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Comparative Ultrastructure of Ilpumbyeo, a High-Quality Japonica Rice, and Its Mutant, Suweon 464: Scanning and Transmission Electron Microscopy Studies

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A new rice mutant Suweon 464 (S-464), which has extreme contrast in cooking quality and physicochemical properties from those of its mother variety Ilpumbyeo (IP), revealed striking differences in ultrastructure in in situ, fractured whole grain, and isolated starch preparation. In scanning electron microscopy (SEM), compound starch granules (CSG) in whole grains of IP were readily split during fracturing, whereas those in S-464 were structurally intact and were enclosed within a sac-like structure tolerant of fracturing. In isolated preparation, IP starch consisted entirely of individual starch granules, whereas S-464 starch consisted of mostly large CSG enclosed within the sac, preventing the release of the individuals. In transmission electron microscopy (TEM), S-464 starch granules were smaller, solidly "condensed", and highly contrasted, whereas those of IP were larger, loosely "diffused", and less contrasted. The boundaries of amyloplasts and starch granules in S-464 were coated with a thin proteinaceous layer, presumed to be the counterpart of the sac that enclosed the CSG observed in SEM.

KEYWORDS: New rice mutant; ultrastructure; SEM; TEM; rice endosperm; compound starch granule; individual starch granule; amyloplast

INTRODUCTION

As in many other Asian countries, cultivated rice (*Oryza sativa* L.) is the major agricultural crop in Korea, and its cereal has been the principal food for its people since ancient times. Lately, the per capita consumption of rice, however, has steadily decreased since the introduction of fast-food products, which incorporate the use of wheat and maize, in the late 1970s. On the contrary, the hypoallergenic property of rice and its traditional nutritional value received increasing interest, which led Korea's rice-breeding program to develop new rice varieties suitable for incorporation into various processed "healthy" food products.

Recently, a new rice variety, Suweon 464 (S-464), was developed by mutation breeding via *N*-methyl-*N*-nitrosourea (MNU) treatment of Ilpumbyeo (IP), a high-quality japonica rice (*1*), at the National Institute of Crop Science, RDA. Preliminary studies indicated that S-464 had unsuitable properties for traditional cooked rice due primarily to its high amylose content, but it had promising qualities from a nutritional point of view, such as its higher contents of dietary fiber, protein, and lipid (*2*). On the basis of these data, a series of studies has been initiated on the comparison of various aspects of chemical, physicochemical, and ultrastructural characteristics of IP and S-464 to elucidate the factors involved in directing the quality of cooked rice and to exploit the possible utilization of S-464 in other processed food products.

Physicochemical analysis of S-464 revealed that it was unusually high in amylose content, had β-type crystallinity of starch, and had a markedly lower proportion of short chains in the distribution of glucan-chain fraction of debranched starch, all of which would contribute to its unsuitability for ordinary cooked rice (*2*). However, on the basis of its higher contents of fiber, protein, and lipid and unusual properties of starch, the authors suggested that the mutant could be an excellent candidate for other processed food products (*2*).

As a follow-up study of the previous paper (*2*), the ultrastructural analysis of endosperm cells in in situ, fractured whole grain, and isolated starch granules of IP and S-464 was undertaken to investigate how the differences of physicochemical properties and the contents of basic components (starch, protein, lipid, and fiber) existing between the two would be reflected in cells and isolated starch ultrastructure. This paper describes the results of both scanning (SEM) and transmission electron microscopy (TEM) on endosperm cells and starch granules of IP and S-464, which demonstrated striking differ-

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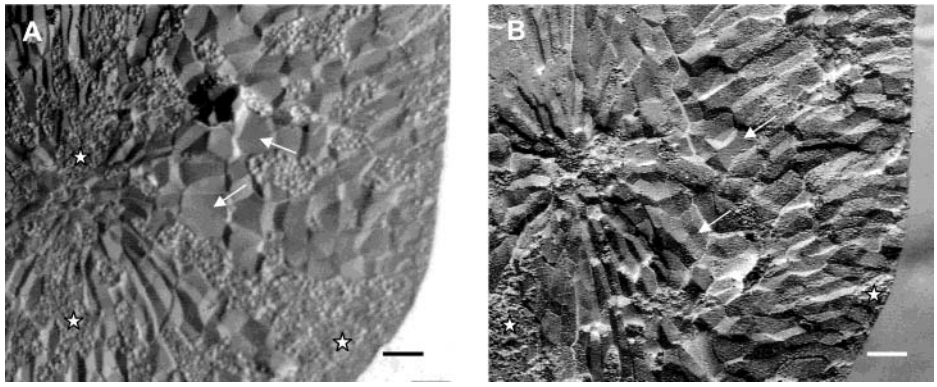

Figure 1. Low magnification view of the transversely fractured midregion of whole grains from IP (**A**) and S-464 (**B**) demonstrating both intercellular (arrows) and intracellular (☆) cleavage planes (scale bar = 100 μm). (**A**) Intercelluarly cleaved cells appeared rectangular in shape, having a smooth and glossy surface, whereas those intracellularly cleaved cells were rough and granular without cellular profile. (**B**) General morphology is similar to that of IP except that the surface of intercellularly cleaved cells were somewhat grainy rather than smooth and glossy.

ences, especially in compound starch granules, amyloplasts, and associated protein structures.

MATERIALS AND METHODS

Plant. Both IP and S-464 rice plants were grown at the experimental field of the National Institute of Crop Science, Suwon, South Korea, during the 2001−2003 growing season. Individual flowers were tagged at anthesis, and grains were harvested at various stages of development. For the ultrastructural study reported in this paper, grains of both IP and S-464 harvested 20 days after flowering (DAF) were used. Rice starch endosperm has been reported to mature ∼20 DAF, and no noticeable differences in endosperm ultrastructure were noted in those harvested at later dates (*3*, *4*).

Specimen Preparation. *SEM*. Two types of specimens were prepared, one for the whole grain of milled rice and the other for the isolated starch granules. For the whole grain, individual grains were fractured in the midregion with a razor blade by applying a slight pressure on the top of the grain. During fracturing, efforts were made to produce no physical contact between the razor blade and the fractured surface of the internal endosperm tissues. Fractured rice grains, with the fractured surface upward, were immediately mounted on the specimen stub and sputter coated with gold before viewing with a JSM 5401 LV (JEOL) scanning electron microscope at 30 kV. Isolated starch granules were prepared according to a method described by Hoover and Sasulski (*5*) involving repeated steeping of rice flour in 0.2% aqueous NaOH solution. The precipitated starch was further treated repeatedly with methanol and then ether to remove lipid (*6*). Starch so obtained was dried under atmosphere, ground into powder, and passed through a 100-mesh sieve. Thoroughly dried starch powder was sprayed on the specimen stubs, sputter coated with gold, and viewed with the same SEM as above.

TEM. Each of the harvested grains of IP and S-464 (20 DAF) was cut with a razor blade in the midregion and one or two 1.0−1.5 mm thick slices, which were semicircular in shape, were used. Each slice was further cut into a number of tissue blocks of ∼1−2 mm^2, each of which included the peripheral and central regions of endosperm. Grains harvested at 20 DAF were already hardened and, therefore, difficult to cut cleanly. Efforts were, however, made each to include both regions. Tissue blocks were then immediately fixed by placing them in a modified Karnovsky's fixative (*7*) containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, for 2 h at room temperature. After two changes of washing, 10 min each, with the same buffer solution, the blocks were postfixed in 1% osmium tetraoxide for 2 h at room temperature. The blocks were en bloc stained overnight in 0.5% aqueous uranyl acetate at 4 °C, dehydrated through an ethanol series from 30 to 100%, and embedded in Spurr's low-viscosity embedding medium (*8*). The quality of thin sections was poor due apparently to the nature of the tissue, the endosperm consisting primarily of starch. The thin sections usually had numerous folds and torn areas as noted by other investigators (*9*). Slightly thicker sections, which had improved quality compared with the thin sections, were cut with a diamond knife and stained with 2% aqueous uranyl acetate for 5 min followed by lead citrate for 2 min. Grids were viewed by Zeiz LEO 906 TEM at 80 kV.

RESULTS AND DISCUSSION

SEM Study of Whole Grain. The transversely fractured surface of the midregion in whole milled grains of both IP and S-464 displayed two types of endosperm cell morphology depending upon the cleavage planes. The cells in areas where the cleavage occurred through intercellular spaces showed their individuality by angled and shaded curvatures and straight lines of cell wall traces (**Figures 1** and **2**). In areas where the cleavage occurred intracellularly, on the other hand, no individual cells can be identified due to the disruption of the cell walls during the process of fracturing. These areas appeared as granular masses consisting of tightly packed starch granules of various sizes and shapes (**Figure 1** and **2**).

Intercellular Cleavage. In IP, the cells cleaved through intercellular spaces, at a low magnification, had evenly flat, smooth, and glossy surfaces with clear cellular profiles (**Figure 1A**). At a higher magnification view, the smooth and glossy surfaces of IP endosperm cells exhibited the presence of tightly packed compound starch granules of various sizes, which were roughly ellipsoidal and/or spherical with smooth angles (**Figure 2A**). Each compound starch granule revealed traces of smaller subunits of about an equal size and similar shape (**Figure 2A**). Apparently, these subunits represent the individual starch granules synthesized in a amyloplast and clustered into a compound starch granule as endosperm cells mature. It has been demonstrated that each compound starch granule consists of a single amyloplast (*10*), which contains 20−60 individual starch granules (*11*, *12*). Smaller granules, occurring often singly, trapped between large compound starch granules may represent smaller compound starch granules or the individuals separated from the latter during the endosperm maturation (**Figure 2A**). All of these granules were embedded in the matrix material made presumably of the remains of degenerating cytoplasmic constituents (cytosol + organelles) retained and storage protein bodies developed during the endosperm maturation (**Figure 2A**). Therefore, no empty spaces between starch granules were present. It is believed that the matrix material filling the spaces between starch granules is reflected in the appearance of the evenly flat, smooth, and glossy surface of IP endosperm cells in intercellular cleavage planes.

In S-464, intercellularly cleaved endosperm cells, at a low magnification, were similar in appearance to those of IP except

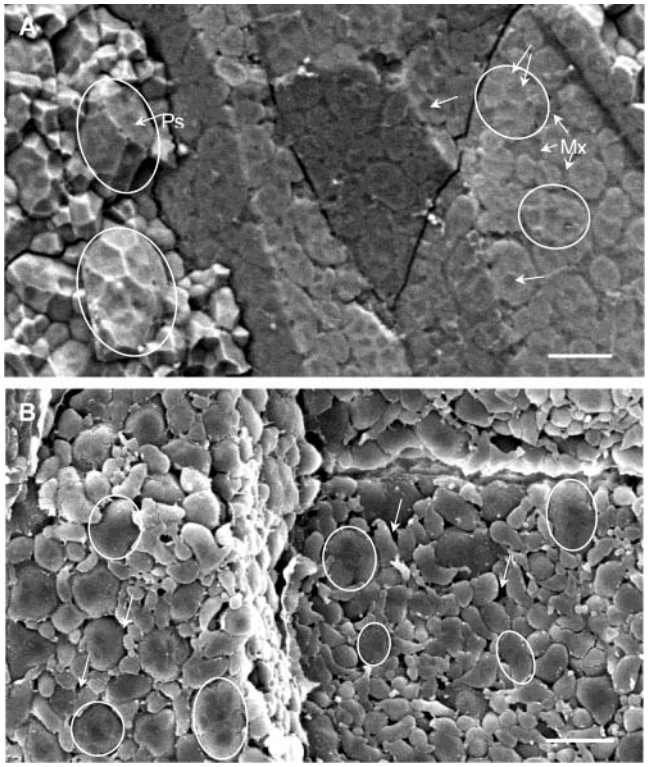

Figure 2. Higher magnification view of intercellularly cleaved endosperm cells in whole grains of IP (**A**) and S-464 (**B**) (scale bar = 10 μm). (**A**) The smooth and glossy surface consists of tightly packed compound starch granules (rings) embedded in matrix material (Mx). The surface of each compound starch granule exhibits the presence of subunits (individual starch granules) by small circular indentations of about the same size (unlabeled arrows). At left, intracellularly cleaved cells exhibiting unsplit (circles) and partially split (Ps) compound starch granules are shown. (**B**) Compound starch granules (rings) of various sizes are not embedded in the matrix material, but each of them is surrounded by air space (arrows) enhancing their contrast and individuality. No structural traces of the presence of the subunits in the surface of compound starch granules are evident. Some of the small granular structures between compound granules may represent protein bodies.

that the flat surfaces were not as smooth as those in IP but were somewhat grainy (**Figure 1B**). At a higher magnification, however, S-464 endosperm cells displayed striking differences from those shown in IP, in that most starch granules, including those large compound starch granules and associated smaller ones, exhibited crisp individual profiles (**Figure 2B**). Unlike those in IP, the S-464 starch granules were surrounded by air spaces instead of the matrix material (**Figure 2B**). Most of these granules had smooth and rounded profiles without sharp angles, producing a pebble-like appearance (**Figure 2B**). Very small, some of which were elongated, granules may represent the protein bodies (PB), such as spherically shaped prolamin PBs and/or irregularly shaped glutelin PBs developed in the cytoplasm of maturing endosperm cells (*3*, *9*). No traces or structural signs of the presence of the subunits (individual starch granules), which occurred in IP, were discernible in the surface of large compound starch granules of S-464 (**Figure 2B**). Instead, randomly oriented hairline cracks were obvious in most of these large compound starch granules (**Figure 2B**), suggesting that they were covered by a coat that is thick enough to mask the traces of individual starch granules and to produce such hairline cracks rather than stripping or splitting under certain physical or mechanical impacts such as fracturing.

Intracellular Cleavage. Intracellular cleavage of IP endosperm produced an uneven and rough surface morphology due to the exposure of unevenly cleaved starch granules of various sizes and shapes (**Figure 3A**). Large unsplit and partially split compound starch granules and small individual starch granules separated from compound granules were clearly demonstrated (**Figure 3A**). All of these starch granules, including those clustered individuals remaining in the compound granules, had sharp angles and edges (**Figure 3A**), which were not conspicuous in intercellular cleavage planes. It is believed that the sharp angles and the edges of the starch granules in the intracellular cleavage planes resulted from the disruption of the matrix material, in which they were embedded, during the fracturing process of the grain. Indeed, these individual starch granules were structurally indistinguishable from those in the isolated starch preparation (compare **Figures 3A** and **4A**). It is obvious that the treatments involved in starch isolation processes would surely have removed the matrix material.

Unlike in IP, the starch granules of S-464 in intracellular cleavage planes showed no noticeable differences from those in intercellular cleavage planes except that they were in cells with ill-defined boundaries (compare **Figures 3B** and **2B**). No partially split compound starch granules exposing internally located clusters of individual granules, which were most common in IP, were present (compare panels **A** and **B** of **Figure 3**). Most of them, including small and large compound starch granules, were highly contrasted and crisp in appearance and had no sharp angles and edges (**Figure 3B**). This suggests that most S-464 starch granules are physically tolerant of the mechanical impact of fracturing, and thus their initial sizes and shapes remained unchanged when endosperm cells were cleaved intracellulally.

SEM Study of Isolated Starch Granules. Because the morphological differences between IP and S-464 in the starch granules occurring in the whole grains were so striking, a study was undertaken to determine whether such differences would persist to isolated and purified forms of starch granules.

In IP, starch granules in the isolated preparations (**Figure 4A**) were similar in size and shape to those of the individual starch granules separated from large compound starch granules shown in intracellular cleavage planes of whole grain. They were polygonal in shape with sharp angles and edges (**Figure 4A**). However, no whole or partially split compound granules, which were most common in intracellularly cleaved whole grains (**Figure 3A**), occurred, indicating that the entire population of compound starch granules was completely dissociated and that individual starch granules accumulated in them as tightly packed clusters were released freely during the isolation processes.

In S-464, isolated starch preparation, unlike in IP, consisted of two types of starch granule populations on the basis of their sizes and shapes; one consists of smaller rounded or slightly angular granules, apparently representing individual starch granules (**Figure 4B**), and the other, the major population of the two, consists of extremely large voluminous bodies surrounded by a coat appearing as sac-like structures (**Figure 4B**). On the basis of their general morphology and relative size comparison to individual starch granules, it is apparent that the large voluminous bodies represent the compound starch granules derived from single amyloplasts. The voluminous bodies are also morphologically indistinguishable from those of compound starch granules occurring in the S-464 endosperm of whole grain (compare **Figures 3B** and **4B**), indicating that they are, indeed, the same entity, the compound starch granules. This indicates that the compound starch granules of S-464 in the endosperm

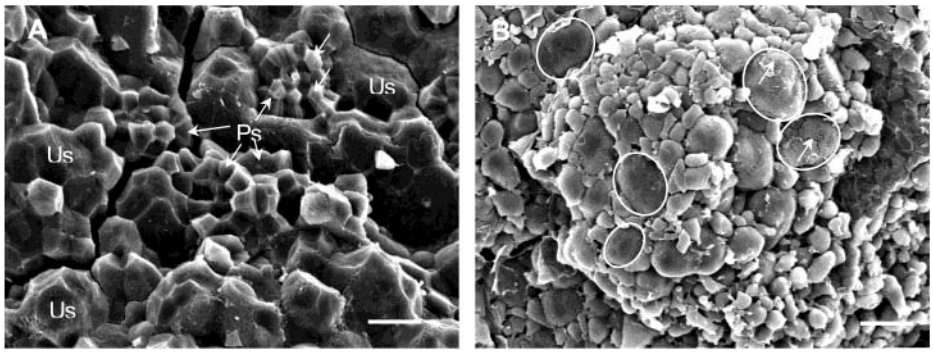

Figure 3. Higher magnification view of intracellularly cleaved endosperm cells in whole grains of IP (**A**) and S-464 (**B**) (scale bar = 10 μm). (**A**) Unsplit (Us) and partially split (Ps) compound starch granules exposing the presence of individual starch granules with sharp angles and edges (arrows) are clearly demonstrated. (**B**) No split compound starch granules are present. Most starch granules (rings) have no sharp angles or edges but appear as rounded sac-like structures often with hair-line fissures (arrows) on the surface. Some of the small bodies between large compound starch granules may represent protein bodies.

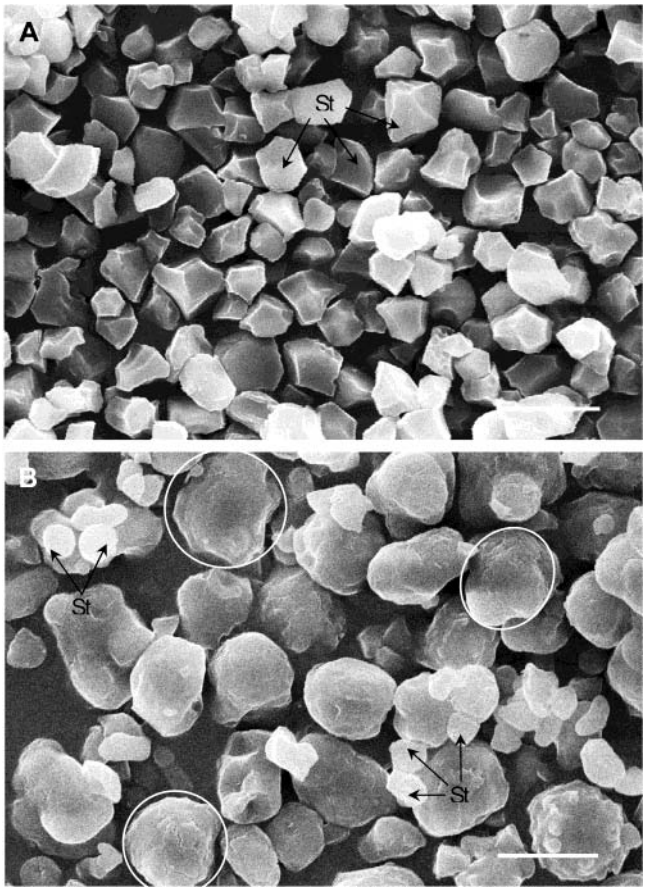

Figure 4. Isolated starch granules from IP (**A**) and S-464 (**B**) (scale bar = 10 μm). (**A**) Entire population consists of individual starch granules (St) of similar sizes that are polygonal in shape with sharp angles and edges. (**B**) Two types of population, one composed of large voluminous compound starch granules (rings) and the other composed of small individual starch granules (St), are shown. All of these granules are rounded in shape with no sharp angles or edges. Individual small granules are much smaller than those in IP.

of whole grains have not been structurally altered by the harsh treatments received during the starch isolation and defatting processes, including grinding and steeping in NaOH, ethanol, and ether solutions, etc. It seems, therefore, that the coat of sac-like structure surrounding each of the compound starch granules in both whole grain and isolated preparation (**Figures 3B** and **4B**) must play a major role in retaining their structural integrity by being mechanically firm and rigid enough to prevent the splitting and subsequent release of individual granules accumulated in them.

In the nature of the coat, it seems that the amyloplast membrane must be involved in the formation, because each compound starch granule is derived from a single amyloplast (*10*−*13*). It is assumed that the coat is a complex structure of amyloplast membrane associated with unidentified material formed during the maturation process of the S-464 starch endosperm. It is hypothesized that the S-464 starch, which has significant differences in physicochemical properties, such as amylose content, X-ray diffraction pattern, and relative crystallinity, from those of IP starch and other components, such as fiber, protein, and lipid (*2*), which are unusually higher in S-464 than those in IP, may interact with amyloplast membrane during endosperm maturation, resulting in the formation of a structural barrier, the coat. The starch granule-associated protein (*12*, *14*) as well as starch lipid or embraced lipid (*15*, *16*) and unusually higher contents of crude fiber and material represented by ash in S-464 (*2*) could be strong candidates for participating in the formation of the coat (see details under TEM Studies). The coat, thus formed, would apparently be able to withstand the chemical as well as physical treatments received during the processes of starch isolation, enabling the compound starch granules to retain their structural integrity. It should be added that among several different rice varieties examined in our laboratory, including japonica, indica, and waxy rices, the structurally undissociated compound starch granules in the isolated preparation of respective rice occurred only in S-464, whereas that of the other rices consisted solely of well separated individual starch granules (*17*) as shown in IP (**Figure 4A**). The capability of retaining structurally intact compound starch granules after the various harsh treatments, therefore, appears to be unique, among other rices, to S-464, and it may be a major determinant in the contribution to the characteristic qualities of its cooked rice, such as poor gelatinization, lower swelling power, and higher hardness (*2*). It is obvious that the compound starch granules enclosed within the coat that can survive through even the isolation processes would limit the entrance of water and subsequent absorption, resulting in the cooked rice qualities of S-464 mentioned.

TEM Studies. The general morphologies of the caryopsis coat of IP and S-464 rice grains harvested at 20 DAF were similar to that of other rices reported previously by others (*18*, *19*), consisting mostly of crushed cell layers of pericarp and seed coat. The present study is concentrated on the observation

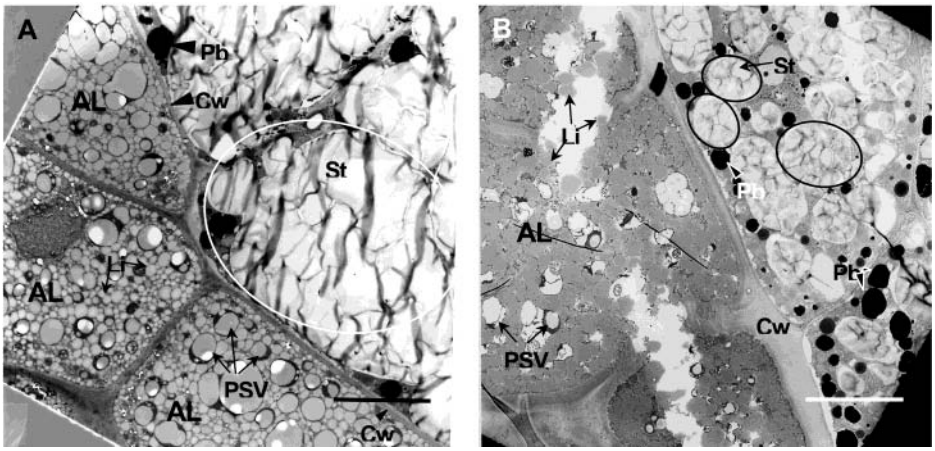

Figure 5. Low magnification view demonstrating aleurone cell layer and subaleurone starch endosperm cells from IP (**A**) and S-464 (**B**) (scale bar = 10 μm). (**A**) Three densely stained aleurone cells (AL) packed primarily with lipid bodies (Li) of various sizes and aleurone protein storage vacuoles (PSV) are shown. PSV are more densely populated than those in aleurone cells of S-464. Large amyloplast (ring) packed with fluffy starch granules (St) in a subaleurone starch endosperm is shown. Darkly stained protein bodies (Pb) are concentrated near the cell wall (Cw). (**B**) Lipid bodies (Li) in aleurone cells were much larger than those in IP, and aleurone protein storage vacuoles (PSV) are sparsely distributed. Cell walls (Cw) between aleurone cell themselves and between aleurone and subaleurone starch endosperm cells appear to be thicker than those in IP. Protein bodies (Pb) occurring not only near the cell wall but also in the cytoplasm around the amyloplast (rings) are shown. The sizes of amyloplasts (rings) as well as individual starch granules (St) within them are much smaller than those in IP.

of starch endosperm cells in which the major constituents of rice, starch, protein, and lipid are synthesized and stored, becoming the major body of the rice grain at maturity. The aleurone, the outermost layer of endosperm tissue, is included in this paper, because conspicuous differences in cell components between IP and S-464 did occur.

Aleurone Layer. In both IP and S-464, the aleurone consisted of two to four layers of cuboidal cells, which have densely packed cytoplasmic components (**Figure 6**). Like aleurone cells in other cereals (*20*), aleurone protein storage vacuoles (*21*), formerly called aleurone grains (*18*, *20*), and lipid bodies of various sizes were the major components of aleurone cells (**Figure 5**). Other usual organelles such as mitochondria, endoplasmic reticulum, and plastids also occurred, but they were few and located at the cell periphery, suggesting that they were functionally not very active (*18*). The nuclei were usually centrally located (**Figure 5A**). One of the major differences between IP and S-464 was that in S-464 the individual lipid bodies were substantially larger in size and the protein storage vacuoles were considerably less in distribution as compared to those in IP (**Figure 5**). It has been well-known that the protein storage vacuoles in cereal aleurone cells represent a type of lysosomes containing hydrolytic enzymes that break down reserves in the starch endosperm when germination is triggered (*21*, *22*). Poor germination rates and slow growth of young seedlings right after germination seen in S-464 (data not shown) as compared to those in IP may be a reflection of sparsely distributed protein storage vacuoles. In addition, the cell walls between aleurone cells themselves and between aleurone cells and subaleurone starch endosperm cells in S-464 appeared to be somewhat thicker than those in IP (**Figure 5B**). It is not certain, however, to what extent these thicker cell walls in S-464 would have contributed to the differences in basic composition and physicochemical properties that exist between IP and S-464, such as higher fiber and hemicellulose contents and poorer solubility and gelatinization shown in S-464 (*2*). The effect should be minimal, if present, because no noticeable differences in the cell wall thickness were detected in most of the starch endosperm cells observed. Furthermore, most aleurone layers, in which cell wall thickness was detected, should have been removed from the milled rice from which compositional and physicochemical data were obtained (*2*).

Starch Endosperm. As previously reported for other rices (*9*, *18*), the starch endosperm of both IP and S-464 was divided into two regions, the subaleurone, the two layers of relatively well preserved cells located just underneath the aleurone layer, and the central region consisting of the rest of the starch endosperm. The subaleurone region is characterized by a large number of densely stained protein bodies (PBs) of both types, the spherical prolamine PBs (PB-I) and the irregularly shaped glutelin PBs (PB-II) (*3*, *9*, *23*), which were concentrated primarily near the cell wall and scattered randomly around amyloplasts (**Figures 6** and **7**). PBs also occurred in the central region but were less numerous than those in the subaleurone region. Regardless of the region, the starch endosperm of both IP and S-464 consisted of cells loaded with starch granules in the amyloplasts, which were morphologically still identifiable at this stage of development (20 DAF) by their general configuration and starch content (**Figures 6** and **7**). Each amyloplast was filled with numerous individual starch granules tightly packed together, which would become a compound starch granule at maturity in the grain. No thylakoid membrane or stroma material was, however, detected in any of the amyloplasts encountered, suggesting that they were fully matured in terms of starch synthesis.

IP. One of the most striking differences between the endosperm cells of IP and those of S-464 was the appearance of starch granules in the amyloplasts. IP starch granules appeared as semicircular bodies of various sizes and shapes, which had smooth and somewhat "stretched" or diffusive morphology (**Figures 6A** and **7A**). Starch granules in each amyloplast were tightly pressed to each other, causing some of them to fuse with adjacent ones and resulting in difficulties to distinguish the individuals (**Figure 6A**). Amyloplasts loaded with these starch granules were also tightly packed in each endosperm cell, occupying most of the volume of the cell (**Figure 6A**). The double membrane of amyloplasts occurring in younger endosperm cells was not discernible, suggesting that it had degenerated or disintegrated during the course of endosperm maturation. The cell wall between endosperm cells retained its

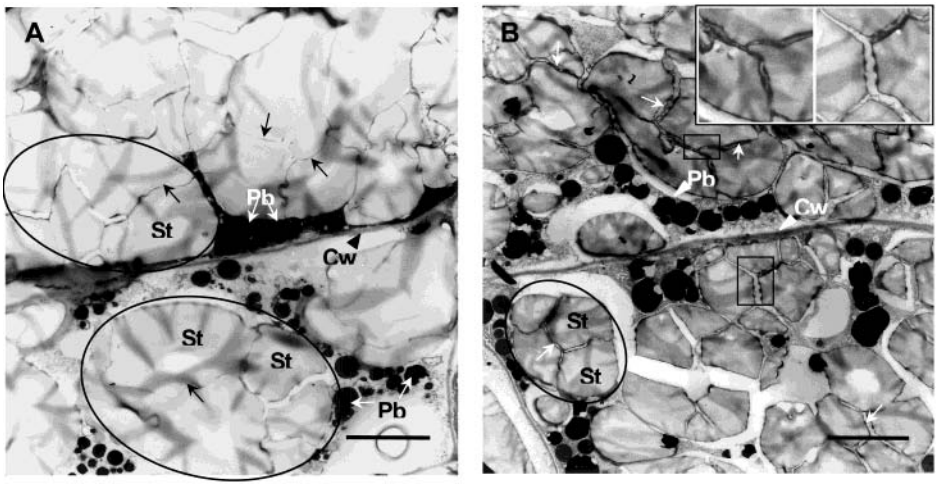

Figure 6. Higher magnification view of two subaleurone starch endosperm cells from IP (**A**) and S-464 (**B**) (scale bar = 5 μm). (**A**) Amyloplasts (rings) and individual starch granules (St) within them are larger than those in S-464. Large fluffy starch granules are tightly packed together, causing some of them to fuse with each other (unlabeled arrows). Protein bodies (Pb) concentrated near the cell wall penetrate between amyloplasts. (**B**) Starch granules (St) are solidly "condensed" in appearance, exhibiting enhanced contrast and individuality by the presence of intergranular spaces (unlabeled arrows) between them. The outer boundary of amyloplasts and individual starch granules are coated with a thin electron-dense layer decorated with a number of crescent-shaped granular material (see insets) that have the same electron density as those of nearby protein bodies (Pb). (Insets) Higher magnification of the square boxes in the main figure showing the structural details of the thin electron-dense layer surrounding the outer boundary of starch granules and amyloplasts.

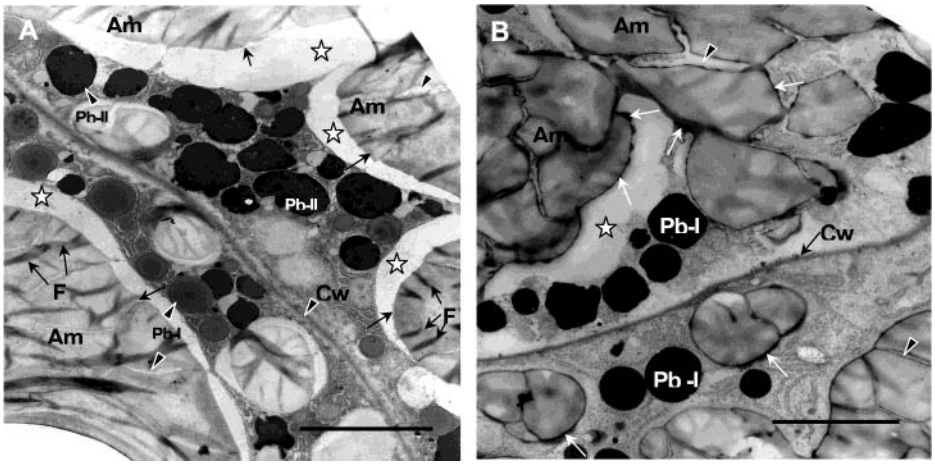

Figure 7. Structural details of the boundaries of amyloplasts and individual starch granules from IP (**A**) and S-464 (**B**) (scale bar = 5 μm). (**A**) The boundaries (unlabeled arrows) of amyloplasts (Am) and intergranular spaces (unlabeled arrowheads) show no particular structural entity. The wide empty spaces (☆) between each of amyloplasts and surrounding cytoplasm are the separation artifacts produced during thin-sectioning of the tissue blocks. Dark stripes (F) running irregularly through starch granules represent folded areas of starch sections. (**B**) Thin electron-dense layer decorated with crescent-shaped granules (unlabeled arrows) surrounding amyloplasts (Am) and intergranular spaces (unlabeled arrowheads) are clearly shown. The electron density of the layer is about the same as those of nearby protein bodies (Pb) in the cytoplasm.

structural entity (**Figures 6A** and **7A**), however. The densely stained PBs, which were more common near the cell wall, often penetrated into the spaces between amyloplasts, helping in distinguishing individual amyloplasts (**Figure 6A**).

S-464. Amyloplasts in S-464 were much smaller in size and less densely packed than those in IP, giving them clearer individual profiles (**Figures 5B**, **6B**, and **7B**). The starch granules in amyloplasts, which were also smaller than those in IP, were more electron-dense and solidly "condensed" in appearance, enhancing their contrast and individuality (**Figures 5B** and **6B**). Unlike those in IP, starch granules were rarely fused with adjacent ones, but were separated discretely, producing intergranular spaces between them (**Figures 6B** and **7B**). It has been generally accepted that starch granules are organized into amorphous and crystalline regions in which the crystalline region is primarily composed of amylopectin, whereas the amylose is interspersed with amylopection in amorphous region (*24*−*26*). Starches that contain amylose and lipids, such as rice starch, form amylose−lipid complexes that are known to reinforce the granule structure (*27*, *28*). It is assumed therefore that the solidly condensed appearance of S-464 starch granules is the reflection of the higher content of amylose in S-464 starch, which would reinforce its granules structurally more tightly than those of IP starch, which appeared somewhat stretched and diffusive.

The outer boundaries of amyloplasts and of individual starch granules within them in S-464 were coated with a thin densely stained layer decorated often with a number of small crescent-shaped granular material that had the same electron density as those of nearby protein bodies (**Figures 6B** and **7B**). This layer may contain some of the structurally uncharacterized proteins and lipids, such as the starch granule-associated protein, the

waxy gene product (*14*, *29*), and starch-associated lipid, the embraced lipid (*15*, *16*). The starch granule-associated protein has been demonstrated to be involved in amylose synthesis and related directly to amylose content (*14*, *30*). High-amylose rice contained more starch granule-associated protein than lower amylose rice (*31*). The starch-associated lipid, which affects some physicochemical properties of rice starch (*15*, *16*), is complexed mainly with amylose (*32*). S-464 starch, which has a greatly higher amylose content than IP starch (*2*), should, therefore, contain much more of this protein and lipid than IP starch. Higher rigidity, lower swelling power, and poorer gelatinization of S-464 rice, compared with those of IP rice, therefore could be attributed to the presence of high amounts of starch granule-associated protein and embraced lipid, which may inhibit the swelling potential of the granules as suggested by Sandhya Rani and Bhattacharya (*31*) and Hamaker et al. (*33*). Ultrastructurally, such high amounts of the protein and lipid and of crude fiber and ash material (*2*) present in S-464 could have been reflected in the electron-dense layers and/or granules as shown in **Figures 6B** and **7B**. It is not certain, however, whether this electron-dense layer corresponds to the coat of the sac-like structures surrounding the compound starch granules observed in SEM studies of whole grain (**Figure 3B**) or of isolated starch preparation (**Figure 4B**). However, because this is the only structural feature that could be accountable to the coat observed in this TEM study, it is suggested that the layer represents the coat, at least in part.

It appears, therefore, that the electron-dense layer associated with S-464 starch granules and the compositional differences of starch itself existing between S-464 and IP rices (*2*) play a major role in determining the characteristic S-464 starch morphology, the smaller, condensed, and highly contrasting appearance.

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