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Rapid Communication

Morphometric Analysis of Rice Seed Protein Bodies[1]

Implication for a Significant Contribution of Prolamine to the Total Protein Content of Rice Endosperm

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Electron microscopic observation of thin sections of rice (*Oryza sativa* L.) endosperm revealed two types of protein bodies (PBs): spherical and irregular-shaped ones. Immunocytochemical localization studies using antibodies raised against purified glutelins, prolamines, and globulins indicated that the prolamines were localized in the spherical PB, whereas the irregular-shaped PB contained glutelins and globulins. We counted and measured the surface area and the relative volume of 2303 PBs randomly selected from two different developmental stages and from different locations within the endosperm. The ratio of spherical to irregular-shaped PBs was 1:1.6. Double-label immunogold electron microscopic localization indicated that the globulins represented about 18% of the surface area of the irregular-shaped PBs. Based on our morphometric analysis, we estimate the relative contribution of glutelin as 53%, that of prolamine as 35%, and that of globulin as 12% of the total seed protein.

Seed storage proteins can be divided into four classes based on their solubility properties. Albumins are water soluble, globulins are salt soluble, prolamines are soluble in aqueous alcohol solutions, and glutelins are soluble in alkali or acid (Shotwell and Larkins, 1989). Rice (*Oryza sativa* L.), a staple food crop for millions of people worldwide, has a seed protein content ranging from 5 to 12% (Villareal and Juliano, 1978). The major storage proteins found in rice are the glutelins, which according to previous studies, account for 80% or more of the total seed protein (Tecson et al., 1971; Juliano, 1972; Villareal and Juliano, 1978). The remaining 20% is divided as follows: albumins, 1 to 5%; globulins, 4 to 15%; and prolamines, 2 to 8% (Houston et al., 1968). It has been demonstrated that the relative contributions of each of the solubility classes of seed proteins can be influenced by genotype, growing conditions, and the analytical methods employed (Sugimoto et al., 1986; Ogawa et al., 1987; Huebner et al., 1990).

The biosynthesis of rice glutelins and prolamines has been studied in detail by several researchers (Yamagata et al., 1982; Luthe, 1983; Wen and Luthe, 1985; Krishnan and Okita, 1986; Li and Okita, 1993). Glutelins are first synthesized as precursor proteins of 51 to 57 kD. These precursors are then proteolytically processed into α subunits of 34 to 39 kD and β subunits of 21 to 22 kD. The end products are stored in specialized structures called PBs. A previous electron microscopic study (Krishnan et al., 1986) found that glutelins are processed through the Golgi apparatus and are deposited in irregular-shaped PBs (PB type I). Rice prolamines have molecular masses in the 10- to 17-kD range (Mandac and Juliano, 1978; Kim and Okita, 1988; Masumura et al., 1990) and accumulate within distensions of the rough ER (Yamagata et al., 1982; Krishnan et al., 1986). They are stored in spherical PBs (PB type II). Recently, the relative contribution of prolamines to the total protein content of rice seeds has been re-examined. Traditionally, prolamines have been extracted with 70% ethanol. However, a study by Sugimoto et al. (1986) found that, when extracted with 55% *n*-propanol, rice seeds yielded four times more prolamine than when extracted with 70% ethanol. More recently, Li and Okita (1993), using a quantitative immunoblot technique, calculated that the level of glutelin in rice seeds was only 30% higher than the level of prolamine. These results suggest that prolamine levels have been significantly underestimated and call into question the validity of the long-accepted values found in the literature for the various classes of rice seed storage proteins.

In this study, we employed EM and immunocytochemistry to identify and measure PBs in rice seeds. We used the technique of computer-assisted morphometric analysis to quantify the relative contribution of glutelins, globulins, and prolamines to total seed protein.

MATERIALS AND METHODS

Chemicals

^{125}I-labeled protein A (8.9 μCi/μg) was purchased from New England Nuclear. Acrylamide, *bis*-acrylamide, and SDS-molecular-weight markers were purchased from Bio-Rad. Spurr's resin and glutaraldehyde were purchased from Polysciences, Inc. (Warrington, PA). Protein A-gold (5

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Abbreviation: PB, protein body.

and 20 nm diameter) and most other reagents were purchased from Sigma.

Plant Material

Rice (*Oryza sativa* L. cv Lamont) plants were grown in an environment-controlled growth chamber. Rice plants were grown in 25-cm plastic pots containing commercial garden soil. The photoperiod was 14-h day (30°C) and 10-h night (25°C), and the plants were fertilized every week with Peter's soluble fertilizer (Allentown, PA) to which we added supplemental ferrous-chelate and micronutrients.

Purification of Glutelins and Prolamine Proteins

Glutelins and prolamines from rice flour were extracted and partially purified essentially as described by Krishnan and Okita (1986). The partially purified glutelins and prolamines were fractionated on preparative SDS-PAGE gels. Gel slices containing the 34- to 37-kD α-glutelin subunits, the 21- to 22-kD β-glutelin subunits, and the 12-kD prolamines were excised. Proteins from the gel slices were electro-eluted, and the purity of the eluted proteins was verified by SDS-PAGE (Laemmli, 1970).

Antibodies to the gel-purified α- and β-glutelin subunits and prolamines were raised in rabbits essentially as described by Krishnan and Okita (1986). The purification of rice seed globulins and antibody production to the purified globulins has been described previously (Krishnan et al., 1992). Western blotting to nitrocellulose filter (0.45-μm pore size) was essentially as described (Burnett, 1981). All of the antibodies were employed at a dilution of 1:200. The immunoreactive polypeptides were identified by incubating the nitrocellulose with 1 μCi of ^{125}I-protein A followed by autoradiography using a Cronex (Dupont, MA) intensifying screen.

Tissue Preparation and Immunocytochemical Labeling

Rice seeds (20 and 25 DAF) were removed from the middle of the panicles and cut into 2- to 3-mm cubes with a razor blade and immediately fixed in 2.5% (v/v) glutaraldehyde buffered at pH 7.2 with 50 mM sodium phosphate. The tissue was processed for immunocytochemical transmission EM essentially as described earlier (Krishnan et al., 1986, 1992).

Morphometric Analysis

Nineteen electron micrographs were randomly selected for analysis. All were taken at a magnification of 1650× and all were printed on 8 × 10 inch photographic paper at a final magnification of 4500×. The images were digitized on a Summagraphics (Fairfield, CT) Bit Pad digitizing tablet. Area measurements of PBs were made using an electronic planimeter (Summagraphics). Computerized analysis was carried out using SIGMA-SCAN version 3.90 scientific measurement software (Jandel Scientific, Sausalito, CA). PBs were identified using immunocytochemical labeling combined with characteristic PB shape and stainability.

RESULTS AND DISCUSSION

We have purified the rice seed α- and β-glutelin subunits and the 12-kD prolamine by preparative SDS-PAGE. These proteins were employed to raise polyclonal antibodies in rabbits. The specificity of the antibodies raised against the rice seed glutelins, prolamines, and globulins was examined by western blot analysis. All three antibodies showed high specificity with no detectable cross-reactivity to any other proteins (Fig. 1). The rice seed glutelins recognized both the subunits as well as the 51-kD polypeptide. This high-molecular-weight protein has been shown to be the precursor for the rice glutelins (Yamagata et al., 1982; Wen and Luthe, 1985; Krishnan and Okita, 1986). The 25-kD globulin and the 12-kD prolamine antibodies exhibited strong reactivity to their respective polypeptides with very little cross-reactivity to other proteins (Fig. 1).

We further examined the specificity of the rice seed protein antibodies by immunocytochemical methods. When thin sections of rice endosperm tissue were examined in the electron microscope, two different types of PBs were identifiable: spherical PBs and irregular-shaped PBs (Fig. 2A). Antibodies raised against the gel-purified 12-kD prolamine antibodies specifically reacted with the spherical PBs as shown by the intense labeling with protein A-gold particles (Fig. 2B). Double-immunogold localization studies indicated that the glutelins and globulins were localized in the irregular-shaped PBs. As reported earlier (Krishnan

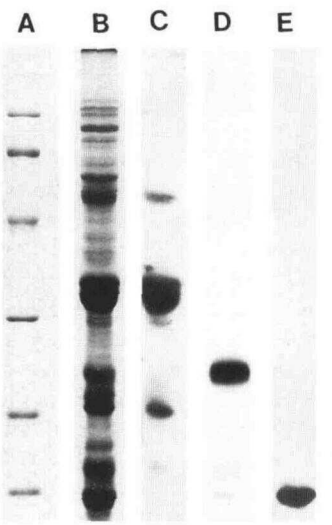

Figure 1. Western blot analysis showing the specificity of rice seed storage protein antibodies. Total protein (50 μg) from mature rice seeds was fractionated on a 12.5% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. Lane C, Incubated with glutelin antibodies; lane D, incubated with 25-kD rice globulin antibodies; lane E, incubated with 12-kD prolamine antibodies. The proteins reacting with the antibodies were identified by incubating the nitrocellulose with ^{125}I-labeled protein A followed by autoradiography. Lanes A and B, Coomassie brilliant blue-stained pattern of molecular weight marker proteins (A, from top to bottom: phosphorylase *b*, 97,400; BSA, 66,200; ovalbumin, 42,699; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400) and rice total seed proteins (B).

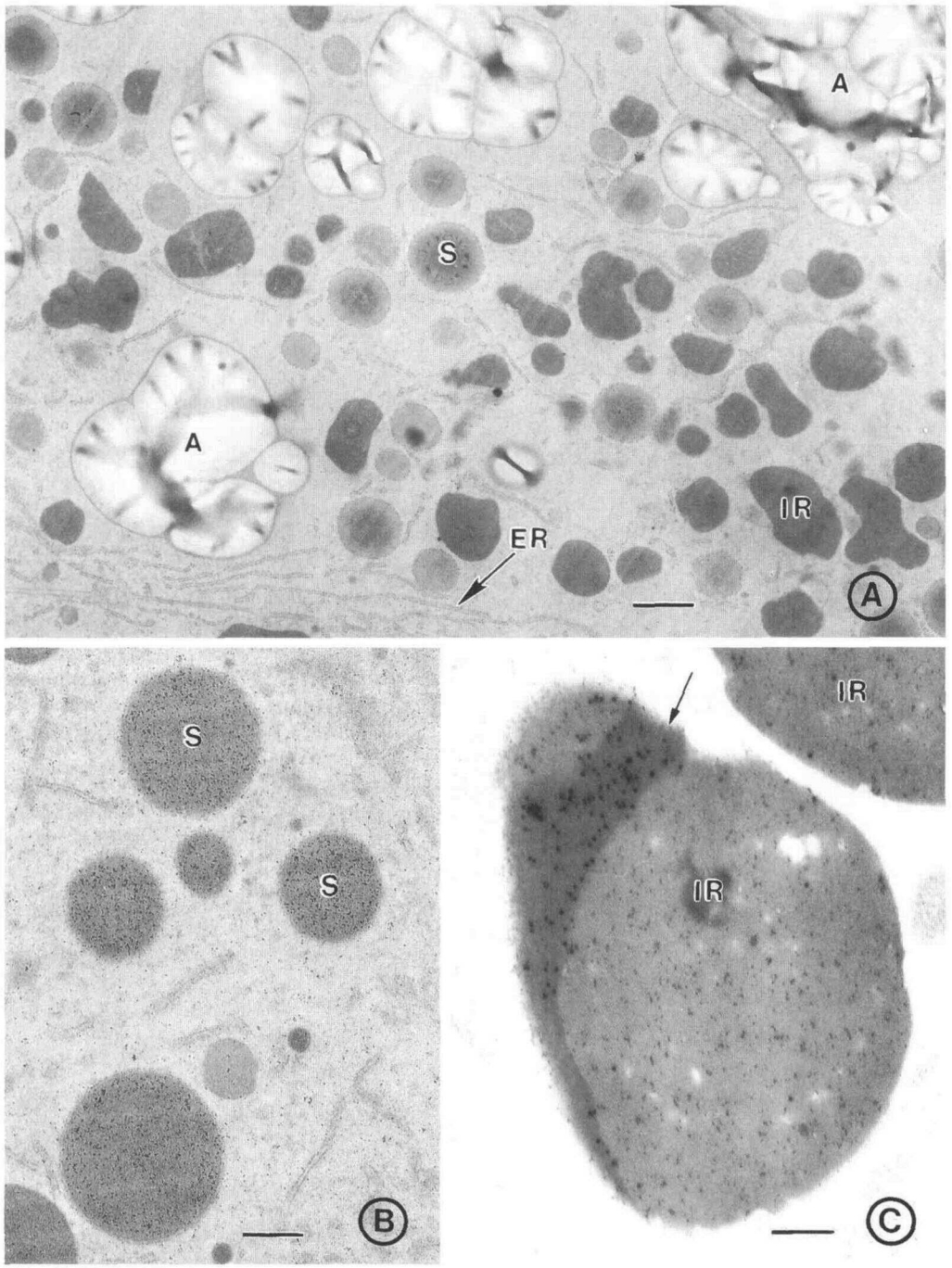

Figure 2. A, Low-magnification view of rice endosperm harvested 20 d after anthesis. The endosperm contains two types of PBs: spherical (S) and irregular shaped (IR). Note that some of the spherical PBs contain a dark-staining central core. The arrow points to RER. A, Amyloplast. Bar = 1.5 μm. B and C, Immunocytochemical localization of prolamines, glutelins, and globulins in rice endosperm cells. Prolamines were localized within the spherical PBs (B), whereas the glutelins and globulins were found in the irregular-shaped PBs (C). The glutelins were tagged with 5-nm gold particles, and the globulins were marked with 20-nm gold particles. The arrow points to the discrete pocket where the globulins are localized. This micrograph was intentionally underexposed to reveal the gold particles, which were hard to see under normal exposure due to the dark-staining nature of the irregular-shaped PBs. Bar in B = 0.7 μm; bar in C = 0.3 μm.

et al., 1992), the globulins were found in discrete pockets within the irregular-shaped PBs (Fig. 2C).

We reasoned, by measuring the relative surface area occupied by the two types of protein bodies, that we could estimate with some accuracy the relative contribution of the different classes of storage proteins to the total protein content of rice seed. Therefore, we employed a morphometric approach to address this issue. Thin sections were obtained from two late-endosperm, developmental stages (20 and 25 DAF) for this study. We identified 2303 PBs that were photographed from 19 different locations of the endosperm. When all 2303 PBs were categorized and counted, it was found that glutelins represented 61.3% (1412) of the total number of PBs and prolamines accounted for the remaining 38.7% (891). Using an electronic planimeter, the area of each of the 2303 PBs was then measured. These data are shown in Table I and can be summarized as follows: based on PB area, the glutelins constituted 65.4% of the total seed protein and the prolamines constituted 34.6% of the total seed protein. To extrapolate these measurements to represent volume, we applied the Delesse principle, which states that the area density (area fraction) of profiles in two-dimensional sections is an unbiased estimator of volume density (volume fraction) in a three-dimensional structure (Weibel, 1973). Using this principle, we determined that in the typical endosperm cell of our rice cultivar, glutelin PBs occupied 7.91% of the volume of the cell, whereas prolamine PBs accounted for 4.18% of the volume.

Table I. *Morphometric analysis of rice seed PBs*

Using computer-assisted electron microscopic morphometry of thin sections of PBs, the relative contribution of glutelin and prolamine was calculated using total number of PBs and relative surface area of 2303 PBs. The field numbers represent low magnification, electron microscopic fields.

Field No.	Total No. of PBs/Field		Total Area of PBs	
	Glutelin	Prolamine	Glutelin	Prolamine
			μm^2	
1	61	25	151.65	50.49
2	57	37	128.14	73.62
3	106	59	241.96	134.85
4	64	37	135.38	72.35
5	60	39	135.90	86.65
6	65	49	164.25	102.65
7	86	48	204.87	104.49
8	73	50	152.81	80.57
9	78	37	168.75	89.02
10	89	47	221.62	79.68
11	69	38	126.41	70.85
12	116	86	183.51	118.14
13	104	103	209.07	153.21
14	71	70	167.36	135.01
15	87	57	406.73	112.29
16	56	39	126.67	94.72
17	52	17	96.73	32.96
18	62	32	126.94	65.48
19	56	21	92.47	56.15
Total	1412	891	3241.22	1713.20
	61.3%	38.7%	65.4%	34.6%

As we reported previously, glutelin PBs often contain significant amounts of globulins (Krishnan et al., 1992). To obtain an approximation of just how much of a glutelin PB was actually globulin, we took advantage of the double-label immunogold electron microscopic localization technique and selected several irregular-shaped PBs in which the globulin segments were clearly delineated, as shown in Figure 2C. We measured the area of these segments and found that, on average, 82% of the PB was glutelin and 18% was globulin. When we applied this ratio to total endosperm protein, we concluded that glutelins actually accounted for just 53% of total protein. This observation adds quantitative reinforcement to our general conclusion that glutelins account for considerably less than 80% of total protein, the value that is frequently cited in the literature.

Several studies on the storage protein composition of rice seeds have identified glutelins as the most abundant component, with prolamines being minor constituents (Tecson et al., 1971; Juliano, 1972; Villareal and Juliano, 1978). These earlier studies were subject to some limitations. The classification of seed proteins on the basis of their solubility properties is variable and is influenced by analytical procedure and the conditions used during the extraction. For example, SDS-PAGE analysis of the four solubility groups of proteins revealed that rice prolamines remain a major contaminant in the glutelin fraction (Krishnan and Okita, 1986). Most of the earlier studies used 70% ethanol to extract prolamines from rice seeds. However, Sugimoto et al. (1986) employed 55% *n*-propanol to extract rice prolamines. This procedure yielded higher recovery of prolamines, and the authors estimated that the prolamines may account for 20 to 30% of the total rice seed proteins. This represents only a small molar excess of glutelins over prolamines. Recently, Li and Okita (1993), using the quantitative immunoblot technique, reported that rice prolamine contribution to the total seed protein content has been underestimated. They calculated that the molar ratio of glutelins to prolamines in rice M-201 was 1.2 in 25-d-old seeds as opposed to 2.6 for the same rice cultivar estimated by differential extraction and HPLC quantification (Huebner et al., 1990). However, our morphometric study indicates that, on a mole basis, there is at least twice as much prolamine as glutelin. Even though there is a discrepancy in the relative molar ratio of glutelin and prolamine between our study and previous biochemical studies, it is nevertheless evident that the relative contribution of rice glutelin to the total protein content of rice seed has been substantially overestimated in previous studies.

The presence of different-shaped PBs in rice endosperm was reported as early as 1967 (Mitsuda et al., 1967). Wu and Chen (1978) recognized round and angular-shaped PBs in rice endosperm. These correspond to the spherical and irregular-shaped PBs that we report in this study. They reported that the spherical PBs contained mostly glutelins. However, several studies have clearly shown that the spherical PBs accumulate mostly prolamines (Tanaka et al., 1980; Yamagata et al., 1982; Krishnan et al., 1986). It is interesting that these authors also reported that the number of spherical PBs far exceeded the number of irregular-

shaped PBs. They calculated a ratio between the spherical and irregular-shaped PBs as 4:1. This is in contrast to our present study, in which the ratio of spherical PBs to irregular-shaped PBs was 1:1.6. This discrepancy could be due to the fact that Wu and Chen's conclusion was based on counting only a few PBs. We, however, counted 2303 PBs representing two developmental stages and from different areas within the endosperm.

In summary, based on ultrastructural morphometry, the storage protein content of the endosperm of rice cv Lamont was approximately 12%. The reader should keep in mind that this value excludes embryo and pericarp and the minor albumin fraction. Of this total protein, approximately 53% was glutelin, 35% was prolamine, and 12% was globulin. On a molar basis, the rice prolamines represent about twice the amount of glutelins. Thus, our study demonstrates that rice glutelin accounts for a significantly lower proportion of the total protein content then has been previously reported. Our results also corroborate the recent biochemical findings, which indicate that the prolamines, along with the glutelins, are the predominant rice seed storage proteins.

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LITERATURE CITED

Burnett WN (1981) Western blotting: electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem **112:** 195–203

Houston DF, Iwasaki T, Mohammad A, Chen L (1968) Radial distribution of protein by solubility classes in the milled rice kernel. J Agric Food Chem **5:** 720–724

Huebner FR, Bietz JA, Juliano BO (1990) Rice cultivar identification by high-performance liquid chromatography of endosperm proteins. Cereal Chem **67:** 129–135

Juliano BO (1972) The rice caryopsis and its composition. *In* DF Houston, ed, Rice: Chemistry and Technology. American Association of Cereal Chemists, St. Paul, MN, pp 16–74

Kim WT, Okita TW (1988) Structure, expression, and heterogeneity of the rice seed prolamines. Plant Physiol **88:** 649–655

Krishnan HB, Franceschi VR, Okita TW (1986) Immunochemical studies on the role of the Golgi complex in protein body formation in rice seeds. Planta **169:** 471–480

Krishnan HB, Okita TW (1986) Structural relationships among rice glutelin polypeptides. Plant Physiol **81:** 748–753

Krishnan HB, White JA, Pueppke SG (1992) Characterization and localization of rice (*Oryza sativa* L.) seed globulins. Plant Sci **81:** 1–11

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Li X, Okita TW (1993) Accumulation of prolamines and glutelins during rice seed development: a quantitative evaluation. Plant Cell Physiol **34:** 385–390

Luthe DS (1983) Storage protein accumulation in developing rice (*Oryza sativa* L.) seeds. Plant Sci Lett **32:** 147–158

Mandac BE, Juliano BO (1978) Properties of prolamin in mature and developing rice grain. Phytochemistry **17:** 611–614

Masumura T, Hibino T, Kidzu K, Mitsukawa N, Tanaka K, Fujii S (1990) Cloning and characterization of a cDNA encoding a rice 13 kDa prolamin. Mol Gen Genet **221:** 1–7

Mitsuda H, Yasumoto K, Murakami K, Kusano T, Kishida H (1967) Studies on the proteinaceous subcellular particles in rice endosperm: electron microscopy and isolation. Agric Biol Chem **31:** 293–296

Ogawa M, Kumamaru T, Satoh H, Iwata N, Omura T, Kasai Z, Tanaka K (1987) Purification of protein body-I of rice seed and its polypeptide composition. Plant Cell Physiol **28:** 1517–1527

Shotwell MA, Larkins BA (1989) The molecular biology and biochemistry of seed storage proteins. *In* A Marcus, ed, The Biochemistry of Plants, Vol 15: A Comprehensive Treatise. Academic Press, San Diego, CA, pp 297–345

Sugimoto T, Tanaka K, Kasai Z (1986) Improved extraction of rice prolamin. Agric Biol Chem **50:** 2409–2411

Tanaka K, Sugimoto T, Ogawa M, Kasai Z (1980) Isolation and characterization of two types of protein bodies in rice endosperm. Agric Biol Chem **44:** 1633–1639

Tecson EM, Esmama BV, Lontok LP, Juliano BO (1971) Studies on the extraction and composition of rice endosperm glutelin and prolamin. Cereal Chem **48:** 169–181

Villareal RM, Juliano BO (1978) Properties of glutelin from mature and developing rice grain. Phytochemistry **17:** 177–182

Weibel ER (1973) Stereological techniques for electron microscopic morphometry. *In* MA Hayat, ed, Principles and Techniques of Electron Microscopy, Vol 3. Van Nostrand Reinhold, New York, pp 237–296

Wen TN, Luthe DS (1985) Biochemical characterization of rice glutelin. Plant Physiol **78:** 172–177

Wu HK, Chen JT (1978) Protein bodies in developing rice grain. Proc Natl Sci Council Repub China **3:** 281–292

Yamagata H, Sugimoto T, Tanaka K, Kasai Z (1982) Biosynthesis of storage proteins in developing rice seeds. Plant Physiol **70:** 1094–1100