

Rice caryopsis development II: Dynamic changes in the endosperm

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High-Impact Article

Abstract The rice endosperm plays crucial roles in nourishing the embryo during embryogenesis and seed germination. Although previous studies have provided the general information about rice endosperm, a systematic investigation throughout the entire endosperm developmental process is still lacking. In this study, we examined in detail rice endosperm development on a daily basis throughout the 30-day period of post-fertilization development. We observed that coenocytic nuclear division occurred in the first 2 days after pollination (DAP), cellularization occurred between 3 and 5 DAP, differentiation of the aleurone and starchy endosperm occurred between 6 and 9 DAP, and accumulation of storage products occurred concurrently with the aleurone/starchy endosperm differentiation from 6 DAP onwards and was accomplished by 21 DAP. Changes in cytoplasmic membrane permeability, possibly caused by programmed cell death, were observed in the central region of the starchy endosperm at 8 DAP, and expanded to the whole starchy endosperm at

21 DAP when the aleurone is the only living component in the endosperm. Further, we observed that a distinct multi-layered dorsal aleurone formed near the dorsal vascular bundle, while the single- or occasionally two-cell layered aleurone was located in the lateral and ventral positions of endosperm. Our results provide in detail the dynamic changes in mitotic divisions, cellularization, cell differentiation, storage product accumulation, and programmed cell death that occur during rice endosperm development.

Keywords: Caryopsis; differentiation; endosperm; programmed cell death; rice

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INTRODUCTION

The triploid endosperm, which originates from fertilized polar nuclei in the embryo sac, is the major storage tissue in the rice caryopsis (Becraft 2001; Olsen and Becraft 2013). The endosperm functions to nurture the embryo during the early stages of seed development. In cereals that have persistent endosperms, it also functions as a food reserve to nourish the growing seedling during germination (Olsen and Becraft 2013; Zhang and Wang 2015). Moreover, cereal endosperms are the most important global sources of food for humans and livestock, and also provide raw materials for manufactured goods and biofuels (Juliano 1993).

Morphological studies performed in the past have showed that rice endosperm development shares some common characteristics with other cereal crops (Hoshikawa 1967a, 1967b, 1967c, 1967d, 1968; Brown et al. 1996; Becraft 2001; Becraft and Yi 2011; Gu et al. 2001; Wang et al. 2012; Zhang and Wang 2015). The formation of a rice endosperm involves successive stages of coenocytic nuclear division (Brown et al. 1996; Wang et al. 2012), cellularization (Brown et al. 1996; Olsen 2004; Wang et al. 2012), differentiation of an inner starchy endosperm and an outer aleurone layer (Olsen 2004; Becraft and Yi 2011; Wang et al. 2012), and differential accumulation of storage products (Krishnan and Dayanandan 2003; Becraft and Yi 2011). It has been noted that, unlike most other cereals, the rice endosperm does not have distinct transfer cells that are featured with cell wall

ingrowth (Zee 1972; Bosnes et al. 1992; Opanowicz et al. 2011).

Rice endosperm consists of an inner starchy endosperm and an outer aleurone layer (Hoshikawa 1967d, 1967e; Brown et al. 1996; Becraft 2001, 2007; Krishnan and Dayanandan 2003). It has been reported that aleurone and starchy endosperm become distinct from each other on 5 DAP (Hoshikawa 1967d; Krishnan et al. 2001). All cells in the starchy endosperm are dead in mature seeds owing to programmed cell death (PCD), while cells in the aleurone layer remain alive (Olsen 2004; Sabelli and Larkins 2009; Wang et al. 2012; Kobayashi et al. 2013; Domínguez and Cejudo 2014). The aleurone and embryo together account for only about 7% of the weight of the caryopsis, but these tissues contain approximately 80% of the total proteins, 80% of the lipids, 90% of the vitamin B1, and 60% of the iron in the caryopsis. In contrast, the starchy endosperm, as the major energy source of white rice, contains mostly starch and a small amount of proteins (Juliano 1993; Becraft and Yi 2011).

Molecular genetic studies in recent years have established a fundamental understanding of the regulation of rice endosperm development. In particular, some transcription factors expressed in the rice caryopsis are now known to play essential roles in endosperm development. OsMADS29 negatively regulates degeneration of the nucellus and of the nucellar projection tissue; suppressed expression of OsMADS29 resulted in delayed degradation of these tissues

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and caused defective endosperm development (Yang et al. 2012; Yin and Xue 2012). The NF-Y family transcription factor NF-YB1, which is expressed primarily in the dorsal side of the rice aleurone, plays a critical role in regulating the expression of several sucrose transporters, allowing sucrose to be loaded to the developing starchy endosperm (Bai et al. 2016). In addition, several genes regulating aleurone differentiation have been identified in rice. The plant-specific calpain-like cysteine proteinase ADAXIALIZED LEAF1 (ADL1) and the receptor-like kinase OsCR4 function as positive regulators for aleurone differentiation (Hibara et al. 2009; Pu et al. 2012), while the bZIP zinc finger transcription factor RISBZ1 and the DOF zinc finger transcription factor RPBF regulate both storage protein biosynthesis and the differentiation of the aleurone (Kawakatsu et al. 2009).

In this study, we examined the growth and development of the rice endosperm on a daily basis, from the first day of anthesis to the formation of a mature caryopsis. No attention was paid here to embryo development, as there

is already sufficient knowledge available about this topic (Jones and Rost 1989). Our results enabled us to classify the rice endosperm development into four stages based on landmark events. The stages are as follows: coenocyte, cellularization, storage product accumulation, and maturation. Dynamic changes occurring in these individual stages are described in detail.

RESULTS

The growth of rice caryopsis

To investigate the general features of rice caryopsis growth, the lengths, widths and thicknesses of rice caryopses were measured on a daily basis throughout the course of caryopsis development. We observed that the elongation of the caryopsis occurred rapidly from 1 DAP, and reached its maximum length on 6 DAP (Figure 1A), while the expansion of caryopsis width and thickness occurred mainly from 4 DAP

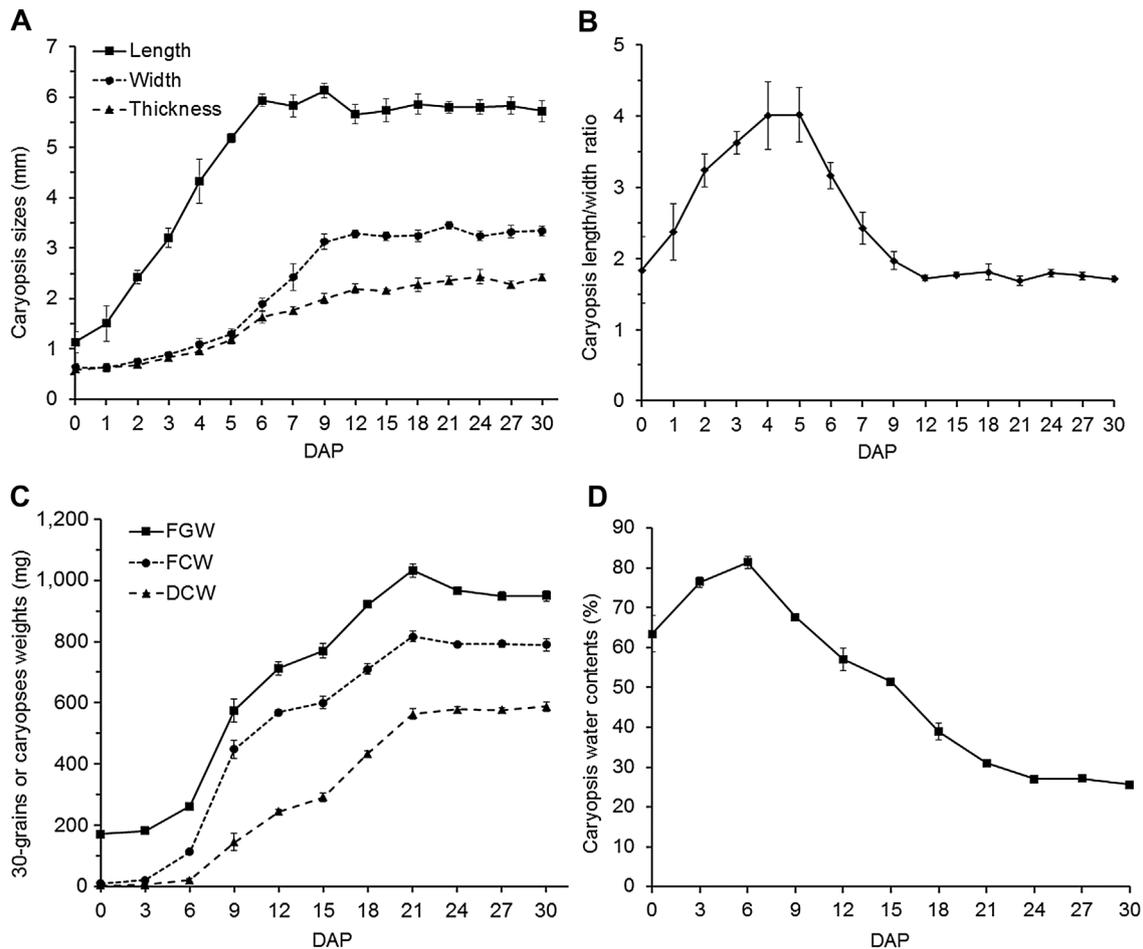


Figure 1. Rice grain and caryopsis growth: from anthesis to maturity

(A) The growth of the rice caryopsis in the length, width, and thickness. (B) The change in the length/width ratio during caryopsis development. Values for (A) and (B) are means \pm s.d. ($n = 5$ each). (C) Changes in the weights of grains and caryopses during development. Data showed the total weights of 30 grains or caryopses. FGW, fresh grain weight (with lemma and palea included); FCW, fresh caryopsis weight; DCW, dry caryopsis weight. (D) The water content in the caryopsis during development. Values for (C) and (D) are means \pm s.d. ($n = 30$ each).

onwards, reaching their maxima at 9 and 12 DAP, respectively (Figure 1A). Consequently, the maximal length/width ratio was seen between 4 and 5 DAP (Figure 1B). No major increases were observed in any of these three dimensions following 12 DAP (Figure 1A).

The successive changes in the fresh grain weight (FGW, with the palea and lemma included), fresh caryopsis weight (FCW), and dry caryopsis weight (DCW) were also measured throughout the post-fertilization caryopsis developmental process. We observed that a slow increase in FGW and FCW began to occur from 3 to 6 DAP, and a rapid increase in these weights was observed between 6 and 9 DAP, followed by another period of slow increase that lasted until 21 DAP (Figure 1C). No further increase was observed in these two weights after 21 DAP, instead, a slight decrease was seen. This decrease was associated with water loss from 21 to 30 DAP. For the DCW trait, very little increase was seen in the first 6 days. A steady increase was observed from 6 to 21 DAP, and no further increase was detected from 21 DAP onwards (Figure 1C). As seen in Figure 1D, the highest water content was observed on 6 DAP, which was followed by a gradual decrease.

Endosperm development

We performed cytohistologic analysis of rice endosperm development on a daily basis using London Resin (LR) White-embedded caryopses. The periodic acid-Schiff (PAS) reagent used in this study stains polysaccharides including cell walls and starch grains to red, and Coomassie Bright Blue (CBB) was used to counter stain the proteins to blue. This allowed us to divide the rice endosperm development into four stages: I: coenocyte (1 to 2 DAP); II: cellularization (3 to 5 DAP); III: storage product accumulation (6 to 21 DAP); IV: maturation (22 to 30 DAP). Since aleurone/starchy endosperm differentiation coincides with storage product biosynthesis, we classified the aleurone/starchy endosperm differentiation process (6 to 9 DAP) to the storage product accumulation stage. This classification differs from those published previously (Olsen et al. 1992; Brown et al. 1996; Sabelli and Larkins 2009; Wang et al. 2012), and the time needed for each stage is defined more accurately. The cytomorphological changes that occurred in these four stages are described below.

Stage I: Coenocyte (1 to 2 DAP)

From 1 to 2 DAP, the development of the rice endosperm is coenocytic, that is, characterized by rapid and synchronized endosperm nuclear divisions that lack cytokinesis. All nuclei produced together with their surrounding cytoplasm (indicated by arrowheads; Figure 2A, A'), were localized to the periphery of the embryo sac, while the central region of the embryo sac was occupied by a central vacuole (cv) (Figure 2A, A'). No storage product accumulation was observed in the endosperm at this stage (Figure 2A, A').

Stage II: Cellularization (3 to 5 DAP)

Cellularization of the rice endosperm (indicated by redlines; Figure 2) started on 3 DAP, and was completed by 5 DAP (Figure 2B–D, 2B'–D'). Newly formed anticlinal (indicated by arrows), then periclinal (indicated by red arrowheads), cell walls were observed at the periphery of the central vacuole (Figure 2B, B'), which compartmentalized adjacent coenocytic nuclei (indicated by black arrowheads; Figure 2B'). The

cellularization progressed gradually from the periphery towards the center of the endosperm (Figure 2B–D, 2B'–D'). Five to seven layers of cellularized endosperm cells, with no obvious starch grain accumulation, were observed by 4 DAP (Figure 2C, C'). By 5 DAP, the cellularization of the endosperm was completed; the cavity of the embryo sac was completely filled up by thin-wall endosperm cells (Figure 2D, D'). At this stage, small starch grains, stained in red by periodic acid-Schiff (PAS) reagent, located around the endosperm nuclei became recognizable (indicated by arrowheads; Figure 2D, D'). Of note, endosperm cells located at the periphery were usually smaller in size than those in the center, and contained almost no starch grains (indicated by a two-head arrow; Figure 2D'). These periphery cells form the aleurone layer later.

Stage III: Storage product accumulation (6 to 20 DAP)

The morphological differences between cells of the aleurone and those of the starchy endosperm started to become distinct at 6 DAP (Figure 3A, A'). The outer aleurone cells (indicated by red two-head arrows) contain dense cytoplasm and few small starch grains (Figure 3A, A'), differing obviously from the underlying starch grain-containing starchy endosperm cells. Further, the aleurone cells are smaller than the starchy endosperm cells (Figure 3A, A'). At this stage, the boundary of the aleurone and the starchy endosperm was not evident, and the subaleurone cells located between these two tissues exhibited a mixed identity.

The difference between the starchy endosperm (indicated by yellow bars) and the aleurone layer (indicated by red two-head arrows) was more apparent from 7 to 9 DAP, due to the accumulation of different storage products (Figure 3B, C, B', C'). The accumulation of storage protein bodies stained in blue by CBB was detected in the aleurone and the subaleurone from 9 DAP onwards (Figures 3C, C', 4). When examined at 10 to 18 DAP, cells in the aleurone contained mostly protein bodies and lipid bodies, while cells in the starchy endosperm (indicated by yellow bars) contained many starch grains and protein bodies (Figure 4). Cell wall thickening was observed in dorsal aleurone cells from 12 DAP onwards (Figure 4B, C).

One to three layers of subaleurone cells (indicated by green bars), which accumulate both protein bodies and starch grains, were observed in lateral regions of the endosperm at 10 DAP (Figure 4A'), whereas no evident subaleurone cells were observed in the dorsal region (Figure 4A–C). Between 10 and 18 DAP, further accumulation of both starch grains and protein bodies were observed in the subaleurone layer (Figure 4A'–C'). The size and number of the starch grains in the subaleurone were less than those in the starchy endosperm, whereas the subaleurone has a much bigger number of protein bodies compared with starchy endosperm (Figure 4A'–C'). At 18 DAP, the margins of individual protein bodies became less clear (Figure 4C'), suggesting a disintegration of their membranes. These subaleurone cells had thin walls and irregular shapes (Figure 4B', C').

Based on the position of the primary vascular bundle (also called the dorsal vascular bundle) in the rice caryopsis, the endosperm typically defined into three regions, the dorsal (near the dorsal vascular bundle), the ventral (opposite to the dorsal vascular bundle), and the lateral regions (Wu et al. 2016). Histological analyses of transversally sectioned mature caryopses showed that, at the lateral and lower lateral

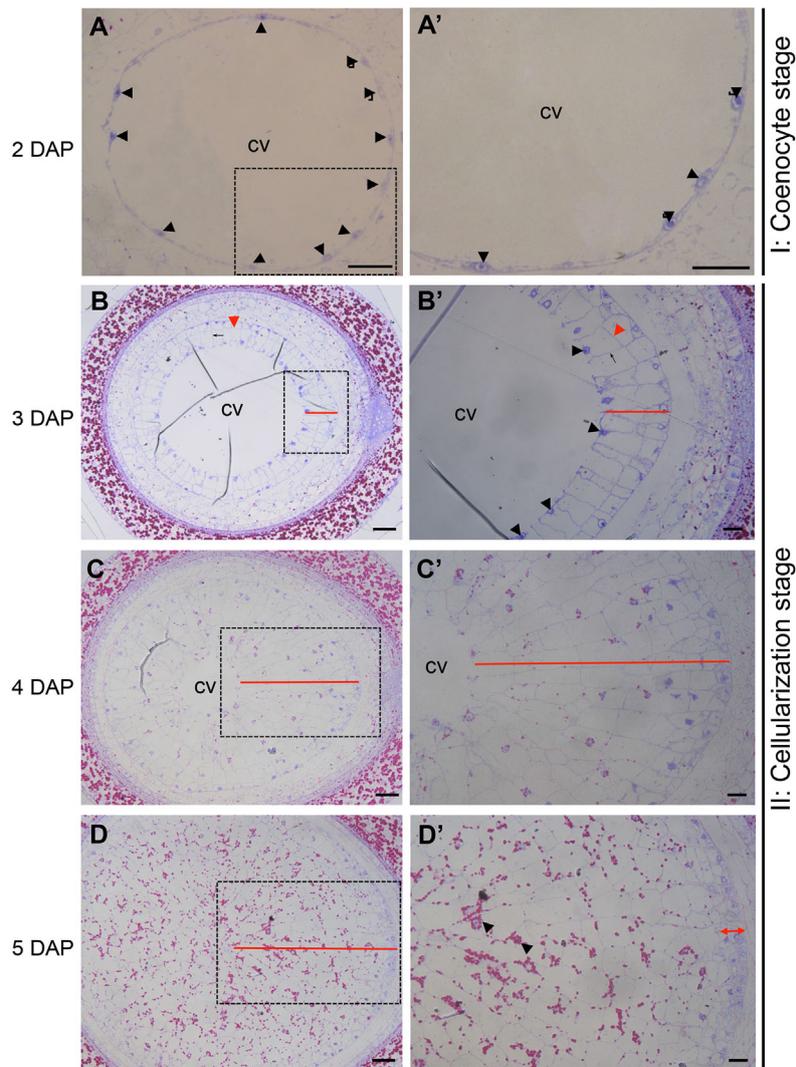


Figure 2. Morphological changes in the rice endosperm at the coenocyte and cellularization stages

Transverse sectioned caryopses collected at 2 (**A, A'**), 3 (**B, B'**), 4 (**C, C'**) and 5 DAP (**D, D'**), with attention focused on the endosperm region. **A'–D'** are the enlarged pictures of the boxed regions in **A–D**, respectively. Note the free endosperm nuclei (indicated by arrowheads) at 2 (**A, A'**) and 3 DAP (**B, B'**), and the newly formed anticlinal cell walls at the periphery of the endosperm. Arrows in **B** and **B'** indicate anticlinal walls, while red arrowheads indicate periclinal cell walls. The central region of the endosperm is occupied by a central vacuole (cv). The endosperm is completely cellularized at 5 DAP (indicated by red lines; **D, D'**). Note that no starch grains were observed in the endosperm at 4 DAP, while at 5 DAP starch grains were recognizable in the inner region of the endosperm (indicated by arrowheads; **D'**). The outer layer of the endosperm (indicated by a two-head red arrow; **D'**) forms the aleurone and subaleurone at a later stage. Scale bar = 50 μm in (**A–D**); 20 μm in (**A'–D'**).

positions, the aleurone had mostly one layer of cells; at the upper lateral and the ventral positions, an average of 1.5 layers of aleurone cells was observed (Figure 5A, B). At the dorsal position, an average of 3.5 layers of aleurone cells, spanning about 20 to 30 cell files, was observed (Figure 5B). No cell wall ingrowth was observed in any of the aleurone cells.

From 5 DAP onwards starch grains started to accumulate in the cellularized endosperm (Figures 2D, 3, 6), suggesting a tight connection between the metabolic and developmental events. The starch biosynthesis initiated from the central region of the endosperm, and then continued gradually towards the periphery (Figures 3, 6). At 6 DAP, abundant

starch grains were observed in cells located at the central region of the endosperm (Figure 6B, B'). Within these cells, most starch grains were located at the periphery, while the central region was occupied by large vacuoles. Active starch biosynthesis occurred throughout the next 4 days (Figure 6C–F, C'–F'). By 9 DAP, cells located in the central region of the endosperm were filled with starch grains (Figure 6E, E'), while cells in the periphery of the endosperm had a much smaller number of starch grains (Figure 3C, C'). We noticed that the sizes of starch grains in cells located at the dorsal side of the starchy endosperm (Figure 3A–C) were usually bigger than those cells located at the lateral regions (Figure 3A'–C').

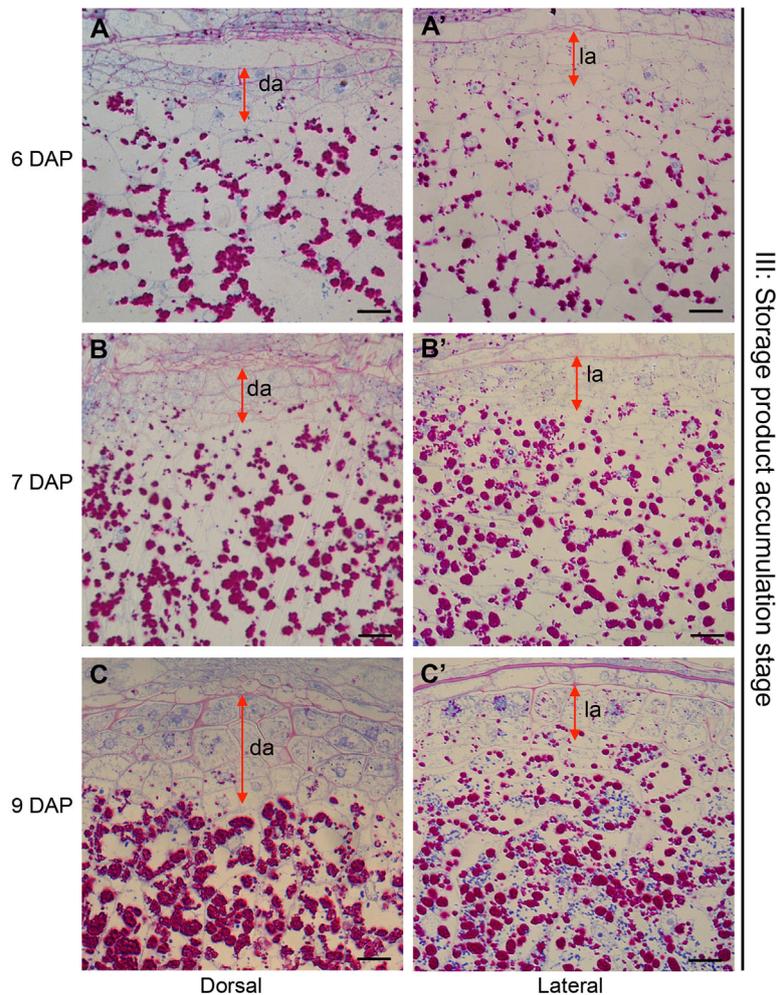


Figure 3. Aleurone/starchy endosperm differentiation in the rice endosperm from 6 to 9 DAP

Morphology of the endosperm at the dorsal (A–C) and lateral (A'–C') positions at 6 (A, A'), 7 (B, B'), and 9 DAP (C, C'). In contrast to the single- or occasionally two-cell layer aleurone at the lateral position, the aleurone at the dorsal position consists of 3 to 4 cell layers. Two-headed red arrows indicate the aleurone layer. da, dorsal aleurone; la, lateral aleurone. Scale bar = 20 μ m.

As indicated by Evans Blue staining in Figure 7, PCD, which led to the loss of cytoplasmic membrane integrity, was first observed in the central region of the starchy endosperm at 8 DAP (indicated by a black arrowhead; Figure 7B). PCD expanded to the periphery of the endosperm in the next few days, and reached the whole starchy endosperm by 18 DAP (Figure 7A–F). In contrast, cells of the surrounding aleurone layer remained alive in the whole process of the endosperm development, as shown by the lack of Evans Blue staining (indicated by a white arrowhead; Figure 7F). Given that active starch biosynthesis is still going on the starchy endosperm, it seems that the loss of integrity of the cytoplasmic membrane in the endosperm did not interfere with starch biosynthesis. We speculate that intra-cellular membranes in nuclei, plastids, mitochondria and endoplasmic reticular are intact and remain functional.

Stage IV: Maturation (21 to 30 DAP)

From 21 DAP onwards, the rice endosperm entered the maturation stage (Figure 8), as no further increases in

caryopsis fresh and dry weights were observed (Figure 1A). Histological studies showed that, during this period, cells in the aleurone layer showed very few changes in morphology, while cells in the starchy endosperm gradually lost their boundaries (Figure 8A–C). Subaleurone cells accumulated a large amount of protein bodies (Figure 8A'–C'). Compound starch grains were formed in the starchy endosperm as the margins of individual starch grains became less clear (Figure 8B–C). At the whole grain level, the crystallization of starch and storage proteins led to the formation of semi-transparent starchy endosperm by the end of the maturation process.

DISCUSSION

In this paper, morphological studies were performed in the rice endosperm throughout the 30-day developmental process. Our data allowed us to classify the rice endosperm development into four developmental stages. Highly dynamic

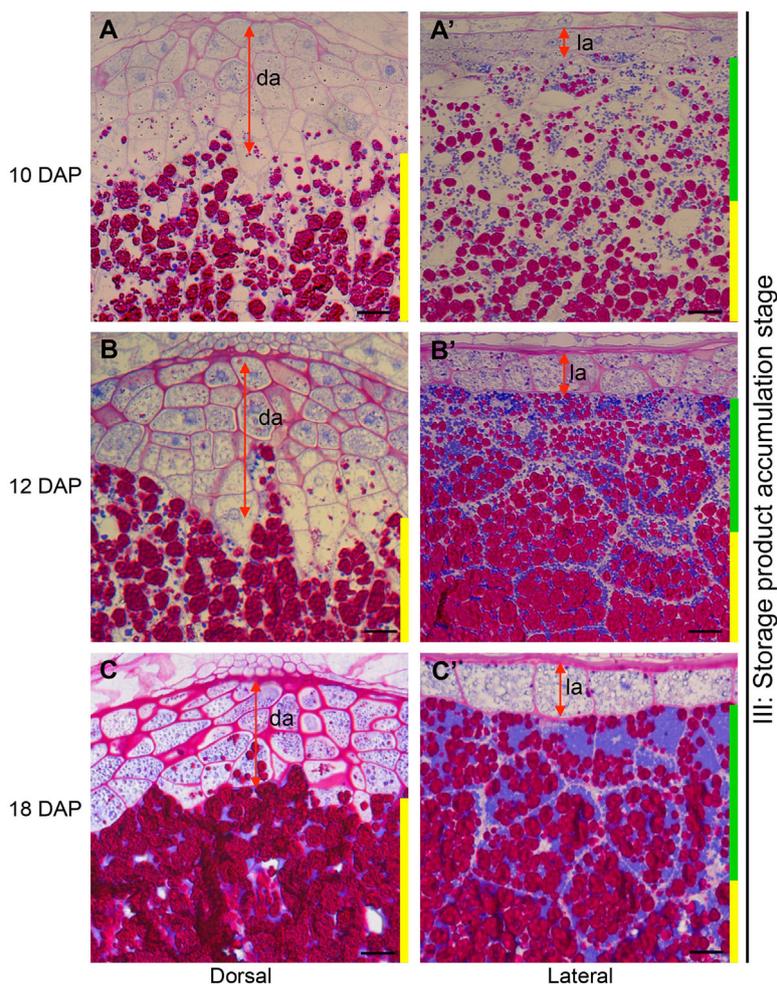


Figure 4. Storage product accumulation in the rice endosperm from 10 to 18 DAP

Morphology of the endosperm at the dorsal (A–C) and lateral (A'–C') positions at 10 (A, A'), 12 (B, B') and 18 DAP (C, C'). Note the rapid accumulation of both starch grains and protein bodies in the subaleurone and the starchy endosperm during these days. Green bars indicated the subaleurone. Yellow bars indicated the starchy endosperm. Two-headed red arrows indicate the aleurone layer. da, dorsal aleurone; la, lateral aleurone. Scale bar = 20 μ m.

changes were observed in the endosperm in nuclear divisions, cellularization, aleurone/starchy endosperm differentiation, storage product accumulation and PCD at different time points.

The general growth and development of the rice endosperm

Our previous studies revealed a dynamic change in different maternal tissues of the rice caryopsis in cell expansion, storage product accumulation, and degeneration after fertilization (Wu et al. 2016). In this study, attention was given to the rice endosperm. We showed firstly that the growth of the caryopsis along the longitudinal axis occurred rapidly after fertilization, reaching its maximum at 6 DAP (Figure 1A), while expansion in width and thickness occurred mainly from 4 DAP onwards, reaching their maxima by 12 DAP (as illustrated in Figure 9).

The rice endosperm is derived from a triploid central nucleus and consists of an outer aleurone and an inner starchy

endosperm (Hoshikawa 1973; Brown et al. 1996; Becraft and Asuncion-Crabb 2000; Becraft 2001, 2007; Becraft and Yi 2011). Further analyses were performed in the rice endosperm on daily basis, allowing us to divide the rice endosperm development into four stages (Figure 9). Compared with the four development stages divided in cereal endosperm (Olsen et al. 1992), Wang et al. (2012) defined the development of rice endosperm into coenocyte (1–2 DAP), cellularization (3–5 DAP), differentiation (5–15 DAP) and maturation (16–30 DAP) stages. In our classification, coenocyte stage (1–2 DAP) and cellularization stage (3–5 DAP) are similar with Wang et al. (2012) and Hoshikawa (1973). However, we classified the endosperm/aleurone differentiation (6–9 DAP) into the storage product accumulation stage (6–20 DAP).

The coenocyte (stage I) lasts only for the first 2 DAP, when mitosis occurs in the endosperm without cytokinesis. The coenocyte stage of rice is similar to that of wheat (Jing et al. 2013), but is significantly shorter than that of barley, in which the coenocyte stage lasts for 5 days (Bosnes et al. 1992). The

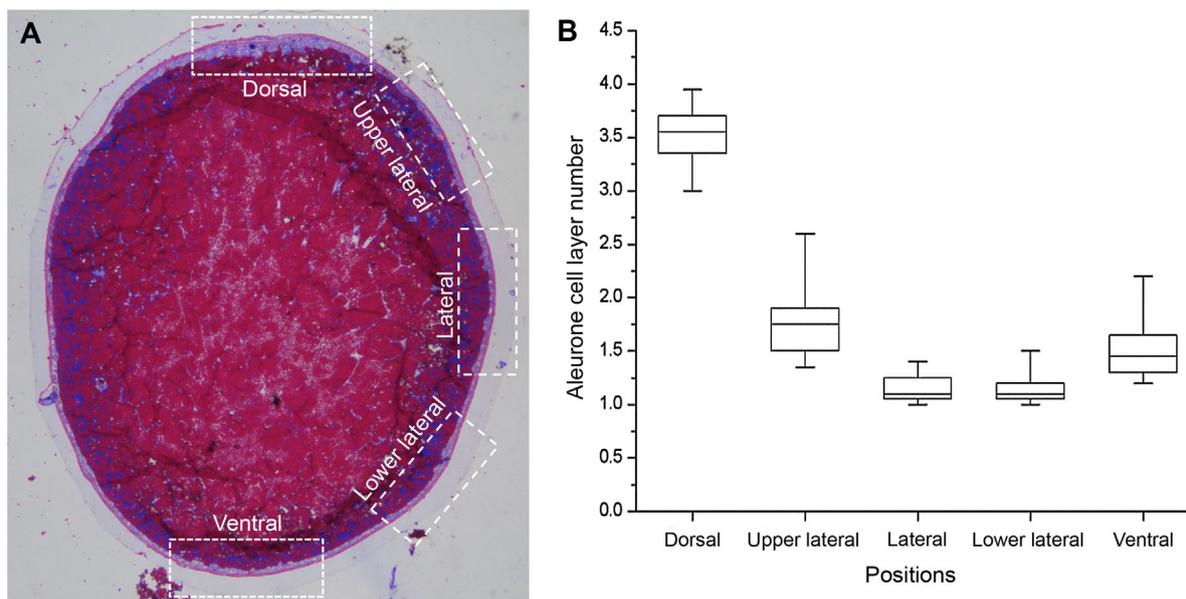


Figure 5. The number of aleurone cell layers in different regions of the mature rice endosperm

(A) A transversely sectioned mature rice caryopsis. Boxes indicate locations (dorsal, upper lateral, lateral, lower lateral, and ventral) examined for the numbers of cell layers in the aleurone. (B) Analysis of cell layer number in the aleurone at different positions of mature endosperm. Twenty cell files were counted in each position, as shown in box plots ($n = 20$ each).

cellularization of the rice endosperm (stage II) is achieved in the next 3 days (from 3 to 5 DAP), generating an endosperm with 12–15 cell layers from the center to periphery. Barley endosperm cellularization is much slower, being completed at 9 DAP (Bosnes et al. 1992). A rapid accumulation of starch grains in the rice starchy endosperm (stage III) occurs from 5 DAP onwards (Figures 2D, 3, 4, 6), immediately following the completion of endosperm cellularization. The tight connection between endosperm cellularization and storage product accumulation suggests the potential importance of endosperm cellularization in storage product accumulation, as proposed before (Hoshikawa 1973; Brown et al. 1996). We speculate that the major factor driving the longitudinal extension of caryopsis between 0 and 6 DAP is the intrinsic force of cell expansion, while the major drive of transverse expansion of caryopsis between 5 and 12 DAP is water and nutrient uptake, since expansions in the width and thickness correlate with the rapid storage product accumulation in the endosperm during this period (Figures 2–4, 6). It has been shown in another paper in this issue that the period of the most abundant starch grain accumulation in maternal tissues occurs at 5 DAP, and degradation of these starch grains occurs afterwards (Wu et al. 2016). In this study we observed that the major phase of storage product accumulation occurred between 6 and 21 DAP. Most likely, the starch in the pericarp is remobilized to the developing endosperm.

It has been shown previously in *Arabidopsis* that endosperm cellularization is critical for embryo development (Hehenberger et al. 2012). Further, downregulation of the *OsCycB1;1* expression in rice led to abnormal caryopses with an enlarged embryo and a defective endosperm (Guo et al. 2010). It is plausible that cellularized endosperms provide a superior matrix that facilitates sugar loading from maternal tissues to

the endosperm for starch biosynthesis. The potential mechanism underlying these suppositions remains to be elucidated.

The differentiation of the aleurone and the starchy endosperm occurs in the early phase of storage product accumulation (stage III). It is known that cells in the aleurone accumulate mainly lipids, proteins, vitamins, and minerals, while cells in the starchy endosperm mainly accumulate starch and storage proteins (Juliano 1993; Ogawa et al. 2002; Becraft 2007; Becraft and Yi 2011; Iwai et al. 2012). Further, the aleurone in rice is critical for sugar loading to the starchy endosperm, and is also critical for the release of amylase, which degrades the starch in the starchy endosperm during seed germination (Becraft 2001; Olsen and Becraft 2013; Zheng and Wang 2015; Bai et al. 2016).

From 21 DAP onwards, the rice endosperm enters the maturation (stage IV), when no increase in fresh and dry caryopsis weights was observed. Instead, a slight decrease in dry caryopsis weight was detected, suggesting that the 21 DAP represents an obvious turning point from a storage product accumulation phase to a phase of seed maturation and desiccation. Cell biological studies showed that during this stage the starchy endosperm loses its cellular structure and starchy endosperm crystallization occurs. These events correlate with the degeneration of most of the maternal tissues including the dorsal vascular bundles, as reported previously (Wu et al. 2016).

PCD in the rice endosperm

During rice caryopsis development, PCD occurs initially in the central region of the starchy endosperm at 8 DAP and continues towards the periphery. Eventually, in the mature caryopsis, all of the cells in the starchy endosperm are dead, as has been reported recently (Kobayashi et al. 2013). However,

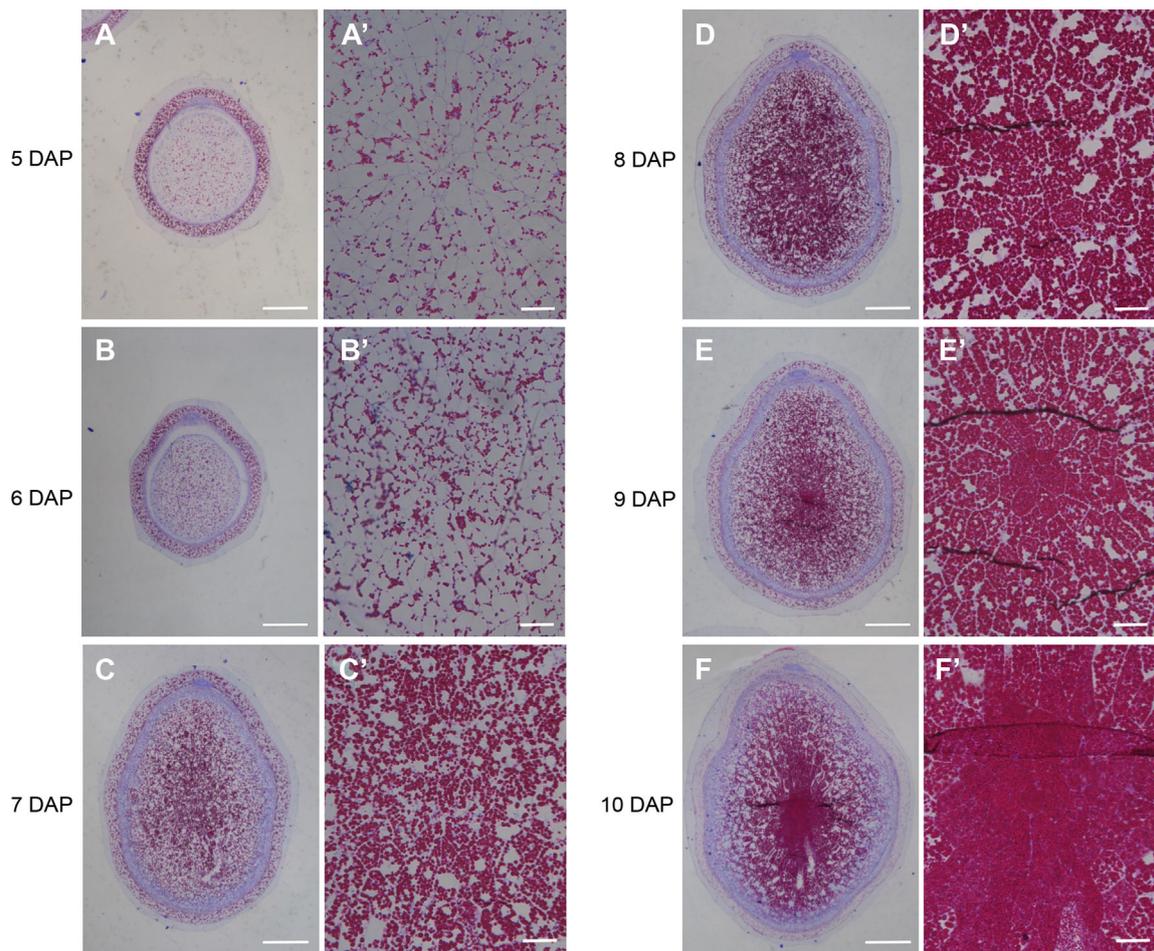


Figure 6. Progressive starch grain accumulations in rice endosperm

The whole caryopsis (A–F) and the central region of endosperm (A'–F') were stained by PAS and CBB at 5 (A, A'), 6 (B, B'), 7 (C, C'), 8 (D, D'), 9 (E, E'), and 10 DAP (F, F'). Note the rapid accumulation of starch grains during this period, especially from 7 to 10 DAP. Scale bar = 5 mm in (A–F); 50 μ m in (A'–F').

cells in the aleurone remain viable, and undergo PCD only during seed germination (Fath et al. 2000). PCD in maize endosperm starts in the central region of the endosperm at 16 DAP, and continues towards the upper region and then the basal region of the kernel. In contrast, in the wheat endosperm, it has been reported that PCD is mostly a random process (Young and Gallie 1999, 2000). It is interesting to note that PCD in rice occurs simultaneously with the rapid accumulation of starch grains (Figures 3, 6), indicating that PCD does not interfere with starch biosynthesis. Further, PCD in the rice endosperm occurs in a non-lytic manner, meaning that its cell corpses remain non-degenerated until seed germination (Kobayashi et al. 2013; Hautegeem et al. 2015). Most likely, PCD in rice endosperm causes only a partial degeneration of cytoplasmic membranes, while nuclei and organelles remain functional during this process, allowing starch biosynthesis to occur in a large endosperm compartment with a shared cytoplasm. This supposition is supported by the fact that no nuclear DNA degradation was observed in rice endosperms in DNA laddering and TUNEL labeling assays (Wei et al. 2002; Li et al. 2004; Kobayashi et al. 2013).

The differentiation of the aleurone and the starchy endosperm

The differentiation of an outer aleurone and an inner starchy endosperm in the rice endosperm was visible from 6 DAP onwards, producing two different tissues with distinct morphology, metabolic activities and cell fates, as reported previously (Hoshikawa 1967d). We observed that the aleurone has different numbers of cell layers at different positions in rice endosperm. A distinct multi-layered dorsal aleurone formed near the dorsal vascular bundle, while the single- or occasionally two-cell layered aleurone was located in the lateral and ventral positions of endosperm. The same phenomenon was also observed by Hoshikawa (1967d). Moreover, the number of aleurone cell layers at different positions is constant in the different rice varieties (Hoshikawa 1967e). In most cereals, such as maize, wheat and sorghum, the aleurone is single cell layer, while it comprises three cell layers in barley (Kent and Evers 1994). Interestingly, *Brachypodium distachyon*, a model system for the temperate small grain cereals, peripheral aleurone showed one to four irregular cell layers (Opanowicz et al. 2011; Hands et al. 2012),

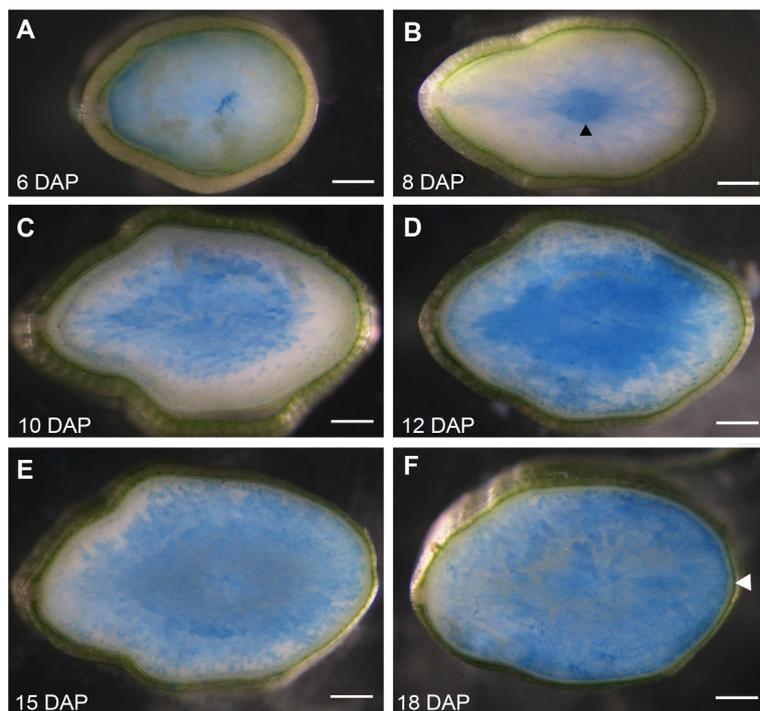


Figure 7. Progressive PCD in the rice endosperm

Rice endosperms collected at 6 (A), 8 (B), 10 (C), 12 (D), 15 (E), and 18 DAP (F) were stained by Evans Blue (able to penetrate cells with compromised membrane integrity). PCD was first observed at the central region of the starchy endosperm at 8 DAP (indicated by a black arrowhead; B), expanded to the periphery of the endosperm in the next few days, and reached the whole starchy endosperm by 18 DAP. In contrast, cells in the aleurone layer (indicated by a white arrowhead; F) remained alive throughout the entire developmental process. Scale bar = 0.5 mm.

suggesting that a highly differentiated and organized peripheral aleurone was domesticated for enhancing the grain storage properties or seed germination (Hands et al. 2012).

The dorsal aleurone in rice endosperm, which is located next to the dorsal vascular bundle, has on average 3.5 cell layers (Figure 5), and is expected to act together with the dorsal vascular bundle and nucellar projection to facilitate the loading of sugars and water to the developing endosperm (Sreenivasulu et al. 2011; Wu et al. 2016). This idea is supported by dye-loading experiments performed in rice (Krishnan and Dayanandan 2003). No orthologs of *ZmMRP-1* encoding an atypical single-repeat MYB transcription factor to regulate the differentiation of transfer cells in maize basal endosperm transfer cell layer (BETL) was identified in both rice and *Brachypodium*, suggesting that a distinctive modified aleurone region was absent in their endosperm. *Brachypodium distachyon* endosperm is bounded by a continuous layer of aleurone cells without a modified aleurone region (Opanowicz et al. 2011; Hands et al. 2012). Although rice endosperm lacks the BETL or the modified aleurone, their dorsal aleurone cells resemble to a certain extent transfer cells (also known as pigment strand cells; Zee and O'Brien 1970) in maize BETL and wheat modified aleurone, based on their locations and functions (Kent and Evers 1994; Gómez et al. 2009; Becraft and Yi 2011; Sreenivasulu et al. 2011; Thiel 2014; Bai et al. 2016). It is interesting to note that, different from transfer cells with

cell wall ingrowths as in the modified aleurone of most cereals, cells in the dorsal aleurone of rice do not have cell wall ingrowths. It has been shown recently that an NF-YB1 transcription factor expressed primarily in the dorsal aleurone regulates the expression of three sucrose transporters in the aleurone, allowing sucrose to be loaded directly to the starchy endosperm (Bai et al. 2016). Whether other regions of the aleurone are also involved in nutrient and/or water delivery to the endosperm remains to be determined.

Recently, several transcription factors involved in aleurone differentiation have been identified in maize (Becraft et al. 1996; Lid et al. 2002; Shen et al. 2003; Yi et al. 2011). Mutation of *NAKED ENDOSPERM (NKD)*, which encodes an INDETERMINATE1-domain containing transcription factor, led to the formation of an endosperm with a multi-cell layer aleurone (Yi et al. 2015). In rice, suppressed expression of two transcription factors, the basic leucine zipper factor *RISBZ1* and the prolamin box-binding factor *RPBF*, leads to reduced expression of several aleurone differentiation regulatory genes such as *CR4*, *DEK1*, and *SAL1* and leads to the formation of endosperms with multi-cell layered aleurone (Kawakatsu et al. 2009). These transcription factors may function in a complex regulatory network for cell fate specification in the aleurone. Given that the aleurone and starchy endosperm share the same developmental origin, further genetic studies will be needed to elucidate how the distinct cell fates of the aleurone and the starchy endosperm are established.

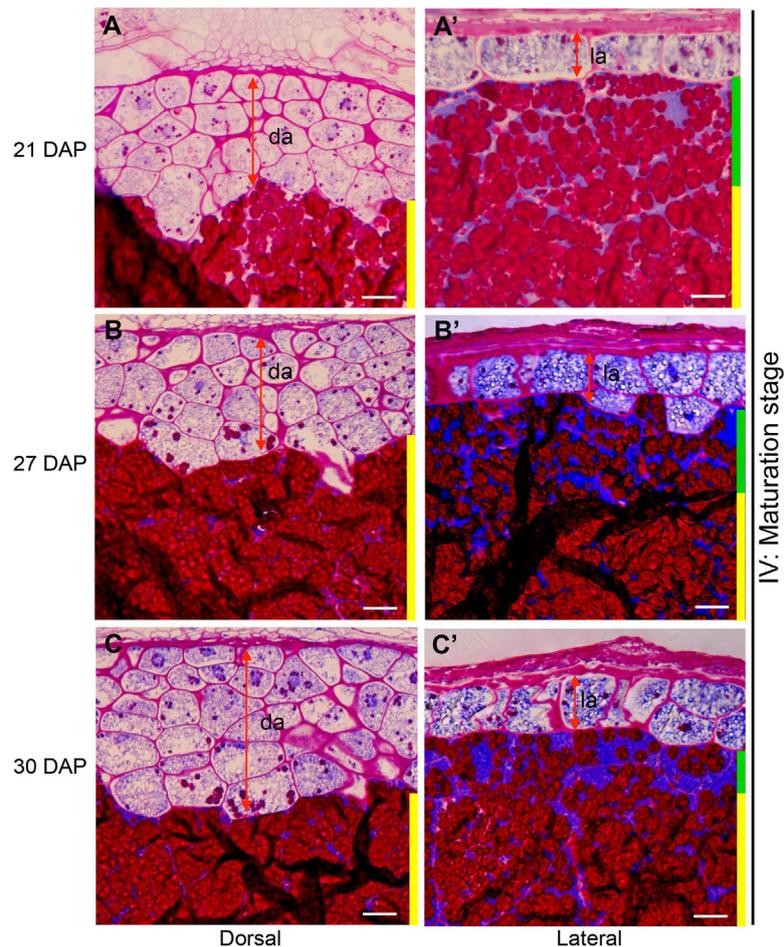


Figure 8. Maturation stage in the rice endosperm

Morphology of the rice endosperm at the dorsal (A–C) and lateral (A'–C') positions at 21 (A, A'), 27 (B, B'), and 30 DAP (C, C'). Note that aleurone cells showed very few changes in morphology, while cells in the starchy endosperm gradually lost their boundaries. Green bars indicated the subaleurone. Yellow bars indicated the starchy endosperm. Two-headed red arrows indicate the aleurone layer. da, dorsal aleurone; la, lateral aleurone. Scale bar = 20 μm .

In summary, we showed in detail in this study that the development of the rice endosperm undergoes a series of highly dynamic processes including coenocytic nuclear divisions, cellularization, cell differentiation, storage product accumulation, and PCD, leading to the formation of a functional endosperm. This detailed descriptive study establishes a high-resolution foundation for further investigations of cereal endosperm development.

MATERIALS AND METHODS

Plant material

Rice plants (*Oryza sativa* L. ssp. *Japonica*, cultivar Zhonghua 11) were grown in the experimental fields located at the Institute of Botany of the Chinese Academy of Sciences in Beijing, China. Under the field conditions used for growing rice, the time from anthesis to production of a mature grain was 30 ± 2 days. The anthesis time of the individual spikelets was marked on the surface of the hull, and subsequent caryopsis samples

were collected daily throughout the 30-day developmental process, as reported previously (Wu et al. 2016).

Weight measurement

Grains (with palea and lemma included) and caryopses (both palea and lemma were removed) were harvested at 1- to 3-day intervals from the start date of anthesis, and at least 30 grains or caryopses were collected for each time point and used for the measurement of fresh and dry weights using an analytical balance. To measure the dry weight and the water content, caryopses were harvested and weighed first for the fresh weights. These samples were then placed in small aluminum specimen boxes and heated at 105 °C for 3 h, and then cooled to room temperature prior to being weighed again.

Size measurement

Caryopses were harvested at 1- to 3-day intervals from the start date of anthesis, and five caryopses were collected for each time point and used for the measurement of the length, width, and thickness of caryopsis using a vernier caliper.

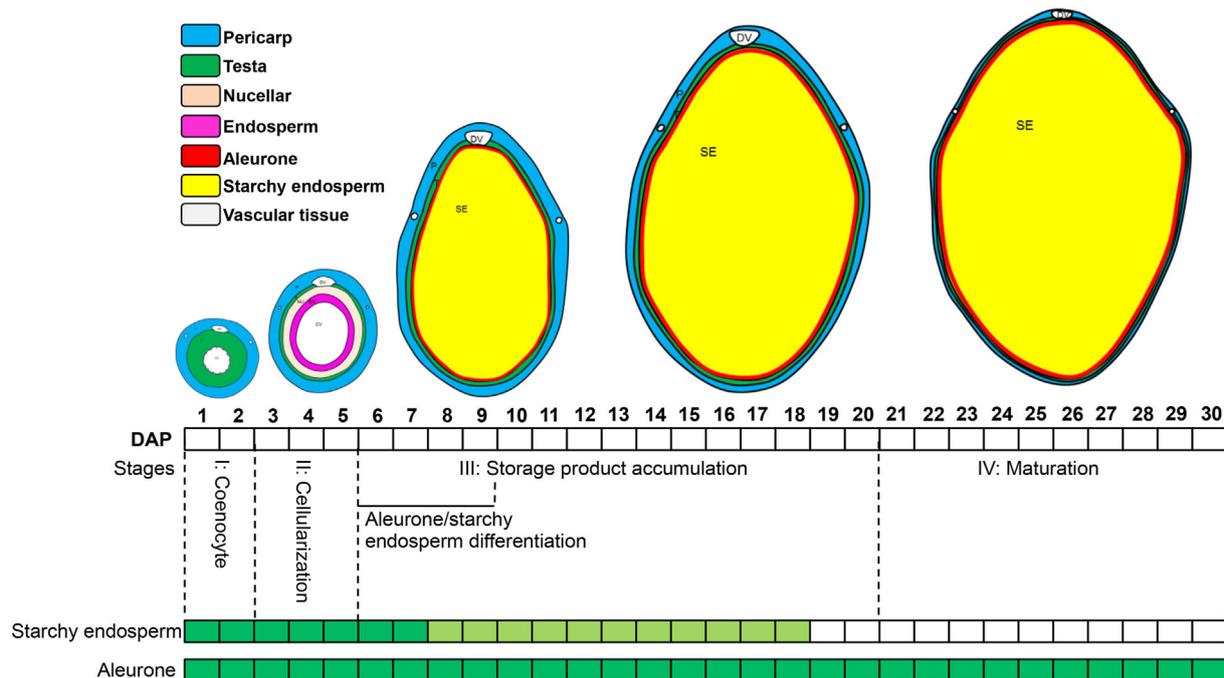


Figure 9. Schematic illustration of the dynamic changes of the rice endosperm

Changes in endosperm tissues at transversal sections during caryopsis development are illustrated. The drawing is roughly in scale with the relative sizes of the rice caryopsis at the different days labeled underneath. Major tissues are color-coded. Endosperm development is divided into four developmental stages: Stage I, coenocyte (1 to 2 DAP); Stage II, cellularization (3 to 5 DAP); Stage III, storage product accumulation (6 to 20 DAP); and Stage IV: maturation (21 to 30 DAP). Note that the aleurone/starchy endosperm differentiation overlaps with the storage product accumulation stage. Boxes underneath the drawing represent individual DAP. Boxes filled in dark green indicate endosperm cells that are alive, boxes in pale green indicate endosperm cells are undergoing PCD, and boxes lacking color represent cells that have lost their membrane integrity. CV, central vacuole; DV, dorsal vascular tissue; EN, endosperm; NU, nucellar; P, pericarp; SE, starchy endosperm; T, testa.

Microscopy

For the semi-thin sections, caryopses collected on different DAP were sectioned transversely into 2 mm slices using a razor blade and then fixed immediately in a modified FAA solution (Liu et al. 1993) for 24 h. These were then dehydrated in an alcohol series (50%, 70%, 80%, 95%, 100%) for 1 h at each concentration step, and then infiltrated in LR White (London Resin Company, UK) using 2:1, 1:1, and 1:2 mixed ethanol: LR White ratios for 2 h each, followed by two infiltration with pure LR White for 4 h or overnight each time. These samples were polymerized in capsules at 60 °C for 24 h. Semi-thin sections (1.25 µm) were cut using a glass knife on a Leica ultramicrotome (EM UC7, Leica Microsystems), stained with periodic acid-Schiff (PAS) reagent as reported previously (Schneider 1981), and counter-stained with 0.1% Coomassie Bright Blue (CBB) for 20 min. They were then washed in running water and subsequently rinsed 3 times in double distilled water prior to mounting with a cover slip. Pictures were taken under a microscope (Eclipse 80i, Nikon).

Evans blue staining

Thin sections were made by hand of caryopses in different DAP. Sections were taken as thinly as possible from the central point of the caryopsis. Cut sections were immersed in a 0.1% Evans Blue solution for 10 min and then washed three

times with distilled water. Sections were immediately mounted in distilled water and photographed using a microscope (SMZ800, Nikon).

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AUTHOR CONTRIBUTIONS

X.W., J.L. and D.L. designed and performed the experiments. X.W. drafted the first version of the manuscript, J.L. and C.M.L. analyzed the data and revised the manuscript.

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