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Genomic Cloning of 18 kDa Oleosin and Detection of Triacylglycerols and Oleosin Isoforms in Maturing Rice and Postgerminative Seedlings[1]

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Oleosins are hydrophobic proteins localized abundantly in the oil bodies of plant seeds. Two distinct oleosin isoforms of molecular masses 18 and 16 kDa are present in rice oil bodies. These isoforms were found in similar ratio in rice embryos and aleurone layers. To survey potential DNA sequences involved in the activation of oleosin genes, a genomic clone of rice 18 kDa oleosin was sequenced, and its 5′-flanking region was compared with that of the known rice 16 kDa oleosin gene. Corresponding mRNAs of the two rice oleosin isoforms appeared seven days after pollination and vanished in mature seeds. Triacylglycerols and oleosins were accumulated concomitantly in maturing rice reeds in accord with the active assembly of oil bodies, and partly mobilized in postgerminative seedlings. Approximately 60% of the stored triacylglycerols in rice were not utilized: while the majority of oil bodies in embryos were mobilized in five days after imbibition, those in aleurone layers remained intact in postgerminative seedlings.

Key words: aleurone layer, embryo, oil body, oleosin, rice.

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Plant store lipids in intracellular particles as energy sources for a forthcoming active period of metabolism. These lipid particles can be found in seeds (*1, 2*) and pollens (*3*) of angiosperms as well as in the tissues of more primitive plants, such as the megagametophytes of gymnosperms (*4*) and the spores of ferns (*5*). Similar structural organization is also present in mammalian lipoproteins, the vesicles for transportation of TAGs and cholesterol esters in the blood system (*6*).

The stored lipids, triacylglycerols (TAG), in seeds are confined to discrete spherical organelles called oil bodies (*7*). An oil body is 0.5 to 2.5 μm in diameter and contains a TAG matrix surrounded by a monolayer of phospholipids and abundant proteins termed oleosins (*8*). Oleosins are hydrophobic proteins of molecular mass 15 to 26 kDa, depending on the isoforms and plant species in which they occur (*9*). There are at least two isoform classes of oleosins present in seed oil bodies (*10, 11*). The physiological significance of the presence of these two isoforms is still unknown. Oil bodies remain as individual small organelles even after a long period of storage in plant seeds. This stability is a consequence of the steric hindrance and electronegative repulsion provided by oleosins on the surface of oil bodies (*12, 13*).

In rice seeds, oil bodies are present in both embryos and aleurone layers (*14*). Whether the oil bodies in these two tissues are assembled with the same constituents has not been clarified. Two oleosin isoforms of molecular masses 18 and 16 kDa are present in rice oil bodies (*15*). It is not clear how these two oleosin isoforms are localized in the oil bodies of rice embryos and aleurone layers. Whether the two oleosin genes are regulated simultaneously is not known. Neither is it known if the two oleosin isoforms are accumulated concurrently with TAGs during the maturation of rice seeds. In the germinating seeds and postgerminative seedlings, oil bodies are supposed to be degraded for energy supply. However, the priority and efficiency of mobilization of the oil bodies in rice embryos and in aleurone layers have not been studied.

In this study, we examined constituents and oleosin distribution of the oil bodies purified from rice embryos and aleurone layers, respectively. A genomic clone of rice 18 kDa oleosin was sequenced. Several conserved DNA sequences of potential regulation were identified in the 5′-flanking regions of the 18 kDa oleosin gene and that of the known 16 kDa oleosin gene (*16*). Northern hybridization and Western detection revealed the concomitant expression of the two oleosin mRNAs and the concurrent accumulation of the two oleosin isoforms during seed formation, respectively. Interestingly, we found that oil bodies in rice embryos were almost completely mobilized while those in aleurone layers remained intact in postgerminative seedlings.

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Abbreviations: TAGs, triacylglycerols; TLC, thin layer chromatography.

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MATERIALS AND METHODS

Plant Materials—Mature rice (*Oryza sativa* L., Japonica TNG67) was a gift from the Taiwan Agricultural Research Institute. Mature seeds were dehulled and embryos were dissected from endosperms (including the aleurone layers) at 4°C. Embryos and endosperms were separately subjected to oil body preparation immediately.

Preparation of Oil Bodies—Oil bodies extracted from rice were subjected to further purification including two-layer flotation by centrifugation, detergent washing, ionic elution, treatment with a chaotropic agent, and integrity testing with hexane (*17*).

Analyses of TAG Content in TLC—Oil bodies extracted from various stages of maturing seeds were assayed for TAG content. Each sample of 50 μl was extracted with 150 μl of chloroform/methanol (2:1, v/v). After centrifugation, the lower chloroform fraction was collected for the analysis of TAG content. The extract was spotted onto a TLC (thin layer chromatography) plate coated with silica gel. The TLC plate was developed in a solvent system containing hexane:diethyl ether:acetic acid (80:20:2, v/v/v) (*18*). After development and drying, TAG was visualized with iodine.

Analysis of Oleosin Isoforms on SDS-PAGE—The proteins in the oil bodies were resolved on SDS-PAGE (*19*). The sample was mixed with an equal volume of 2×sample buffer according to the suggestion in the Bio-Rad instruction manual, and the mixture was boiled for 5 min. The electrophoresis system consisted of separating and stacking gels containing 12.5 and 4.75% polyacrylamide, respectively. After electrophoresis, the separating gel was stained with Coomassie Blue R-250, then destained.

Western Blotting—Chicken antibodies against rice 18 and 16 kDa oleosins were individually raised and purified according to the protocols described in Chuang *et al.* (*15*). The proteins in the SDS-PAGE gel were transferred to a nitrocellulose membrane for 2 h at 0.25 A in a Bio-Rad Trans-Blot system. The transfer buffer comprised 25 mM Tris, 192 mM glycine (pH 8.3), and 20% methanol. The membrane was blocked with 3% gelatin in Tris-buffered-saline (TBS) containing 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl for 30 min. It was then incubated with the antibodies or pre-immune antibodies (control) diluted with TBS containing 1% gelatin at room temperature for 2 h. The membrane was rinsed with distilled water, then washed twice (10 min each) with TBS containing 0.05% Tween-20 before the addition of the peroxidase-conjugated goat anti-chicken IgG in TBS containing 1% gelatin. After 1 h of incubation, the membrane was briefly rinsed in a large volume of water, then washed twice (10 min each) in TBS containing 0.05% Tween-20. It was then incubated with 4-chloro-1-naphthol containing H_2O_2 for color development (*20*).

Genomic DNA Cloning and Sequencing—A rice genomic library constructed with DNA of *O. sativa* L. (indica, c.v. IR36) was purchased from Clontech. For screening the genomic clone, the library was plated and plaques in each plate were lifted onto nylon membranes (BRL), denatured, neutralized according to the methods described in Sambrook *et al.* (*21*), then fixed by UV Stratalinker (Stratagene). The membranes were prehybridized in a buffer containing 100 μg/ml denatured salmon sperm DNA, 3× Denhardt's solution, 0.5% SDS, 6×SSC, and 50% formamide for 2 h at 42°C, then hybridized in the same prehybridization buffer containing a ^{32}P-labeled probe of 897 nucleotides including the coding sequence of the rice oleosin 18 kDa cDNA clone (Genbank U43931) for 16 h at 42°C. Hybridized membranes were washed twice in 1×SSC, 0.1% SDS at room temperature for 15 min, then exposed to X-ray films. Positive plaques containing the genomic DNA fragment of interest were isolated and the DNA inserts were verified by Southern assay.

The inserts of the genomic DNA were excised *in vivo* into pBK-CMV phagemid, and clones with various lengths of deletion in both directions were obtained for sequencing analyses. Dideoxynucleotide DNA sequencing was carried out using *Taq* DNA polymerase on an automated DNA sequencer (Applied Biosystems Model 373A) according to the manufacturer's instructions.

Isolation of RNA and Northern Blot Analysis—Total RNA from rice embryos in different stages of maturity was extracted according to the method of Sambrook *et al.* (*21*). The isolated RNA was resolved in a 1.2% agarose/formaldehyde gel, blotted onto Zeta-probe blotting membrane (Bio-Rad) and hybridized with the ^{32}P-labeled cDNA probes containing coding sequences of rice 18 or 16 kDa oleosins, respectively (*22*).

Sampling Different Stages of Maturing Rice—Rice plants were grown in an experimental field in Chung-Hsing University, Taiwan. The growing period was from early October to late December in 1996. Flowers were tagged for sampling different stages of developing seeds. Three thousand seeds of each stage were harvested and subjected to preparation of oil bodies for subsequent analyses.

Sampling Different Stages of Postgerminative Seedlings—Mature rice was spread in ten trays for germination at 27°C. One thousand seeds were harvested daily from one of the trays for ten days. The seedlings were harvested and subjected to preparation of oil bodies for subsequent analyses.

Quantitation of TAG Content—An oil body sample of 50 μl was extracted with 150 μl of diethyl ether. After centrifugation, the upper ether fraction was subjected to quantitation of TAG content. The TAG content was quantitated by measuring acyl ester bonds using a serial dilution of known trilinolein concentrations to calibrate a linear standard equation according to the method developed by Dittmer and Wells (*23*).

Electron Microscopy of Oil Bodies—A mature seed or postgerminative seedling of rice and its dissected embryo were separately fixed in 2.5% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.5, overnight. After several rinses with the buffer, each sample was postfixed in 2% OsO_4 in the buffer for 2 h. Dehydration was carried out in a graded ethanol series, and the sample was embedded in LR-White resin. Sections of 75 nm were stained with uranyl acetate and lead citrate, and observed in a Hitach H-300 electron microscope.

RESULTS

Both Oleosin Isoforms Present in Rice Embryos and Aleurone Layers—In mature rice seeds, oil bodies were found in both embryos and aleurone layers. To investigate

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the localization of two oleosin isoforms, oil bodies were purified from rice embryos and aleurone layers, respectively. Proteins extracted from the respective oil bodies were resolved on SDS-PAGE (Fig. 1). The result indicated that both rice oleosin isoforms were present in embryos and aleurone layers in a similar ratio. Oil bodies purified from both embryos and aleurone layers were mainly composed of TAGs. Based on the analysis of acyl ester bonds, embryos contained 34% of the total TAGs in rice seeds, and thus possessed less oil content than aleurone layers.

Conserved Sequences Found in the Promoters of Rice Oleosin Genes—To survey potential DNA sequences involved in the activation of oleosin genes, a genomic clone of rice 18 kDa oleosin was sequenced, and its 5′-flanking region was compared with that of the known rice 16 kDa oleosin gene. Several conserved sequences were found between these two promoter regions of oleosins (Fig. 2). RY repeat (CATGCANG), a potential regulatory element of cereal storage protein genes (*24*), was present between −150 and −300 bp in both rice oleosin genes. A tetranucleotide sequence, CATC, which has been found in the 5′-flanking regions of many cereal storage protein genes (*25*), was repeatedly present in both rice oleosin genes. Three CATC segments were located between −800 and −1100 bp of these two oleosin genes, while five additional copies were found within 550 bp upstream of the coding region of the 18 kDa oleosin gene. In addition to the above common regulatory elements of storage protein genes, three conserved sequences of 8-10 bp located at similar positions (approximately −150, −360, and −480 bp) were found within 500 bp upstream of the coding regions in both rice oleosin genes.

Detection of mRNAs of Oleosin Isoforms in Maturing Rice—To investigate the expression of oleosin genes in the maturing oil bodies, RNA was extracted from rice embryos at different stages of maturity and subjected to Northern blot analysis using probes derived from cDNA clones of rice 18 and 16 kDa oleosins (Fig. 3). The results revealed that mRNAs of the two rice oleosin isoforms were initially

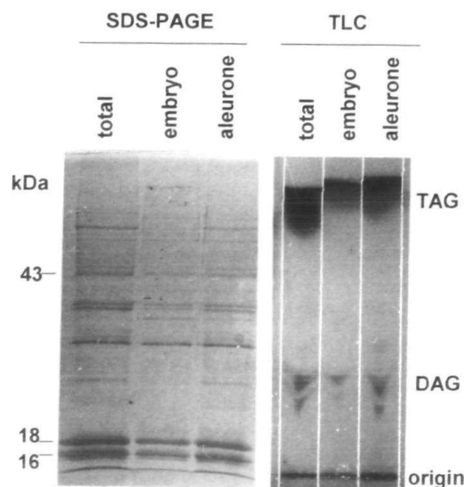

Fig. 1. **Proteins and lipids of oil bodies extracted from rice embryos and aleurone layers.** (Left) Proteins extracted from whole seeds, embryos, and endosperms including aleurone layers were separately resolved by 12.5% SDS-PAGE with loading samples adjusted to represent amounts derived from equal quantities of the seed extract. Labels on the right indicate the molecular masses of rice oleosins (18 and 16 kDa) and a 43 kDa marker protein, ovalbumin. (Right) Lipids extracted from the same samples were resolved by TLC with a solvent system of hexane : diethyl ether: acetic acid (80: 20:2, v/v/v). The positions of TAG (triacylglycerols) and DAG (diacylglycerols) are indicated in the right margin.

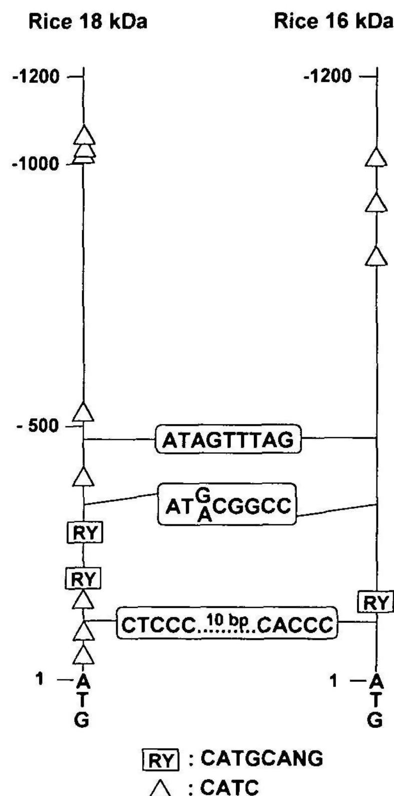

Fig. 2. **Comparison of the 5′-flanking regions of rice 18 and 16 kDa oleosin genes.** Three conserved sequences located at similar positions in the 5′-flanking regions of rice 18 and 16 kDa oleosin genes are boxed in the middle. Two potential regulatory elements of cereal storage protein genes, RY repeat and CATC tetranucleotide, are indicated in various positions in which they occur. The numbers represent bp upstream of the coding regions of the two oleosin genes.

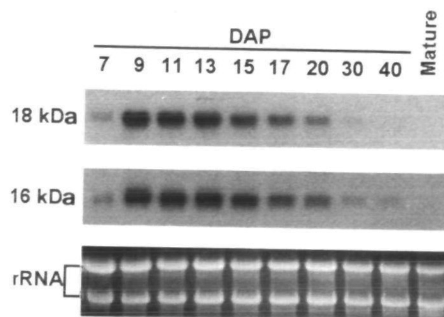

Fig. 3. **Northern hybridization of the two oleosin mRNAs in seed formation.** Total RNAs isolated from different stages of seed formation were resolved in a 1.2% agarose/formaldehyde gel, blotted onto Zeta-probe blotting membrane and hybridized with the ^{32}P-labeled cDNA probes containing coding sequences of rice 18 or 16 kDa oleosins. The loading samples were adjusted to represent amounts derived from equal quantities of the seed extract. The ethidium bromide-stained agarose gel used to prepare the blot is shown at the bottom. DAP represents days after pollination.

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expressed seven days after pollination, highly accumulated in the following week, gradually diminished in the successive stages, and completely degraded in mature seeds.

Concomitant Accumulation of TAGs and Oleosins in Maturing Rice—Maturing rice seeds started to accumulate TAGs approximately one week after pollination and completed 90% of TAG synthesis in another two weeks (Fig. 4). Both rice oleosin isoforms (18 and 16 kDa) were initially accumulated nine days after pollination (two days later than the accumulation of TAGs). The accumulation of the two oleosin isoforms was further detected by immunoassay using antibodies against each isoform. The expression of 16 kDa oleosin appeared to be slightly stronger or earlier than that of 18 kDa oleosin in the early stages of oil body formation (one to two weeks after pollination). However, a similar abundance of the two oleosin isoforms was found in the oil bodies of mature seeds. Basically, TAGs and oleosins were concomitantly accumulated in maturing rice seeds in accord with the active assembly of oil bodies.

Mobilization of TAGs and Oleosins in Postgerminative Seedlings—Stored TAGs were partly mobilized in the postgerminative seedlings of rice. Based on the analysis of acyl ester bonds, 38% of the seed TAGs were mobilized concurrently with the two oleosin isoforms within five days after imbibition (Fig. 5). Hereafter, the remaining 62% of TAGs and oleosin isoforms remained largely unutilized in the following five days. The mobilization of the two oleosin isoforms was further detected on immunoassaying using antibodies against 18 and 16 kDa oleosins, respectively.

Drastic Difference between Utilization of Oil Bodies in Rice Embryos and Alurone Layers in Postgerminative Seedlings—In the postgerminative seedlings of rice, the oil bodies in embryos were nearly exhausted within five days after imbibition, while those in the aleurone layers remained unutilized as observed under an electron microscope (Fig. 6). Growing rice seedlings in the dark for ten days did not provoke the mobilization of oil bodies in aleurone layers (data not shown). No obvious embryo could

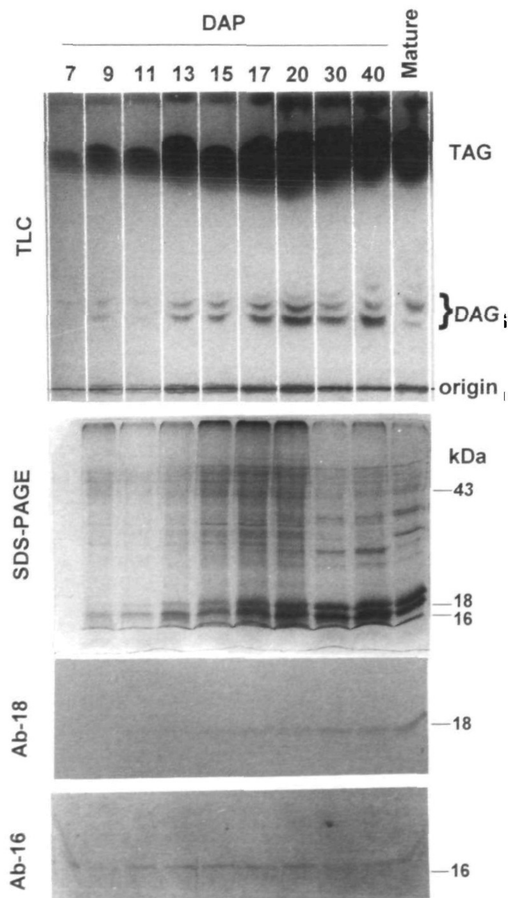

Fig. 4. **Analyses of TAGs and oleosin isoforms in seed formation.** TAGs and rice oleosin isoforms extracted from different stages of seed formation were resolved by TLC and SDS-PAGE, respectively. The conditions used for TLC and SDS-PAGE, and the labels on the right are the same as those in Fig. 1. Duplicate SDS-PAGE gels were transferred to two pieces of nitrocellulose membrane and subjected to immuno-assays using antibodies raised against rice 18 kDa (Ab-18) and 16 kDa (Ab-16) oleosins. DAP represents days after pollination.

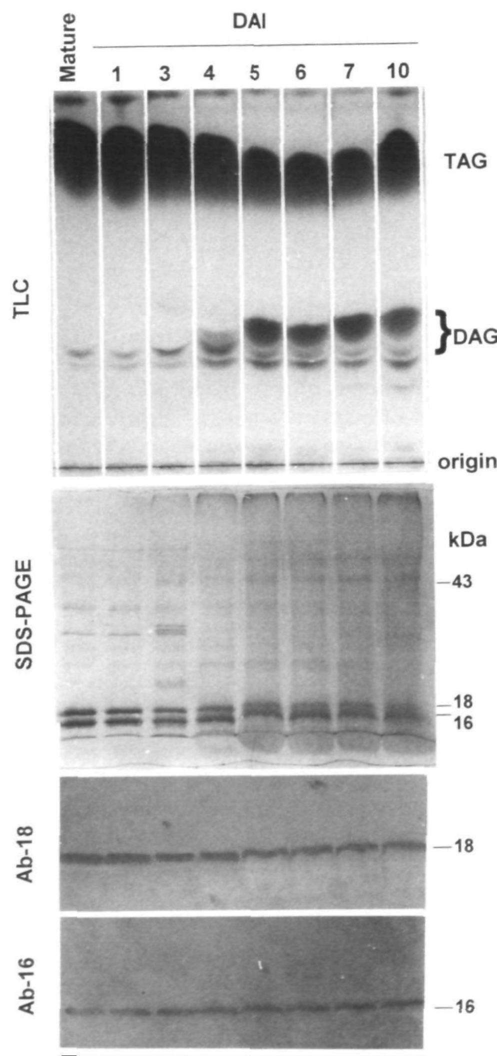

Fig. 5. **Analyses of TAGs and oleosin isoforms in seed germination.** TAGs and rice oleosin isoforms extracted from different stages of seed germination and postgerminative growth were resolved by TLC and SDS-PAGE, respectively. The conditions used for TLC and SDS-PAGE, and the labels on the right are the same as those in Fig. 1. Duplicate SDS-PAGE gels were transferred to two pieces of nitrocellulose membrane and subjected to immuno-assays using antibodies raised against rice 18 and 16 kDa oleosins. DAI represents days after imbibition.

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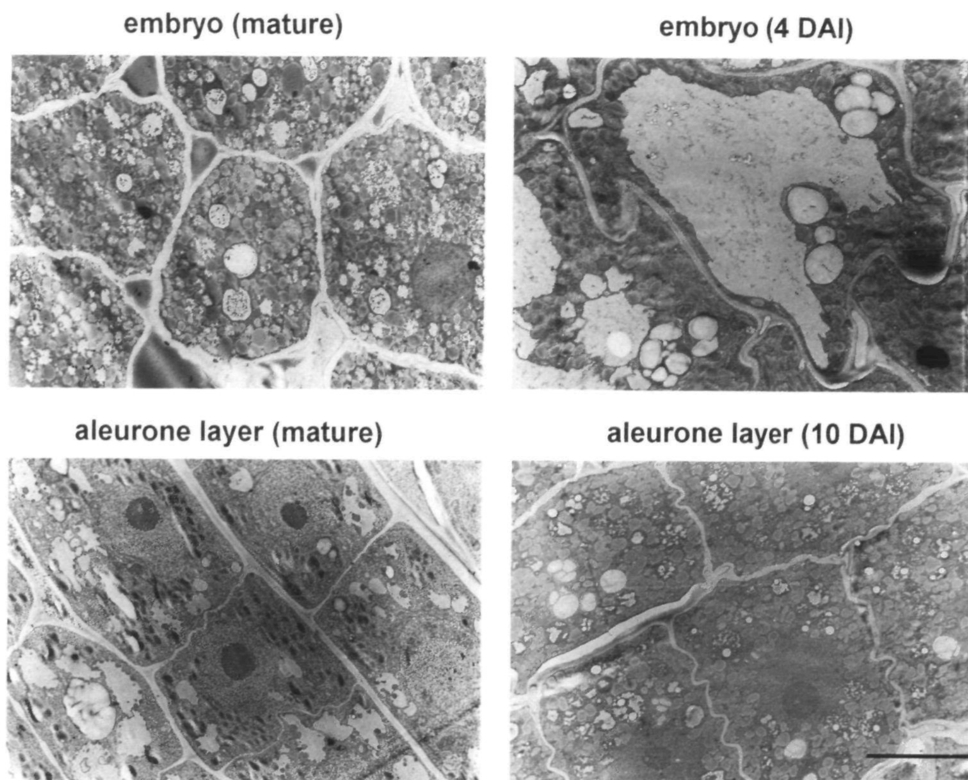

Fig. 6. **Electron microscopy of the rice embryo and the aleurone layer of a mature seed and a postgerminative seedling.** Samples of a mature rice seed and a postgerminative seedling were fixed in 2.5% glutaraldehyde and postfixed in 2% OsO_4. The embryo and aleurone layer of each sample were sectioned at 75 nm thickness and photographed under an electron microscope. The most abundant gray spherical particles of 0.5-1 μm in diameter are oil bodies. All photos are of the same magnification. Bars represent 5 μm. DAI represents days after imbibition.

be dissected from the seedlings five days after imbibition, whereas the oil bodies in aleurone layers remained undigested in the following five more days in our observation. This observation was in agreement with the consumption of 38% of TAGs in the postgerminative seedlings. It seems that the remaining 62% of TAGs and oleosin isoforms were predominantly located in the aleurone layers.

DISCUSSION

Three conserved sequences as well as two potential regulatory elements of cereal storage protein genes, RY repeat and CATC tetranucleotide, were found in the 5′-flanking regions of rice oleosin 18 and 16 kDa genes. Therefore, it was predicted that the two oleosin genes were regulated simultaneously. Northern hybridization of the mRNA and immuno-detection of the oleosin isoforms extracted from different stages of seed maturation were in agreement with the above prediction. However, the two oleosin isoforms were accumulated at slightly different rates during seed maturation. Presumably, oil bodies assembled at different stages of maturity comprised various combinations of the two oleosin isoforms. Whether the variation in oleosin composition of oil bodies in rice seeds is associated with any biological function remains to be studied.

TAGs and the two oleosin isoforms were accumulated concomitantly during the formation of rice seeds, and mobilized concurrently after germination (Figs. 4 and 5). Our observation was in agreement with the previous studies on the concomitant accumulation of TAGs and oleosins in the developing seeds of maize (*11*), soybean (*26*), rapeseed (*8*), sunflower (*27*), and sesame (*28*). This concomitant accumulation of TAGs and oleosins in all the species examined may imply the unique and essential stabilization of oleosins on the surface of oil bodies.

It is well-documented that oil bodies are present in rice embryos and aleurone layers (*14*). In this study, we found both oleosin isoforms present in a similar ratio in embryos and aleurone layers (Fig. 1). Meanwhile, oil bodies of similar diameter (0.5-1 μm) were found in these two tissues (Fig. 6), being mainly composed of TAGs (Fig. 1). In light of the above analyses and observation, we found no difference between the oil bodies in embryos and those in aleurone layers in the size and composition of the organelles. However, oil bodies in rice embryos were almost completely mobilized, while those in aleurone layers remained unutilized in postgerminative seedlings. It remains to be seen if oil bodies in aleurone layers perform biological function(s) other than energy storage.

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