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PROJECT SUMMARY

This project was directed at determining mechanisms involved in storage of intrinsic and foreign storage proteins in seeds. Seeds constitute the majority of direct and indirect food. Understanding how seeds store proteins is important to design approaches to improve the quality of seed proteins through biotechnology. In the Israeli part of this project we have conducted investigations to elucidate the mechanisms involved in assembling wheat storage proteins into ER-derived protein bodies. The results obtained have shown how domains of storage protein molecules are critical in the assembly of protein bodies. In the US side of this project the fate of foreign and engineered proteins expressed in seeds has been investigated. Engineering seed proteins offers the prospect of improving the quality of crops. Many foreign proteins are unstable when expressed in transgenic seeds. The results obtained have demonstrated that sequestering foreign proteins in the ER or ER-derived protein bodies stabilizes the proteins permitting their accumulation. The collaboration conducted in this project has advanced the understanding how protein bodies are assembled and the potential to use the ER and protein bodies to store engineered proteins that can enhance the composition of seeds.

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**OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL,
INCLUDING A DESCRIPTION AND EXPLANATION OF ANY
DEVIATION FROM THEM.**

Research objectives of funded proposal; The processes of protein translation, folding, and posttranslational modifications may result in improperly formed products. Eukaryotic cells have evolved quality control mechanisms that mediate the recognition and disposal of malformed or misfolded proteins. We have two model systems in which a modified variant of the seed storage protein phaseolin (HiMet phaseolin and gliadin) is expressed as an unstable and/or misfolded/malassembled polypeptides. Preliminary evidence from electron microscopic immunocytochemical studies had suggested that the disposal of misfolded proteins in plants occurs in a post-golgi compartment, which is mostly likely the vacuole. This is in marked contrast to animal cells where the site of misfolded protein disposal is in a pregolgi compartment. Central to this proposal is this apparent difference in the mechanisms that plant and animal cells use to dispose of misfolded proteins; it may constitute a basic difference in the cell biology of members of these two kingdoms. Using a transgenic tobacco system we will examine the over-expression misfolded storage proteins. We will determine the intracellular site where the misfolded proteins are degraded. We will determine if misfolded proteins interact with binding protein (BiP) prior to its transport for disposal as is the case for animal cells. We will determine if BiP is transported to the vacuole with misfolded proteins, and whether the BiP interacting with the misfolded protein are posttranslationally modified. The proposed work will provide information about basic mechanisms of how plant cells mediate posttranslational protein-quality control in the secretory system.

Deviations from research plan: The Israeli partner's research effort retained the focus and objectives. In addition to the basic objectives additional elaborations were undertaken to leverage additional information from the project. These were directed at examining the process of assembly of gliadins into higher order structures in both wheat and in transgenic plants. Even with the completion of the project there is still much more that remains to be done and materials that still have not been fully analyzed. For instance the Beltsville and Weizmann partners still have double transgenic plants co-expressing organelle markers and gliadins that remain to be analyzed. The Beltsville partner expanded the project to not include all the original objectives but exploit new directions that grew from the initial project that in turn have solved the original problem. In the original objectives we proposed to determine the site and process of misfolded protein degradation. We demonstrated that plants use progression to the vacuole to degrade a

model misfolded protein and that this process is different from endoplasmic reticulum-localized process shown in animal cells. We exploited methods to retain proteins in prevacuolar compartments that showed that other methods for impeding progression to the vacuole also impeded disposal. Specifically, we added the maize prolamine protein zein to expand on our objectives with the wheat prolamine protein gliadin. Zeins proved easier to immunolocalize and by using this as a model system we were able to show that although prolamines are retained in the ER via assembly the resulting ER-derived protein bodies also are targeted to, and sequestered within the vacuole by autophagy.

We were also puzzled by the inability of adding ER retention sequences to stabilize proteins. The results we obtained clearly showed that retaining proteins in the ER increased their stability. However the same set of experiments also showed that proteins retained from the ER eventually exit, progress to the vacuole, and are then degraded. This raises questions on how the information of the ER retention sequence is utilized and its suitability to induce foreign protein retention in the ER. Using a model system of an intrinsic plant protein that possesses an ER retention sequence, the protease SH-EP we were able to show that ER-retention sequences can be post-translationally processed. Proteases are normally found in either the vacuole or as secreted proteins. That this protein possessed an ER-retention indicated that it would likely be modified in some way. Our results confirmed this and showed for the first time that an ER-retention sequence can be posttranslationally processed. This is a unique result and further research needs to be done to examine whether this type of processing is also found in all other eukaryotes that use the conserved ER-retention sequence. If this result proves to broadly apply to all eukaryotes then this will provide a means to explain the manipulation of ER protein content, especially in situations such as in plants where they respond to abiotic stress and recovery by large scale changes in ER protein content.

We also added one additional system to our evaluation of creating new protein containing compartments. We have conducted experiments that have shown that co-suppressing one of the two major families of soybean storage proteins induces the formation of protein bodies. Unlike the prolamine containing protein bodies we previously studied in tobacco, the soybean protein bodies do not require insertion of new genes and are not based on the accumulation of hydrophobic self-assembling prolamine proteins.

EVALUATION OF THE RESEARCH ACHIEVEMENTS AS RELATES TO THE ORIGINAL RESEARCH PROPOSAL AND ITS OBJECTIVES

We have achieved the original objective as defined as establishing the mechanism(s) of unstable protein disposal in transgenic seeds. We have shown that the site where foreign proteins are degraded is the vacuole. This result is different from comparable experiments conducted in animal cells where foreign and misfolded protein disposal can occur in the ER. Critical experiments we have conducted where proteins are retained in the ER in seed cells shows that the ER and ER-derived compartment is actually a safe site for protein accumulation and disposal occurs only when the protein progresses beyond the vacuole. The stability of proteins in the ER and ER-derived compartments means that ER folding and processing events can be used in assembling foreign proteins expressed in transgenic seeds. With the expansion of this project into other allied areas of inquiry, we have broadened our accomplishments to identify a suitable means to accumulate foreign proteins in transgenic seeds in the short-term-lower concentrations by use of ER-derived retention sequences and at much higher concentration and stability by inducing the formation ER-derived protein bodies.

CONCLUSIONS AND POTENTIAL BENEFIT TO AGRICULTURE

The modification of seed composition by biotechnological procedures offers the prospect to alter the composition of the products of major crops. The modifications possible extend to both quality and quantity as well as in the production of novel products. Improvements in amino composition by expression of foreign proteins can improve the diet of people and suitability of crops for animal feed. One major limitation in use of this technology is the stability of the products of the introduced genes. The newly synthesized proteins may have a brief half life being degraded shortly after formation while in other examples the proteins are more stable until the end of maturation. In this project we have identified as the primary site of degradation of these posttranslationally unstable proteins. Further we have shown that transport to the vacuole can occur by direct progression through the endomembrane system as well as by autophagic internalization. This gives the vacuole multiple mechanisms to sequester foreign proteins and mediate their disposal. Our results show that in order to have the best chance to accumulate mass quantities of foreign proteins it is essential to keep those proteins from being sequestered in the vacuole.

We have shown that proteins previously identified as unstable in transgenic plants can exhibit increased stability by impeding progression to the vacuole by either inducing retention in the ER or by assembling the proteins into ER-derived protein bodies. Our experiments have served as model for other scientists who have also used ER retention

sequences to increase foreign protein content in plants. For instance it has been shown that adding ER retention sequences increases content of immunogens suitable for immunizing people. The results we obtained has been a resource for other investigators seeking to increase protein content in transgenic plants.

Our results show that in order to accumulate large quantities of protein the foreign proteins must be accumulated in a large intracellular compartment. Our initial studies of protein bodies induced by prolamine protein synthesis indicated that it is quite feasible to synthesize and accumulate large quantities of foreign proteins in transgenic seeds. However these induced protein bodies proved to be unstable being degraded at the end of maturation. In the last project of this study we can now show that transgenic soybeans can be used as a basis for modifying seed composition by creating a new protein-containing compartment. What separates the transgenic soybean model from the previously used tobacco model is that the induced protein bodies are stable and persist through maturation and into germination. Transgenic soybeans can be used as protein factories to accumulate mass quantities of foreign proteins. Our results show that it is feasible to produce proteins that might be otherwise unstable in a stable cellular compartment and under circumstances where the costs of production are very low. Management of soybeans as transgenic agricultural systems is already well established and a significant fraction of the total US soybean crop is already transgenic with phenotypes of Round-Up resistance or producing BT, an anti-insect protein.

COLLABORATION

One of the most important and advantageous aspects of BARD support is the collaboration. The Beltsville and Weizmann laboratories have integrated many different aspects of this research into a joint project that has brought both laboratories very close together in objective and friendship if not distance. The PIs Galili and Herman have had reciprocal sabbatical visits to the other's laboratories. Dr. Galili spent a year at Beltsville and Dr. Herman has spent four months at Weizmann. Funding other than BARD supported these visits, but it is the BARD grant that is the justification for these sabbatical visits. In addition Dr. Galili's senior technician Ms. Hanna Levonony visited Beltsville for 6 weeks and was trained in advanced EM techniques and conducted some research. A Weizmann graduate student supported on the BARD, Yuval Shimoni also visited Beltsville for a week during the term of the grant. But it was the multiple visits of both PIs Herman and Galili that were essential for the collaboration. Dr. Herman visited the Weizmann five times during the grant supported by various sources in addition to the

BARD. With the use of the Weizmann guest house and the government rate tickets to Israel Dr. Herman was able to visit the Weizmann and stay for a useful period time while keeping the associated costs quite low. Dr. Galili visited Beltsville twice in addition to his year long sabbatical.

Collaborations are far more than visits, it is conducting joint experiments and pursuing and publishing together. In this regard both the Weizmann and Beltsville laboratories have engaged in significant collaboration. Both PIs have done research in the other's laboratories. Dr. Herman worked at the Weizmann crossing transgenic tobacco plants expressing foreign marker proteins with those expressing wheat storage proteins for the purpose examining the trafficking of storage proteins. Dr. Galili worked on cloning proteins useful for tracking the progression of storage proteins. This work is continuing even after the termination of the grant and we expect to publish joint papers on this research. Dr. Herman has also contributed several cDNA probes to Dr. Galili's laboratory that encode a variety of wheat proteins useful to track the progression of storage proteins. These probes are currently being used to examine the process of protein body ontogeny in wheat endosperm and will be part of future publications.

BARD also offered the opportunity for Dr. Herman to visit other investigators in Israel and to present seminars. Most important of these is the visit to Tel Aviv University and discussions with Prof. Adina Breiman that resulted in establishing another productive collaboration that is currently yielding its first publications. It is my hope that the collaboration between the Herman and Galili laboratories will be seen as exactly the type of interaction that BARD is supposed to facilitate and support.

BELTSVILLE RESEARCH

SUMMARY

Numerous examples of posttranslationally unstable foreign proteins have been identified by protein expression in transgenic plants. This project has focused on a detailed examination of newly synthesized proteins that exit the endoplasmic reticulum by either progression through the endomembrane system or by assembly into ER-derived protein bodies converge in the seed protein storage vacuole (PSV) for degradation. The destabilized seed storage protein HiMet-phaseolin produced by disrupting the primary sequence with an insertion, is posttranslationally unstable. Adding the ER-retention sequences K/HDEL indicates that the HiMet-phaseolin is stable in the ER/Golgi but is degraded on progression to the PSV. Prolamin maize zein proteins are assembled into protein bodies that are secreted into, and are transiently stable in the cytoplasm. Protein bodies assembled in seed cells are then sequestered in the PSV by apparently selective autophagy and degraded. The sequestration of materials observed within the PSV indicates that there are multiple mechanisms of sequestration that result in proteins resident in the cytoplasm, apoplast and endomembrane system being transferred to the PSV for degradation. The functional role of the PSV in degrading cellular materials from cellular and extracellular locations has important implications in producing high levels of foreign proteins in plant cells. Engineering plants to sequester proteins in a prevacuolar compartment appears to be a suitable strategy for increasing the accumulation of foreign proteins expressed in transgenic plants. A model for this concept is the cosuppression of β -conglycinin in transgenic soybeans. The transgenic seeds store primarily the 11s storage protein glycinin which although is entirely a vacuolar protein in wild type soybeans is present at high concentrations as proglycinin sequestered in ER-derived protein bodies. Unlike the maize prolamine protein bodies produced in transgenic tobacco seeds, the proglycinin protein bodies of transgenic soybeans persist through seed maturation and are present on germination. The stability of the protein bodies in transgenic soybeans makes this an important model to design seeds in order to sequester proteins in a prevacuolar compartment where they will not be sensitive to proteolytic degradation.

INTRODUCTION

Modification of seeds through genetic engineering offers a direct approach to produce improved crops with enhanced characteristics. For example, increased sulfur amino acid content of soybeans and increased lysine amino acid of cereals are sought after objectives that may be achieved with the expression of foreign genes in transgenic plants. Other possible uses of genetic engineering of crops include adding proteins to immunize against illness, adding high value proteins that can be accumulated and recovered for other uses, and producing seeds with altered characteristics that may improve their quality and utility as primary food substances and as products to be used in the processed food industry. There have been many experiments to produce transgenic seeds expressing foreign proteins in model plants (see Galili and Herman 1996 for review) and more recently in crops (see Shimoni et al 1997 for example). Although many of these experiments have been successful there are also many reports of foreign proteins although synthesized at high levels, are not accumulated due to posttranslational instability (Hoffman et al 1988; Ohtani et al 1991; Coleman et al 1996).

Posttranslational protein degradation has been observed as the consequence of stress-induced damage, genetic damage and disease and as the consequence of the induced expression of foreign and/or engineered proteins in transgenic organisms.

During the course of this project we investigated the process of foreign and engineered protein degradation by a variety of approaches. We report a series of results using transgenic tobacco and soybean seeds as model systems to investigate the site(s) of posttranslational protein degradation. We demonstrate that the seed protein storage vacuole (PSV) is capable of degrading proteins that are initially accumulated in the endomembrane system, cytoplasm and extracellular space and are then sequestered in the PSV by secondary processes. This degradation is selective. Our results show that there are multiple pathways of protein sequestration in the PSV that apparently possesses enzyme(s) capable of degrading some proteins while simultaneously accumulating endogenous storage proteins. In addition to the primary goal our investigations also lead us into completing secondary projects that we thought we lead us to further understanding how proteins are degraded after translation. We investigated the biology of BiP, or Binding Protein that is a molecular chaperone of eukaryotes implicated in the misfolded protein response. The study of this protein was a major goal of both the Herman and Bennett laboratories. As we will detail further although we extensively characterized BiP we were able to rule out a significant contribution by this protein in the posttranslational degradation of engineered seed proteins. We also obtained parallel travel support from the

Japanese Ministry of Education (Monbusho) to jointly investigate with Prof. Takao Minamikawa a protease that possesses an ER-retention sequence. Such a protease would seem an obvious candidate to mediate posttranslational protein degradation. However as we will detail instead of finding a role for this protein in posttranslational protein degradation we instead found a new and novel form of posttranslational processing of the ER-retention sequence. We also have investigated to formation of protein bodies and ER-derived vacuoles in both wheat and maize which is central to the objectives of Dr. Galili's part of the project. Much of this was done either in direct cooperation with Dr. Galili or with his input and comment.

This report describes the various projects we have conducted and the results and implications of the data. The research accomplished has clearly shown that proteins synthesized in transgenic plants can be stabilized by retention in the ER or by assembling the proteins into an ER-derived compartment. Conversely the research has also shown that proteins are highly susceptible to degradation on entering the vacuole that can occur by either progression through the endomembrane system or by autophagy of ER-derived and assembled protein bodies

PRESENTATIONS OF BELTSVILLE DATA RESULTING FROM THE PROJECT

During the course of this project we have presented our results as research publication and have also discussed the results at some length in several reviews published in *Advances in Structural Research*, *Advances in Botanical Sciences* and *Plant Cell*. The results of this project have been presented as abstracts/posters at several national (US) meetings including the American Society for Plant Physiology, American Society for Cell Biology and American Society for Biochemistry and Molecular Biology. The results have also been a major part of numerous invited seminars in the US, UK, Italy, Japan and Israel. The results have also been presented as invited presentations at the Society of Experimental Biology meeting in St. Andrews, Scotland, 5th International Botanical Microscopy meeting in Oxford, UK, 9th International Meeting of the Cell and Tissue Culture Society held in Jerusalem, Israel, Endomembrane System in Plants in York, UK, and US-Japan vacuole seminar held in Hawaii in 1999.

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DEGRADATION OF POSTTRANSLATIONALLY UNSTABLE PROTEINS OCCURS IN THE VACUOLE

High Methionine (HiMet) phaseolin and selective degradation of a distorted protein by progression through the endomembrane system

In 1988 Hoffman et al published the results of an attempt to increase the sulfur amino acid content of a model legume storage protein, β -phaseolin. Phaseolin like other legume storage proteins is relatively deficient in sulfur-containing amino acids and introducing this trait into legumes, especially soybeans, would be a great advantage in its utility as animal feed. A β -phaseolin cDNA was modified by inserting a short oligonucleotide encoding an α -helical polypeptide into an α -helical domain of phaseolin (Fig 1). The mutant, termed HiMet-phaseolin, along with a wild-type control was expressed in transgenic tobacco seeds under the control of the phaseolin seed-specific promoter. Both the wild type and HiMet genes were expressed at similar levels and both proteins were synthesized *in vivo* at high levels. However, while wild-type phaseolin was accumulated in the seed protein storage vacuole, the HiMet mutant was not accumulated and appeared to be degraded within several hours after its synthesis. An explanation of why HiMet is unstable emerged when the crystal structure of β -phaseolin was elucidated (Lawrence et al 1994). It appears that the modification used to produce HiMet lengthened an α -helical domain in a major ring structure that probably produced a large-scale distortion of the protein (Fig. 1). Although distorted HiMet does form apparently correct trimeric oligomers in the ER lumen and it is also cotranslationally glycosylated (Hoffman et al 1988). Immunogold localization of HiMet and wild-type phaseolin in transgenic tobacco seeds showed that the wild type is accumulated within the PSVs while in contrast the PSVs of the HiMet-phaseolin expressing seeds exhibited no immunolabel. Based on the literature on the disposal of misfolded proteins in animal cells

Immunogold labeling of HiMet was observed in small dense vesicles that appear to be identical to the Golgi-apparatus derived secretion vesicles that carry storage proteins to the maturing PSV. The results of these experiments were interpreted to indicate that HiMet is postrtranslationally unstable in one or more compartments of the endomembrane system within a few hours of the protein's synthesis.

Retarding posttranslationally unstable HiMet in the ER promotes accumulation of the protein

In order to determine cellular site of HiMet phaseolin degradation Pueyo et al (1995) added K/HDEL ER-retention sequences to the carboxy-terminus of the HiMet sequence. The concept of this experiment was to retain HiMet in the ER/nuclear envelope lumen in order to test whether the protein was degraded by the ER-localized quality control mechanism or whether HiMet was degraded in a post-ER compartment. The concept of this experiment is to directly test whether HiMet is degraded within the ER or in a post-ER compartment. If HiMet was degraded in the ER as might be expected based on analogy with animal and yeast cell studies on misfolded protein degradation then retaining HiMet within the ER by adding HDEL should not alter the protein's stability. However if HiMet was degraded in a post-ER compartment, then adding an ER-retention sequence make a large difference in increasing the protein's abundance by keeping the protein from its degradation site. Shown in figure 1 is a diagrammatic representation showing the position and sequence of the modifications that were used to produce HiMet with K/HDEL. The crystal structure of b-phaseolin has been elucidated and the position of the added retention sequence would appear to be well displayed for recognition by the ER-retention sequence receptor (ERD-1).

The results of this experiment showed that the addition of K/HDEL to HiMet resulted in increased stability and accumulation of the protein. Subcellular fractionation lysates on sucrose gradients from maturing seeds expressing HiMet indicated that all of the protein is contained in a membrane-bound fraction. Previous use of this technique with another seed protein, phytohemagglutinin also modified with a KDEL ER-retention sequence showed that this membrane fraction is enriched in microsomes derived from the ER (Herman et al 1990). Almost all of the HiMet protein accumulated as the consequence of adding HDEL was contained in the ER-derived microsome fraction. This indicates that adding an ER-retention sequence can stabilize a posttranslationally unstable protein and implies that the ER is not the site of degradation. A parallel control with wild-type phaseolin also modified with the addition of HDEL was considerably upregulated in the ER-derived microsomes compared to the unmodified wild type. This confirms that HDEL sequence was correctly displayed and utilized for ER-retention.

In order to further characterize the intracellular sites that sequester stabilized HiMet-HDEL the protein was localized in maturing seeds using EM-immunogold technique. The localization of the HiMet-HDEL showed most of the protein is contained within the ER lumen (Fig. 2A). HiMet-HDEL is apparently transport competent since it was also

localized in the Golgi apparatus and secretion vesicles (Fig. 2A, D). This indicates that HiMet (HDEL) is transport competent and is stable immediately upon exit from the ER. The Golgi secretion vesicles deliver storage proteins to the maturing protein storage vacuole (PSV) (see Larkins and Herman 1999 for review). In contrast to wild type phaseolin (Fig. 2C) HiMet was not accumulated in PSVs (Fig. 2D) indicating that the protein is degraded after its deposition. The difference in stability of wild-type phaseolin and HiMet in tobacco PSVs indicates that the tobacco PSV possesses active proteases capable of selectively degrading the destabilized HiMet while not affecting the wild-type protein. It is this apparent selective activity that it is the basis of the posttranslational instability of one protein while simultaneously permitting the accumulation of other storage proteins in the same compartment. Our results indicate that the resistance of storage proteins to PSV resident proteases during seed maturation must have a structural component. The alteration of the tertiary structure by the HiMet insertion into phaseolin's structure and the resulting structural distortion appears to have eliminated the intrinsic resistance of phaseolin to PSV resident proteases.

Autophagy of cytosolic protein bodies and degradation of cereal prolamins expressed in transgenic plants

Cereal seed prolamins storage proteins such as maize zeins and wheat gliadins are sequestered in cytosolic protein bodies that are directly assembled by, and derived from, the ER (See Galili and Herman 1997; Herman and Larkins 1999 for reviews). Prolamins are hydrophobic proteins that accrete in the ER lumen. Although prolamins are insoluble in an aqueous environment the assembly of the protein accretions is more complex involving specific protein-protein interactions as well as the assistance of ER chaperone proteins (Li et al 1993) that results in the formation of a protein body that can include domains that sequester specific prolamins (Lending and Larkins 1989). Protein bodies may remain attached to the ER such as occurs in maize (Lending and Larkins 1989), or become detached to produce a population of cytoplasmic protein bodies. The cytosolic protein bodies may either remain in the cytoplasm or become stored in the vacuole as in wheat endosperm as the consequence of autophagy (Levanony et al 1992).

Zeins have been expressed in transgenic plants. Among the four types zeins tested only β -zein is accumulated at high levels when expressed alone in transgenic plants (Ohtani et al 1991; Hoffman et al 1987; Bagga et al 1995). The other zeins are either very unstable such as α -zein or accumulated at low levels (Williamson et al 1988). We investigated the site of zein degradation. Coexpression of α and γ -zein in tobacco seeds

results in enhanced stability of the unstable α -zein (Coleman et al 1996). Similar experiments with the β and δ -zeins result increased stability of the δ -zein (Bagga et al 1997). The cytoplasmic protein bodies appear to be stable with a matrix of γ -zein that contains embedded locules of α -zein (Fig 3). The zein-containing protein bodies are also sequestered in the PSV (Figs 3 and 4) (Coleman et al 1996, Bagga et al 1997). The protein bodies sequestered within PSV appear to be partially degraded and possess a lower concentration of immunolabeled α -zein compared to the cytoplasmic-localized protein body (Fig. 4). These results indicate that zein-containing protein bodies are stable as long as the organelle remains in the cytoplasm but is labile to the action of PSV-localized proteases once the protein body is sequestered within the PSV by autophagy.

This result is important because it shows a convergence of mechanisms with HiMet's instability. In both situations HiMet and α -zein as postrationally unstable by previously unknown mechanisms. However by inducing these unstable proteins to remain in the ER by attaching a H/KDEL sequence or by inducing sequestration into an ER-derived protein body these proteins are stable in this environment. Degradation of zeins in the PSV after autophagy parallels similar results of degradation after delivery of HiMet to the PSV by progression through the endomembrane system.

Induced formation of protein bodies in transgenic soybeans by cosuppression of β -conglycinin

Fatty Acid Desaturase2 (FAD2) cosuppression in transgenic soybeans driven by the 7S conglycinin storage protein promoter results in a shift of seed oil desaturation to favor the accumulation of high levels of oleic acid. The use of the conglycinin promoter for cosuppression reduces the accumulation of conglycinin and promotes the accumulation of proglycinin and precursors of other vacuolar proteins, notably the major soybean allergen P34. Structural analysis of maturing transgenic soybean seeds indicates that the process of storage protein accumulation is altered. In normal soybeans storage proteins are deposited in pre-existing vacuoles by Golgi-derived vesicles (Herman et al 1985; Galili and Herman 1997 and Herman and Larkins 1999 for reviews). In contrast, transgenic soybeans that exhibit suppressed conglycinin synthesis accumulate ER-derived protein bodies that sequester storage proteins with the result that the pattern of storage protein accumulation resembles that described in cereal seeds. Although such protein bodies superficially resemble a second population of vacuoles these organelles appear to constitute a monocot-type process of nonvacuolar storage protein accumulation in the form of precursor proteins. Cytoplasmic protein bodies can be a stable environment to accumulate

posttranslationally unstable proteins as was shown with zeins (Coleman et al, 1996). Transgenic soybean seeds cosuppressing the conglycinin promoter accumulate massive quantities of ER derived proteins that may prove to be a suitable compartment for accumulating unstable engineered proteins. The shift from vacuolar to cytoplasmic protein body storage protein sequestration by suppression of one type of storage protein promoter activity suggests a possible origin of monocot-type storage protein accumulation.

Suppressing conglycinin expression promotes accumulation of ER-derived protein bodies

The accumulation of precursor forms of glycinin and other PSV proteins without down-regulating the processing enzyme indicates that proteins must be sequestered in some prevacuolar endomembrane compartment(s). Under normal circumstances precursor proteins are primarily observed in the ER by immunocytochemistry (see Herman et al 1984 for an example), or in isolated ER-derived microsomes (Chrispeels et al 1982ab). Protein resident half-life in the ER-lumen has been shown to be several hours (Chrispeels et al 1982b). Precursor proteins on exiting from the ER are apparently rapidly translocated through the Golgi (Chrispeels 1983) and then on to the PSV where processing to the mature form quickly occurs through the action of processing enzyme in the acidic conditions of the vacuolar sap. The stability of precursor forms in the transgenic soybeans suggests that these proteins must be compartmentalized in a prevacuolar compartment. Conventional EM observations of transgenic seed storage parenchyma cells confirmed that there are significant structural differences between the transgenics and controls. In control soybeans proteins are accumulated in the PSVs and except for some Golgi-derived secretion vesicles, there are no other sites that contain dense deposits of storage proteins. In contrast the transgenic soybeans possess a cytoplasm with abundant electron dense protein bodies (PBs) (Fig. 5A). High magnification examination of protein bodies shows that these structures have a relatively uniform morphology of an amorphous electron dense matrix surrounded by a membrane derived from the rough ER (Fig. 5B).

Protein bodies contain glycinin

The suppression of conglycinin expression results in a transgenic seeds where glycinin has replaced the conglycinin so that the total protein content of the transgenic seed is unchanged compared to the control parent. The presence of large numbers of protein

bodies in the cells of the transgenic seeds suggests that much of the glycinin could be sequestered within this compartment. EM-immunogold assays using a specific antibody for glycinin resulted in a highly specific labeling of the PBs and larger protein-filled bodies (Fig. 6A). The large protein-filled bodies appear to be identical to the protein filled PSVs that normally are formed in soybeans. These are characteristically filled with an amorphous storage protein matrix. Unlike the PBs with the ER-derived limiting membrane, the larger PSVs possess a limiting membrane that does not have bound ribosomes. We have not yet established whether the large PSVs, originate from either aggregation of PBs or by accumulation of protein within a vacuole deposited by endomembrane trafficking or a combination of both.

Protein bodies remain in mature germinated seeds

SDS/PAGE-immunoblot assays of glycinin and P34 assays of extracts prepared from dry seeds shows that precursor forms of proteins remain after the seed is fully mature indicating that some of the proglycinin has not progressed to the vacuole for processing. In order to examine the precursor-containing structures that remain in mature seeds we conducted EM analysis of germinated soybean seeds. Cotyledon pieces were fixed after 18 h of hydration in order to assay material in which mobilization of reserve substances has not yet commenced. Immunogold assays with antiglycinin antibodies label two distinct types of structures, PSVs that appear identical to those of the controls, and PBs (Fig. 6A). The PB structure in germinated seeds is different than the PBs observed in maturing seeds. Although the PB retains a limiting membrane with bound ribosomes, the membrane is expanded creating a much larger space between the included storage protein aggregation and the membrane. The presence of discrete PBs in germinated seeds shows that the PBs are stable in soybean seed cells.

Induced protein bodies lack α -TIP, a PSV tonoplast protein

PSV tonoplasts contain a characteristic abundant integral membrane protein that is an aquaporin (water channel) termed α -TIP (Johnson et al 1989). α -TIP is accumulated in soybean PSV tonoplast during late seed maturation in parallel with the storage proteins and in imbibed seeds it is a very abundant constituent and useful marker of the PSV tonoplast. (Melroy and Herman 1991). Immunogold assay with a specific antibody for α -TIP results in dense labeling of the PSV membrane but a complete absence of labeling on the PB's limiting membrane (Fig. 6B).

Protein body membranes do not contain oil body proteins

Soybean seeds accumulate oil in ER-derived oil bodies that possess a half-unit membrane with a characteristic intrinsic membrane protein termed oleosins. The soybean oleosin promoter possesses many similar structural features with the conglycinin promoter (Rowley and Herman 1996). However immunoassays indicated that oleosin accumulation was not suppressed in the transgenic soybeans (data not shown). With the induced accumulation of protein bodies, transgenic soybeans simultaneously accumulate two structurally distinct ER-derived organelles. We tested whether oleosins might be found in the limiting membrane of the protein bodies. Immunogold assay of oleosin with a highly specific antibody resulted in dense labeling of the oil body membranes but a lack of labeling of the protein body membrane (Fig. 6C). This indicates that the formation of oil bodies and protein bodies and that the processes that drive the assembly of these two structures must be separated and likely represent the product of discrete subdomains of the ER.

VACUOLE-MEDIATED DEGRADATION AS A LIMITING FACTOR IN THE EXPRESSION OF SOME PROTEINS

The vacuole has long been recognized as a lytic compartment that is functionally equivalent to the lysosome of animal cells. Plant vacuoles are unlike lysosomes with a role in storing as well as degrading proteins (Galili and Herman 1997; Herman and Larkins 1999 for reviews). Because specific proteases are expressed after germination to mediate mobilization of storage proteins it has been assumed that maturing seeds accumulating storage proteins are protease deficient. Although appealing from a mechanistic point of view, recent results from our laboratories infer that maturing seeds do contain vacuolar proteases capable of degrading a wide variety of proteins. The results we presented demonstrate that destabilized and otherwise posttranslationally unstable storage proteins are degraded in the maturing PSV. Multiple cellular pathways converge to mediate the sequestration of proteins in the PSV. Unstable proteins can be stabilized by retaining the protein in a protease deficient compartment such as the ER as shown by HiMet (Pueyo et al 1995) and gliadin (Napier et al 1997) and the cytoplasm as shown by the example of protein bodies containing α/γ -zeins (Coleman et al 1996). Preventing proteins from entering the vacuole would appear to be one strategy to improve protein stability. However, preventing translocation of proteins into the vacuole is hindered by a lack of information on how cells mediate protein and organelle sequestration in the

vacuole. An alternative approach may be to impair the proteolytic activity of the PSV allowing it to accumulate not only the storage proteins but also other labile proteins. Little is known about the protease inventory of maturing seed PSV. Identification and characterization of the PSV proteases offers the prospect of producing protease-deficient seeds. Disarming proteases has been used in several expression systems to improve the efficiency of protein accumulation. A similar approach in seeds could provide a basis to improve the ability to engineer changes in crops.

PROTEIN BODIES APPEAR TO BE A SAFE ENVIRONMENT TO ACCUMULATE ENGINEERED PROTEINS

Our results show that protein body formation can be promoted by altering the composition of storage proteins expressed in seeds. We have previously observed that soybeans do not appear to accumulate protein bodies during the course of seed maturation. Indeed the donor cultivar was examined in this study as a control and we could find no evidence of protein body formation in the control seeds. Similarly in other studies of soybean seeds there is no reported instance of protein body accumulation and none could be observed in the published micrographs. Induced protein body formation has been previously observed in transgenic tobacco seeds but this has required the expression of hydrophobic cereal prolamine storage proteins. These proteins are normally localized in ER-derived protein bodies in cereal seeds that appear to form by self-assembly and aggregation that can be replicated in heterologous systems such *Xenopus* oocytes. However the expression of a single type of prolamine while inducing protein bodies, the resulting structure is quite different from the more complex structure that results from the coassembly of different prolamines. The coexpression of α - and γ - zeins result in the formation of protein bodies that appear to be very similar to intrinsic protein bodies of maize (Coleman et al 1996). Recently, Hara-Nishimura et al (1998) reported the presence of protein bodies (termed precursor accumulating vesicles PAC) in maturing pumpkin, which was shown to sequester progobulin (11s storage protein). How the formation of the protein bodies in pumpkin is balanced against conventional endomembrane trafficking in the accumulation of storage proteins remains to be elucidated.

The transgenic soybeans that accumulate ER-derived protein bodies may prove to be a useful starting point for further experiments alter seed protein composition. Expression of heterologous proteins in seeds frequently results in posttranslational degradation of the newly synthesized protein. For instance destabilized phaseolin HiMet, a

7s vicilin-type protein, is posttranslationally unstable (Hoffman et al 1988) with its site of degradation being the forming PSV. Adding ER retention sequences to the destabilized HiMet results in increased stability of HiMet that is sequestered within the ER lumen. Similarly zeins expressed in transgenic tobacco can be posttranslationally unstable but coexpressing two different zeins promotes the formation of ER-derived protein bodies which stabilizes the zeins (Coleman et al 1996), although these protein bodies too are eventually destroyed by autophagy. Taken together these results indicate that the ER and ER-derived protein bodies is an apparently protected site for unstable proteins (see figure 7 for summary information). For example, if such unstable proteins can be cosequestered with proglycinin within ER-derived protein bodies in transgenic soybeans this may provide a means to alter the protein composition of soybean seeds. the stability of protein bodies through seed maturation means that these unstable proteins would still remain in the dry seeds and accumulate in useful quantities. By coexpressing other proteins, perhaps as a glycinin fusion protein with a cleavable spacer it may be possible to configure soybeans to highly express and accumulate foreign proteins that require ER-mediated folding and processing events. This may open up the possibility for soybeans to accumulate novel gene products that would otherwise be difficult to produce in seeds.

OTHER ASPECTS OF THIS PROJECT

Characterization of BiP cDNAs and Expression

BiP (Binding Protein) is a highly conserved molecular chaperone that functions to fold (or refold) nascent and damaged proteins. A resident of the ER lumen, BiP has been shown to be involved in the recognition and disposal of misfolded proteins in yeast and animal cells and is an essential part of the quality control mechanisms of the cell. Under normal circumstances BiP binding to nascent proteins is transient, however BiP has been shown to bind to mutant and misfolded variants of wild-type secretory proteins. Yeast cells appear to monitor the level of misfolded proteins with a transmembrane protein kinase. The luminal domain of this protein appears to detect and bind BiP-protein conjugates and trigger a signal through its cytoplasmic kinase domain (Mori et al 1993, Cox et al 1993 see Shamu et al 1994 for review). This produces a signal cascade that induces the overexpression of BiP that is a characteristic response of cells that express high levels of misfolded proteins either by transgene expression or by induction of chemical reagents such as tunicamycin. The signal transduction from IRE1 is not essential for normal viability of yeast but when mutants deficient in IRE1 are challenged with

reagents that produce misfolded proteins the cells the lack of the IRE1p is lethal (Cox et al 1993). We attempted to clone a plant homologue of the IRE1p but were unsuccessful. Personal communication from Dr. Maarten Chrispeels (UCSD) indicates that his group was successful and we look forward to seeing how this protein may, or may not, be involved in the recognition of misfolded proteins such as HiMet.

Early in this project we cloned and characterized BiP from soybeans with the view of using the legume cDNAs and antibodies produced from the protein as probes to examine the quality control process in more detail. This was a primary objective of the grant proposal as submitted. Three distinct clones were isolated and sequenced which helped substantiate that plants differ from animals and yeast in possessing a number of BiP genes (Kalinski et al 1995). We characterized the expression and regulation of BiP protein accumulation during development in several different organs. A significant observation was that BiP expression is regulated by diurnal periodicity with an excess of 10 fold variation in a 24 hr period. This diurnal variation is present in terminally differentiated cells and is unrelated to growth and development.

In the context of this project we were not able to demonstrate that BiP as a role in the disposal of posttranslationally unstable seed proteins. Our results described in the prior sections that degradation of these proteins occurs in the vacuole rather than the ER eliminates the need for an ER based degradation system. Further attempts to show that HiMet bound BiP more tightly as compared to the wild type β -phaseolin were not successful. Taken together these results suggest and confirm that the ER protein quality control mechanism is not important in either the recognition or turnover in the examples of posttranslationally unstable proteins we studied. Our results should not be interpreted to indicate that the vacuole-based turnover of posttranslationally unstable proteins substitutes for the ER-based quality control system of animals and yeast. There is evidence for an ER quality control system in plants with the best results that of Dr. Vitale's laboratory (DiAmico et al 1992, Pedrezzini et al 1997) on monomeric β -phaseolin. The results from his laboratory provide evidence inferring that this protein is degraded by an ER quality control system using BiP recognition. We view the vacuole and ER turnover systems as operating in parallel providing two different levels of mechanisms that can function to degrade unstable proteins. Of these the vacuole-based system is much broader in that the enzymes resident in the vacuole should be highly active against all proteins except for those selected to survive such a harsh environment. In contrast the ER quality control system appears to be limited with foreign and misfolded (destabilized) proteins passing through without triggering degradation reactions. This makes the ER and its derived

protein body compartment a relatively safe compartment to accumulate posttranslationally unstable proteins.

Processing of KDEL from a protease bearing the ER-retention sequence

SH-EP is a cysteine proteinase from germinated mung bean (*Vigna mungo*) that possesses a carboxyterminal ER retention sequence, KDEL. With its ER retention sequence this protease may be an example of a class of ER resident proteases that is functionally equivalent to the proteases found in lytic compartments. An ER resident protease could be functionally important in understanding the mechanism of posttranslational protein degradation. SH-EP was studied with the goal of determining whether the ER retention sequence has the function of retaining the protein in the ER or whether the retention sequence has some other function involved in the trafficking and activation of the enzyme. In order to examine the function of the ER retention sequence we expressed a full-length cDNA of SH-EP and a minus-KDEL control in insect Sf-9 cells using the baculovirus system. Our observations on the synthesis, processing and trafficking of SH-EP in Sf-9 cells suggest that the KDEL ER-retention sequence is posttranslationally removed either while the protein is still in the ER or immediately after its exit from the ER, resulting in the accumulation of proSH-EP minus its KDEL signal. It is this intermediate form that appears to progress through the endomembrane system and is subsequently processed to form mature active SH-EP. The removal of an ER retention may regulate protein delivery to a functional site and present an alternative role for ER retention sequences in addition to their well established role in maintaining the protein composition of the ER lumen. The results of this study demonstrated that the proteases that possess ER retention sequences do not do so in order to remain as functional proteases in the ER. Instead the ER retention sequence appears to function in regulating the progression of the protein from the ER allowing the inactive precursor to accumulate as a transient zymogen. In the process of conducting this study we discovered an important result, that is that ER retention sequences can be processed and removed. This may have an important role in redirecting ER resident proteins from the ER to other distal locations in the endomembrane system and could be particularly significant in regulating the turnover of ER resident proteins.

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WEIZMANN RESEARCH

SUMMARY

Following synthesis, wheat gliadin storage proteins are deposited into protein bodies inside the endomembrane system in a way that enables not only their efficient accumulation and dehydration during seed maturation, but also their rapid rehydration and degradation during germination. In the present report, we studied the mechanism of gliadin deposition and whether it was controlled by the conformation of these proteins. Although gliadins extracted from mature dry grains are largely insoluble in aqueous solutions, sucrose gradient analysis showed that a considerable amount of the gliadins in developing grains appeared as relatively soluble monomers. Reduction of the intramolecular disulfide bonds in monomeric gliadins *in vitro*, caused their precipitation into insoluble aggregates. In addition, pulse-chase experiments in the absence or presence of reducing agents showed that formation of intramolecular disulfide bonds also played a major role in folding and deposition of the gliadins *in vivo*. Our results showed that following sequestration into the endoplasmic reticulum, the gliadins folded into relatively soluble monomers, which were incompetent for rapid aggregation and could apparently form only relatively weak interactions among themselves. This pattern of deposition was also highly dependent on the conformation of the gliadins as determined by the formation of intramolecular disulfide bonds between conserved cysteines. The contribution of this study to the understanding of the evolution and function of gliadins is discussed.

INTRODUCTION

Wheat storage proteins are co-translationally inserted into the rough endoplasmic reticulum (RER) and are then deposited into protein bodies (PB) at various compartments of the endomembrane system (6). In these PB, the storage proteins survive the process of seed desiccation and are then rehydrated again, degraded and mobilized into the embryo during germination. The major storage proteins in wheat are three closely related classes of alcohol soluble proteins called sulfur (S) rich gliadins; S-poor gliadins and high molecular weight glutenins (HMW-GS). The S-rich gliadins are further subdivided into four subgroups of α , β , γ and aggregated gliadins. All three protein classes contain a domain that is composed of small amino acid sequence repeats, rich in glutamine and proline (14; 22; 24). In the S-poor gliadins, this domain apparently accounts for the majority of the polypeptide. In the HMW-GS, the repetitive domain appears in the center and is flanked by small N and C-terminal non repetitive domains. These non repetitive domains contain several cysteine residues that form intermolecular disulfide bonds between the different glutenin subunits resulting in their polymerization (14; 22; 24). In the S-rich gliadins, the repetitive domain is present in the N-terminal part of the polypeptide. This domain varies between the different S-rich gliadin subclasses in the number and consensus sequences of the repeats, but all of the repeats are thought to be arranged in a β -turn configuration (22; 24). The C-terminal domain of the S-rich gliadins is apparently arranged predominantly in a helix and β -sheet configurations and also contains six to eight highly conserved cysteine residues, which form three to four intramolecular disulfide bonds (22; 24).

Despite extensive studies, the fine structure of wheat gliadins and their mechanism of deposition into PB are still not clearly understood. As gliadins and glutenins extracted from mature grains are largely insoluble in aqueous solutions, it was suggested that these proteins spontaneously precipitate and aggregate into insoluble deposits immediately after insertion into the ER (see ref. 4 for a review). However, in recent years, various lines of evidence suggested that the maturation of wheat storage proteins may not be spontaneous, but rather assisted by molecular chaperones that are present inside the ER (6; 28). Of these molecular chaperones, the binding protein (BiP) was suggested to assist in the general maturation of storage proteins from wheat and other plant species (5; 9; 10; 16; 18; 29). A second molecular chaperone, protein disulfide isomerase (PDI), was suggested to assist in the formation of correct intramolecular disulfide bonds in wheat S-rich gliadins (2).

Nevertheless, it is still unclear whether the molecular chaperones assist only in the folding of the storage proteins or also in various steps of their deposition into PB.

Studying the mechanism of wheat storage protein deposition, we have previously expressed wild-type and modified S-rich gliadins in *Xenopus* oocytes (1). This study demonstrated that following insertion into the ER the gliadins could diffuse rather freely within the organelle for a few hours, suggesting that deposition into PB does not occur by their mere precipitation and aggregation. In addition, density gradient analysis showed that not all of the gliadins were present in dense aggregates, suggesting that the deposition occurs by a slow regulated process. In contrast to wild type proteins, mutant gliadins, lacking conserved cysteines in the C-terminal regions, rapidly aggregated into nondiffusible complexes within the ER, suggesting that intramolecular disulfide bonds played a major role in the deposition of the gliadins. Based on these observations (1), we have presently studied the mechanism of deposition of gliadins in the homologous system of developing wheat grains. We found that not all of the gliadins were present in dense complexes, but a significant amount appeared as relatively soluble monomers. Moreover, we found that the intramolecular disulfide bonds play a major role in the conformation of the gliadins and control the process of their deposition. The relevance of these findings to the function, evolution and possible chaperone-assisted maturation of wheat storage proteins is discussed.

RESULTS

(Figures are included in the attached reprint).

Analysis of storage protein complexes in developing wheat grains

To study the mechanism of wheat storage protein deposition into PB, we first fractionated homogenates from developing grains on 5-20% sucrose gradients. Individual fractions were then reacted in Western blots with anti-HMW-GS and anti γ -gliadin sera. As shown in Fig. 1A, the HMW-GS were entirely present in complexes that sedimented to the bottom of the gradient. A considerable amount of the γ -gliadins also sedimented to the bottom of the sucrose gradients (Fig. 1B), but, in contrast to the HMW-GS, a significant proportion of the gliadins also sedimented in lighter fractions. Although it is difficult to measure the accurate molecular weight of proteins in sucrose gradients, the gliadins sedimenting in the light fractions were apparently monomers, based on the co-sedimentation of protein markers. Thus, for simplicity, these lightly migrating gliadins were defined as "monomeric gliadins" throughout the rest of the study.

Gliadins, extracted from mature dry wheat grains are largely insoluble in aqueous solutions (22), but whether this is related to the structure of the proteins or to their aggregation state is not clear. We therefore wished to test the solubility of the monomeric gliadins from developing grains in an aqueous solution. Developing grains were homogenized in 'aqueous buffer' and upon centrifugation, soluble (supernatant) and insoluble (pellet) proteins were fractionated in SDS PAGE. The gels were then reacted in Western blots with anti HMW-GS and anti gliadin sera. As shown in Fig. 2, while the HMW-GS subunits were insoluble, a significant proportion of the γ -gliadins were soluble. Sucrose gradient fractionation also showed that the soluble γ -gliadins were present entirely as monomers (Fig. 3A).

Reduction of Soluble Gliadins Causes their Aggregation into Insoluble Deposits In vitro

We have previously demonstrated that elimination of conserved cysteines present in the C-terminal region of a wheat γ -gliadin triggered its aggregation into nondiffusable complexes in *Xenopus* oocytes (1). Therefore, we tested whether the presence of a significant amount of gliadins as soluble monomers was related to their conformation as determined by intramolecular disulfide bond formation. Soluble gliadins, extracted from developing wheat grains, were divided into two equal portions and to one portion we added DTT to dissociate their disulfide bonds. The homogenates were then fractionated on 5-20% sucrose gradients and individual fractions were reacted in Western blots with anti γ -gliadin serum. As shown in Fig. 3, the natural soluble gliadins appeared as monomers (panel A), while reduction of the disulfide bonds *in vitro* caused their aggregation into complexes that sedimented to the bottom of the gradient (panel B). Notwithstanding, two cross reacting bands with MWs larger than 43 kDa still appeared as monomers upon reduction (Fig. 3A, bands marked by an asterisk). These proteins are bigger than the expected size of the γ -gliadins and may belong to the related fraction of the S-poor gliadins, which do not possess cysteine residues and therefore are not affected by reducing agents.

As the soluble extract from developing wheat grains apparently contained a mixture of proteins, including molecular chaperones, it was impossible to deduce from Fig. 3 whether aggregation resulted directly from the conformational change of the gliadins upon reduction, or that it was assisted by additional factors. To address this, we tested the change in conformation and aggregation of purified α and γ -gliadins, upon reduction of their intramolecular disulfide bonds. First, purified gliadins were dissolved in SDS sample buffer lacking or containing DTT and the proteins were fractionated by SDS

PAGE. Reduced gliadins migrate slightly slower in such gels apparently due to their extended conformation (2). Indeed, as shown in Fig. 4 the DTT-treated purified gliadins migrated slower than the nontreated ones indicating that the purified gliadins were oxidized and that the DTT reduced their disulfide bonds. Next, the purified gliadins were dissolved in 'aqueous buffer' and soluble gliadins were obtained by centrifugation. Each fraction of soluble gliadins was divided into two equal portions and one of them was supplemented with DTT to reduce the intramolecular disulfide bonds. The reduced or oxidized gliadins were then fractionated on 5-20% sucrose gradients and individual fractions were fractionated by SDS PAGE and stained with coomassie blue. As shown in Fig. 5, both soluble purified α , and γ -gliadins migrated as monomers (panels A, C, E), while reduction with DTT caused their aggregation into complexes that sedimented to the bottom of the gradient (panels B, D, F).

Maturation of Newly Synthesized Gliadins

The mechanism of deposition into protein bodies was studied further by analyzing the maturation of newly synthesized gliadins *in vivo*. Developing grains were pulse labeled with ^{35}S methionine + cysteine either for 10 min, or for 24 h as a control, and their homogenate was separated on a 5-20% sucrose gradient. Individual fractions were then ethanol extracted to enrich for the gliadins, and the ethanol-soluble proteins were fractionated by SDS-PAGE and detected by autoradiography. Notably, a considerable amount of the radioactive gliadins, labeled either for 24 h or for 10 min, sedimented to the bottom of the gradient (Fig. 6, A, B). Western blot analysis of total protein from the same fractions with anti-BiP and anti-PDI sera showed that these two ER-resident molecular chaperones were also enriched in the bottom of the gradient (Fig. 6C). This suggested that sedimentation of the gliadins into the dense fractions may not have been necessarily due to their interactions with each other, but rather due to their association with the ER or with ER-resident molecular chaperones. To eliminate such associations, developing grains were homogenized with 'aqueous buffer' containing 0.5% Triton X-100 and fractionated on 5-20% sucrose gradients. This detergent caused the dissociation of the ER membrane as deduced from the release of most of the PDI and BiP to the top fractions of the gradients (Fig. 6 cf. panels C and F). The Triton X-100 also released a significant amount of monomeric newly synthesized gliadins (pulse labeled for 10 min) that sedimented at the top of the gradient (Fig. 6 cf. panels B and E). Nevertheless, also in the Triton X-100 containing extracts, a small but significant amount of the newly synthesized gliadins was still present in complexes that sedimented to the bottom of the gradient. Analysis of the gliadins labeled for 24 h showed that 0.5% Triton X-100 caused

also some dissociation of complexes into monomers (Fig 6 cf. A to D), but the degree of dissociation was much smaller than that of the newly synthesized gliadins. The results of this experiment implied that a significant amount of the newly synthesized gliadins were present as monomers associated with the ER, while a small amount of these proteins was already present in complexes.

We next studied the assembly of the gliadins during their maturation within the ER. Developing grains were pulse labeled with ^{35}S methionine + cysteine for 10 min and chased for zero, 10 and 50 min. Grains were then homogenized with 'aqueous buffer' containing 0.5% Triton-100 and analyzed on 5-20% sucrose gradients in an identical manner to the experiment shown in Fig. 6E. As shown in Fig. 7, with the maturation of the gliadins, progressively increasing amounts of proteins were present in complexes that sedimented to the bottom of the gradient.

Inhibition of Disulfide Bond Formation in Newly Synthesized Gliadins Causes their Aggregation In Vivo

The results presented in Figs. 3 and 5 showed that *in vitro*, dissociation of the intramolecular disulfide bonds caused the aggregation of monomeric gliadins. We therefore wished to test whether prevention of disulfide bond formation in newly synthesized gliadins would cause their aggregation also *in vivo*. To address this, developing grains were preincubated with 'aqueous buffer' containing 100 mM DTT for 30 min followed by pulse labeling for 10 min with ^{35}S methionine and cysteine. Control grains were preincubated with 'aqueous buffer' alone. Following the labeling, grains were washed extensively in 'aqueous buffer' to remove traces of DTT. *In vivo* reduction of the gliadins was first assessed by their binding to Thiopropyl-sepharose beads which bind to proteins containing free thiol groups. Indeed, while under normal conditions most of the newly synthesized gliadins did not bind to the beads, upon preincubation with DTT about half of the newly synthesized gliadins were bound to the beads, confirming their *in vivo* reduction (data not shown). Homogenates from these grains were then fractionated on 5-20% sucrose gradients, fractions were ethanol extracted, and radioactive proteins in each fraction were analyzed by SDS-PAGE and exposed to a phosphorImager plate. As shown in Fig. 8, pretreatment with DTT, reduced the efficiency of the gliadin labeling. Still, a significantly higher proportion of the gliadins sedimented to the bottom of the gradient, indicating that prevention of disulfide bond formation caused the aggregation of the newly synthesized gliadins *in vivo*. Although some traces of DTT were probably still present inside the cells after the extensive washing, they clearly did not have any *in vitro* effect on the aggregation of the gliadins because upon homogenization of each small grain

slice in 0.8 ml of 'aqueous buffer' the maximal expected concentration of DTT was in the mM range, which is much below the minimal concentration (~1 mM) that can effectively cause gliadin aggregation *in vitro* under these conditions (data not shown).

Insoluble aggregates of reduced gliadins possess different properties than complexes of natural ones

The interactions between gliadins in the insoluble aggregates of the malfolded reduced polypeptides and in the insoluble complexes formed by natural gliadins were studied by the ability of the gliadins to be solubilized from the insoluble pellets upon re-extraction with 'aqueous buffer'. Developing grains were homogenized in 'aqueous buffer' and divided into soluble supernatant and insoluble pellet upon centrifugation (Fig. 9, lanes 1 and 2). The soluble fraction was divided into two fractions, DTT was added to one of them, and the supernatants were then recentrifuged to obtain soluble supernatant and insoluble pellet (Fig. 9, lanes 3, 4 and 5, 6). Insoluble pellets of the natural gliadins (Fig. 9, lane 2) and of the reduced ones (Fig. 9, lane 6) were re-extracted in 'aqueous buffer' and divided again into soluble supernatants and insoluble pellets upon centrifugation (Fig. 9, lanes 7, 8 and 9,10). While a considerable amount of gliadins from the insoluble pellets of the natural gliadins were re-solubilized, no re-solubilization of gliadins was obtained from the insoluble pellets of the reduced polypeptides (Fig. 9, cf. lanes 7, 8 and 9, 10).

DISCUSSION

Maturation of wheat storage proteins

In the present report we have shown that the S-rich gliadins and HMW-GS follow different maturation steps during grain development. The HMW-GS efficiently assemble into relatively large insoluble complexes that sediment to the bottom of the 5-20% sucrose gradients. This observation is in close agreement with previous findings that the HMW-GS are deposited in highly dense PB (20; 21). Assembly of the HMW-GS apparently occurs both by noncovalent interactions as well as by formation of intermolecular disulfide bonds between cysteines present on their N and C-termini (23).

Unlike the HMW-GS, our results show that the S-rich gliadins are synthesized mostly as soluble monomers and a significant amount of them remain as monomers at steady state, in the immature grains. These results support our previous observation (1) that S-rich gliadins can diffuse rather efficiently for several hours in the ER of *Xenopus* oocytes, and also argue against their spontaneous precipitation and aggregation due to polypeptide structure. The presence of monomeric gliadins at steady state in developing grains, and the fact that natural gliadins are largely incompetent for assembly *in vitro*, also suggest that these proteins possess a specific conformation, which does not favor strong protein-protein interactions. This observation is of particular interest taking into account that the S-rich gliadins contain glutamine-rich repeats in their N-termini as well as several glutamine stretches in their C-terminal domain (12; 24). As exposed glutamine residues generally enhance protein oligomerization (26), it is possible that many of these residues are either buried inside the gliadins or favor the formation of hydrogen bonds with water. The involvement hydrogen bonds and other non-covalent interactions in the aggregation of the malfolded reduced gliadins was also supported by our finding that this process was prevented when chaotropic agents or detergents (i.e. potassium thiocyanate, Triton X-100 and SDS) were present in the "aqueous buffer" (data not shown).

Not all of the S-rich gliadins remained as monomers. During maturation, some of the proteins progressively assembled into insoluble complexes that sedimented into the bottom of a 5-20% sucrose gradient (complexes containing at least eight polypeptides based on an average gliadin size of ~30 kDa and on the sedimentation of molecular markers in the gradient, see Fig. 1). Moreover, assembly and precipitation was further greatly enhanced during grain desiccation (data not shown). The assembly and precipitation of the gliadins may result first from increased gliadin concentration within the PB upon grain maturation and later on, from formation of additional hydrogen bonds between the gliadins upon the loss of water from the grains. It is also possible that gliadin

assembly may be furthermore enhanced by a change in the pH, which occurs following their transport from the ER to the vacuoles (6), similar to the case of legume storage proteins (27). Nevertheless, the interactions between the gliadins are presumably relatively weak as these polypeptides can evidently be rapidly rehydrated and degraded upon seed germination.

The present observation that S-rich gliadins are apparently in an equilibrium between monomers and complexes is in agreement with our previous finding that PB containing them possess variable densities, as deduced from sedimentation in metrizamide gradients (20). However, it is impossible to draw a simpler correlation between the density of the PB and the oligomerization state of the gliadins because the dense PB in metrizamide gradients apparently contain both gliadins and HMW-GS (20), while the HMW-GS are sufficient by themselves to form the dense PB (21).

The Intramolecular Disulfide bonds in S-rich Gliadins Stabilize their Conformation and Prevent Their Rapid Aggregation

The high evolutionary conservation of cysteine residues suggests that they are important for the maturation of the S-rich gliadins. However, as the gliadins possess no known function besides storage, the functional role of these cysteines was not clear. In the present report, we showed that the intramolecular disulfide bonds, formed between these cysteines, play a major role in the conformation of the gliadins and in fact also control their deposition by ensuring slow assembly rather than rapid aggregation and precipitation. This was concluded by two lines of evidence: (i) reduction of soluble monomeric gliadins caused their aggregation *in vitro*; and (ii) prevention of disulfide bond formation in newly synthesized gliadins enhanced their aggregation *in vivo*. In this regard, we wish to stress that despite of the fact that both the natural insoluble complexes of the gliadins, as well as the ones formed by the aggregation of malfolded gliadins, sedimented to the bottom of the 5-20% sucrose gradients, the properties of each type of these complexes were entirely different due to the following: (i) the natural complexes contained oxidized gliadins, while the aggregates of the malfolded proteins contained reduced polypeptides; and (ii) when the insoluble pellets of the natural and malfolded reduced gliadins were resuspended in the aqueous buffer, only the natural gliadins could be released as monomers (Fig. 9), suggesting that the interactions responsible for the oligomerization of the natural gliadins were much weaker than those causing the aggregation of the reduced malfolded polypeptides

Is the Deposition of S-rich Gliadins Assisted by Molecular Chaperones?

Although this question was not addressed directly in the present report, our results suggest an indirect role for molecular chaperones in the deposition process, by assisting with the folding of the gliadins to obtain a specific conformation that prevents rapid aggregation. Previous reports have already suggested that folding of the S-rich gliadins, including the formation of correct intramolecular disulfide bonds, is assisted by the molecular chaperones PDI and BiP (2; 9). Moreover, an indirect support for such a role was also recently obtained experimentally in our laboratory. In this experiment, purified S-rich gliadins were reduced and denatured and allowed to refold spontaneously under oxidizing conditions. Formation of intramolecular disulfide bonds under these conditions was incomplete and most of the gliadins were present in insoluble aggregates (data not shown). Another unresolved issue is whether molecular chaperones also assist in the assembly of the gliadins into insoluble complexes. Although the fact that a significant amount of S-rich gliadins appear as soluble monomers at steady state suggests that such an assistance is rather limited, additional studies will be required to solve this issue.

Functional Evolution of Wheat Storage Proteins

The major function of storage proteins is to fix amino acids into a protein-bound form. this enables the accumulation of the amino acids to large amounts during seed maturation and their subsequent mobilization into the germinating embryo upon re-hydration and degradation of the storage proteins. Thus, the composition of amino acids in the coding capacity of the storage proteins should be very similar to that of the free amino acid pool in the seeds. In developing wheat grains, the major free amino acid is glutamine, and indeed the gliadins and glutenins possess a non common amino acid composition in which glutamine is most abundant, averaging between ~30 to 40% (22; 23; 24). Yet, enrichment of glutamines may enhance the oligomerization and aggregation of the storage proteins (26), and hence could interfere with their subsequent rehydration and degradation during germination. In the present report, we have shown that the S-rich gliadins have apparently evolved to obtain a specific conformation that enables their folding into forms that are rather incompetent for tight oligomerization and aggregation despite of their enrichment in glutamine residues. This appears to be controlled by the formation of intramolecular disulfide bonds between cysteine residues that were highly conserved during gliadin evolution.

Wheat storage proteins also contain a class of HMW-GS that contrarily to the gliadins efficiently assemble into large insoluble polymers, linked by noncovalent and intermolecular disulfide bonds (Fig. 1) (13; 23). Notwithstanding, in all wheat species, this class represents only a minor proportion of about 5% of the total storage proteins and

appears to have followed a very interesting evolution: (i) in contrast to the gliadins which appear in multigene families, the HMW-GS are controlled by loci containing only two genes (17); (ii) Due to their high content of over than 40% glutamines, whose CAA/CAG codons can be mutated to TAA/TAG stop codons, the HMW-GS were expected to be silenced at relatively high rates during wheat evolution. Nevertheless, HMW-GS genes are present and active in all of the diploid and polyploid wheat species, and lines lacking these proteins are very rarely observed; and (iii) Some, but clearly not all of the HMW-GS genes appeared to have followed a nonrandom process of diploidization during the polyploidization of wheat (7), apparently due to promoter inactivation (11). Thus, it is tempting to speculate that the HMW-GS were evolved to represent a small fraction of an insoluble core to which the major fraction of the S-rich gliadins can join for the initiation of PB formation. This may have enabled the efficient accumulation of the storage proteins in dense PB within the limiting space of maturing endosperm cells, and their subsequent rapid re-hydration and degradation during germination.

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FIGURE LEGENDS

Fig.1 Sedimentation of Wheat Storage Proteins in Sucrose Gradient - Homogenate from a developing wheat grain was fractionated on 5-20% sucrose gradient. Fractions were separated on SDS-PAGE and subjected to Western blot analysis with anti HMW-GS (A) and anti γ -gliadin (B) sera. The position along the gradient of the following molecular weight marker proteins is indicated at the bottom: cytochrome C (12 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (150 kDa) and catalase (232 kDa). Bands not related, but cross reacting with the anti HMW-GS sera or anti γ -gliadin sera, are marked by an asterisk. P, insoluble pellets that sediment to the bottom of the gradient. The location of molecular weight markers in the SDS gel is indicated on the left.

Fig. 2 Solubility of S-rich gliadins in Aqueous Solutions - Developing wheat grains were ground in 'aqueous buffer' and supernatant (S) and pellet (P) were separated following centrifugation. Equivalent portions from supernatant and pellet were separated on SDS-PAGE followed by Western blot analysis using anti γ -gliadin and anti HMW-GS sera. Bands not related, but cross reacting with the anti HMW-GS sera or anti γ -gliadin sera, are marked by an asterisk. The location of molecular weight markers is indicated on the left.

Fig. 3 *In Vitro* Reduction of Soluble Wheat Grain Extracts, Results in Aggregation of S-rich Gliadins - Soluble gliadin supernatant was loaded on top of a 5-20% sucrose gradient, either lacking (A) or containing (B) DTT. Following ultra centrifugation, fractions were separated on SDS-PAGE and subjected to Western blot analysis using anti γ -gliadin sera. Bands cross reacting with the anti γ -gliadin sera that apparently do not belong to the S-rich gliadins, based on their higher molecular weight are marked by an asterisk. P, insoluble pellets that sediment to the bottom of the gradient. The location of molecular weight markers is indicated on the left.

Fig. 4 Reduction of Intramolecular Disulfide Bonds Alters the Conformation of S-rich Gliadins - A soluble fraction of one g and two a type gliadin groups, were treated with (+) or without (-) 10 mM DTT. Samples were separated on SDS-PAGE and coomassie stained. The location of molecular weight (MW) markers is indicated on the left.

Fig. 5 *In Vitro* Reduction of Purified S-rich Gliadins Results in Their Aggregation - Soluble g, and two a type gliadin groups, were loaded on top of 5-20% sucrose gradients, either lacking (A, C and E) or containing (B, D and F) DTT. Following ultracentrifugation, fractions were separated on SDS-PAGE and coomassie stained. P, insoluble pellets that sediment to the bottom of the gradient. The location of molecular weight (MW) markers is indicated on the left side of each gel.

Fig. 6 Sedimentation of The Bulk and Newly Synthesized Gliadins in Sucrose Gradient - Developing grains were labeled for either one day (A and D) or for 10 min (B and E) with ^{35}S methionine and cysteine. Grains were then gently extracted in an 'aqueous buffer' either containing (A-C) or not containing (D-F) 0.5% Triton X-100. The extracts were then loaded on top of 5-20% sucrose gradients. Following ultracentrifugation and fractionation, gliadin enriched ethanol extracts (A, B, D and E) or total proteins (C and F) were separated on SDS-PAGE. Gels loaded with gliadin enriched extracts, were dried and exposed to film (A, B, D and E). The other two gels were subjected to Western blot analysis, using simultaneously, both anti BiP and anti PDI antibodies (C and F). P, insoluble pellets that sediment to the bottom of the gradient. The location of BiP and PDI is indicated on the right side of panels C and F.

Fig. 7 Pulse Chase Analysis of Gliadin Maturation *In Vivo* - Developing grains were pulse labeled with ^{35}S methionine and cysteine for 10 min, and chased with cold 'aqueous buffer' for 0, 10 and 50 additional min. Grain slices were then gently extracted in a Triton X-100 containing 'aqueous buffer', and layered on top of a 5-20% sucrose gradient. Gliadin enriched fractions were separated on SDS-PAGE and labeled bands were detected by PhosphorImager. P, insoluble pellets that sediment to the bottom of the gradient. A, 0 min chase; B, 10 min chase; C, 50 min chase.

Fig. 8 Reduction of Intramolecular Disulfide Bonds Triggers Gliadin Aggregation *In Vivo* - Developing grains were pre incubated with 'aqueous buffer' (A), or 'aqueous buffer' containing 100 mM DTT (B). After 30 min, the grains were washed and incubated with ^{35}S methionine and cysteine for 10 additional min. Grains were then extracted in a Triton X-100 containing buffer and layered on a 5-20% sucrose gradient. Following fractionation and gliadin extraction, samples were separated on SDS-PAGE, and the labeled bands were detected by PhosphorImager. P, insoluble pellets that sediment to the bottom of the gradient.

Fig. 9 Dissociation of Gliadins From Insoluble Precipitates - Developing wheat grain homogenates were centrifuged to obtain supernatant (S, lane 1) and pellet (P, lane 2). The supernatant (1) was either not supplemented (-DTT) or supplemented with 150 mM DTT (+DTT), and centrifuged again to obtain supernatants and pellets (lanes 3, 4 and 5, 6, respectively). Pellets from both natural gliadins (lane 2) or reduced gliadins (lane 6) were resuspended in 'aqueous buffer', vortexed for 10 min at room temperature and recentrifuged to obtain supernatants and pellets (lanes 7, 8 and 9, 10, respectively). Equal proportions of each one of the treatments were subjected to SDS-PAGE and reacted in Western blots with anti γ -gliadin serum. Bands not related, but cross reacting with the anti γ -gliadin sera, are marked by an asterisk. The location of molecular weight markers is indicated on the left.