracentrifugation and determining the amount of radiolabeled proglycinin in the 9S and 12S fractions.

Fig. 4. Schematic diagram of in vitro assays for glycinin assembly. The procedure for self-assembly of proglycinin monomers synthesized in vitro is shown on the left side of the diagram. Solid circles represent ^3H -labeled proglycinin synthesized at the direction of a glycinin cDNA clone. Open circles represent mature, native glycinin subunits, isolated from seeds, which had undergone post-translational cleavage in vivo to yield disulfide-linked acidic and basic polypeptides. The reassembly assay involves incubation of either proglycinin monomers or trimers with dissociated mature glycinin under conditions favoring hexamer formation. Reproduced with permission from Dickinson et al. (1989).

Fig. 5. Sucrose density gradient profiles of complexes formed by proglycinin trimers and monomers in the reassembly assay. (A) Self-assembled radiolabeled proglycinin trimers were isolated from a sucrose gradient and incubated with dissociated native glycinin subunits. Radiolabeled polypeptides are present only in the 9S fraction, indicating that these polypeptides did not assemble into hexamers. The presence of unlabeled protein in the 12S fraction (dashed line) demonstrates that the mature glycinin subunits had reassembled into hexamers. (B) Same as (A) except that radiolabeled proglycinin monomers were used. The presence of a small amount of radioactively labeled polypeptides in the 12S fraction indicates that some of these monomers assembled into hexamers. Reproduced with permission from Dickinson et al. (1989).

The results of a reassembly experiment are shown in Fig. 5. When 9S proglycinin trimers were tested in this assay, the mature, unlabeled subunits reassembled into the 12S hexamer form, but none of the radiolabeled proglycinin trimers were found in the 12S fraction (Fig. 5A). Instead, these molecules remained as 9S trimers. Proglycinin monomers, however, were able to associate with mature glycinin subunits to form 12S hexamers (Fig. 5B). Note that only a small portion of proglycinin monomers, as measured by the amount of radiolabeled protein, assembled into the hexameric form. The bulk of the radioactivity was found in the 9S fraction, indicating that most of the proglycinin monomers assembled into trimers. Taken together, the results of these two experiments indicate that, although some proglycinin monomers are capable of associating with mature subunits to form a hexamer, assembly of proglycinin appears to be blocked at the trimer stage. This barrier to assembly could be structural in nature, and it is likely that cleavage of proglycinin into its acidic and basic polypeptide components, which normally occurs in protein bodies, results in a conformational change that allows for hexamer formation.

To determine whether proteolytic cleavage of proglycinin trimers would relax any conformational constraints limiting assembly into hexamers, proglycinin synthesized *in vitro* was permitted to form trimers and then treated with the nonspecific protease papain. Mild papain treatment resulted in a slight shift in the sedimentation profile of the trimers, but they clearly persisted as trimers (Fig. 6A). This sedimentation profile also shows that, unlike trimers, monomers are quite sensitive to papain treatment and are rapidly degraded by the protease. Analysis of papain-treated trimers by SDS-PAGE (Fig. 6B) indicated that cleavage occurred at multiple sites in the proglycinin subunits, presumably in regions exposed on the surface of the trimer complex. The sedimentation profile indicates, however, that the basic trimer structure is preserved after proteolysis. As expected, untreated trimers do not assemble into hexamers (Fig. 6C). Use of papain-treated trimers in the reassembly assay resulted in a prom-

Fig. 6. Effect of partial proteolysis on reassembly of proglycinin trimers. (A) Proglycinin was synthesized *in vitro*, allowed to partially self-assemble, then treated with the nonspecific protease papain. The sample was then fractionated by density gradient ultracentrifugation (triangles). Analysis of an untreated aliquot from the assembly reaction (circles) is shown for comparison. (B) Trimer fractions from (A) were subjected to SDS-PAGE and fluorography to assess the extent of proteolysis. Lane 1, untreated trimers; lane 2, papain-treated trimers. Analysis of sedimentation profiles of complexes formed by untreated trimers (C) and papain-treated trimers (D) indicates that partial proteolysis allows for formation of 12S hexamers. Reproduced with permission from Dickinson et al. (1989).

inent 12S peak (Fig. 6D), indicating that proglycinin trimers are capable of forming hexamers subsequent to proteolysis treatment. Although papain treatment does not result in specific cleavage of proglycinin into acidic and basic polypeptides, as is the case in protein bodies, the nonspecific cleavage is apparently sufficient to relax any conformational constraints that interfere with *in vitro* assembly into 12S hexamers.

The assembly of glycinin subunits into the 12S hexameric protein clearly involves proper post-translational processing of the proglycinin precursors. The *in vitro* reassembly assay provides a rapid and sensitive means of determining the extent to which glycinin subunits can be modified through genetic engineering techniques to enhance the nutritional quality of soybean seed protein by the addition of methionine residues. Apparently, the ability of modified subunits to assemble *in vitro* is reflected in seeds of transformed plants (N.C. Nielsen, personal communication). Thus, it is now possible to evaluate methionine-enriched glycinin polypeptides for their ability to assemble into the mature holoprotein within seeds. Subsequent transfer of modified glycinin gene constructions into soybean plants will result in production of seeds with protein of desirable nutritional quality.

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