Cereal Research Communications 42(3), pp. 514–524 (2014)
DOI: 10.1556/CRC.42.2014.3.14

Development of Endosperm Cells and Starch Granules in Common Wheat

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(Received 1 August 2013; Accepted 21 January 2014;
Communicated by R.N. Chibbar)

The objective of the present study was to understand the developmental regularity of wheat endosperm cells at different Days After Pollination (DAP) using microscopic and histochemical methods. Resin semi-thin sections of the endosperm and the enzymatically dissociated Starchy Endosperm Cells (SECs) were observed under a light microscope. The results showed that: (1) SECs were irregular-shaped and had two types of starch granules: large oval-shaped A-type starch granules and small spherical B-type starch granules. (2) The growth shape of SECs was referred to as S-curve and the fastest cell growth period was at 16–24 DAP. (3) The largest increase and growth of A-type starch granules were mainly at 4–16 DAP. B-type starch granules increased rapidly after 16 DAP and made up over 90% of the total starch granules in SEC during the late stage of endosperm development. (4) The nuclei of SEC deformed and degenerated during the middle and late stages of endosperm development and eventually disappeared. However, starch granules still increased and grew after the cell nuclei had degenerated. The investigations showed the development regularity of starch endosperm cells and starch granules, thereby improving the understanding of wheat endosperm development.

Keywords: starch endosperm cell, starch granule, wheat

Abbreviations: DAP – Days After Pollination, PCD – Programmed Cell Death, SD – Standard Deviation, SEC – Starch Endosperm Cell, SEM – Scanning Electron Microscopy, TTC – 2,3,5-Triphenyl-2H-Tetrazolium Chloride

Introduction

Wheat is one of the world's most important cereals in the evolution of human civilization. The endosperm is an important part of the wheat caryopsis and is responsible for more than 85% of its weight. Starch, which accumulates in starch granules, is the main storage carbohydrate and accounts for three-quarters of the dry weight of a wheat caryopsis and provides 20% of the calories in human diet. Finally, the number and size of the starch

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granules has a significant impact on caryopsis weight (Becraft 2001; Sabelli and Larkins 2009a; Uhlmann and Beckles 2010; Wei et al. 2010).

Endosperm develops from the fertilization of two polar nuclei and this development is characterized by four stages: (1) syncytial, a period of nuclear divisions without cytokinesis; (2) cellularization, a period where cytokinesis results in discrete bodies that completely fill the central cell; (3) growth and differentiation, a period where the endosperm develops into different tissues and (4) maturation, a period where storage occurs and desiccation tolerance and dormancy develops (Olsen 1998, 2004). The overall development of wheat endosperm is regulated by the nutrient translocation pathway in the caryopsis (Wang et al. 2012).

Many studies have been conducted on endosperm development, however, they have mainly focused on its configuration. The growth process of SEC and the number, size and percentage changes of starch granules in wheat SECs are not very clear. In this study the differences in starch endosperm cell size and numbers and in the starch granules themselves were investigated at the cellular level during caryopsis development. Results revealed the development regularity of starch endosperm cells and starch granules and improved our understanding of wheat endosperm development.

Materials and Methods

Plant material

Strong-gluten wheat (*Triticum aestivum* L., WanMai33) plants were grown in the experiment fields at Yangzhou University. Caryopses at different days after pollination were collected as the experimental materials.

Cell isolation using cellulase enzymes

The method for isolating SECs was modified from Yang et al. (2002).The endosperm cell walls were dissolved by cellulase enzymes to isolate complete cells. The caryopses were put into Cano reagent (absolute ethanol : glacial acetic : chloroform, 9 : 3 : 1, v/v) for 8 h, then replaced with 70% ethanol and stored in a refrigerator. The pericarp was removed and the endosperm was washed 2–3 times for 0.5–1 h with distilled water. Then the pericarp was treated with 1 M HCl for 1 h and stained with 1% carmine acetate for 2–3 h. The samples were washed, 1 mL 0.3% cellulase enzyme was added (0.1 M acetate buffer, pH 5.5) and then the samples were dissociated in water at 40°C for 3–5 h. The endosperm tissues were diluted 3–4 times with acetate buffer, then 1–2 drops of diluted endosperm tissues were pipetted onto a glass slide and covered with a cover-slip. The samples were examined under a light microscope.

Measurement of SECs and starch granules

Thirty SECs were selected and ruptured to release the starch granules. The starch granules were counted under a Leica DMLS microscope. The long axis, short axis, area and perimeter of the starch endosperm cells and the diameter of the A-type starch granules were

measured using Image J software (Liu et al. 2011). The cell observed under the light microscope was a projection of the material object, so the cell measurements were actually the long axis, the short axis, area and the projection perimeter. The measurements were repeated three times and the Standard Deviation (SD) was calculated using Microsoft Office Excel 2007.

Histochemical assays of developing caryopses

The caryopses at different developmental stages were cut longitudinally from the centre and stained with two dyes. The dehydrogenase in the SECs was stained with 0.5% 2,3,5-Triphenyl-2H-Tetrazolium Chloride (TTC) for 30 min to test the respiration metabolic activity in the SECs. The SECs turned red when dehydrogenase activity was high. Evans blue (0.5%) was used to stain SEC activity and it turned blue when the SEC was dead. The sections were stained at room temperature and photographed under a Leica MZ6 stereomicroscope with Leica CLS (cold light source) 150XE lighting using Canon digital camera PowerShot S50.

Sample observation under the light microscope

The caryopses were cut transversely into pieces that were 1–2 mm in width and length and fixed in 2.5% glutaraldehyde at 4°C for 3 h. Following this, the samples were fixed with 0.5% OsO_4 at 4°C for 3 h, then rinsed with 0.1 M phosphate buffer (pH 7.2) and dehydrated in an ethanol series. After dehydration, the ethanol was replaced by propyleneoxide and low glutinosity Spurr's resin was used to infiltrate and embed the material. A Leica Ultracut rotary microtome was used to cut samples into 1 μm thin sections stained with toluidine blue (TBO) and observed under a Leica DMLS microscope. Photographs were taken by Canon digital camera PowerShot S70.

Sample observation under a Scanning Electron Microscope (SEM)

Mature caryopses were fractured in the middle and cut into 3 mm thick samples. The samples were mounted on aluminum stubs with conducting carbon paint. After they were sputter-coated with gold, the sample fracture plane was observed under a Philips XL-30 ESEM scanning electron microscope.

Results

Endosperm morphogenesis

The free nuclear stage, a period where the nuclei have no cell wall, occurred between 0 and 2 DAP in the wheat endosperm and the interior space of the embryo sac was mainly occupied by a central vacuole (Figs 1a and 1e). The free nuclei divided repeatedly without cytokinesis, which resulted in a large syncytium that lined the periphery of the cell and surrounded the central vacuole. Nuclear division was mainly by mitosis and there were two or more nucleoli in the free nucleus (Fig. 1h).

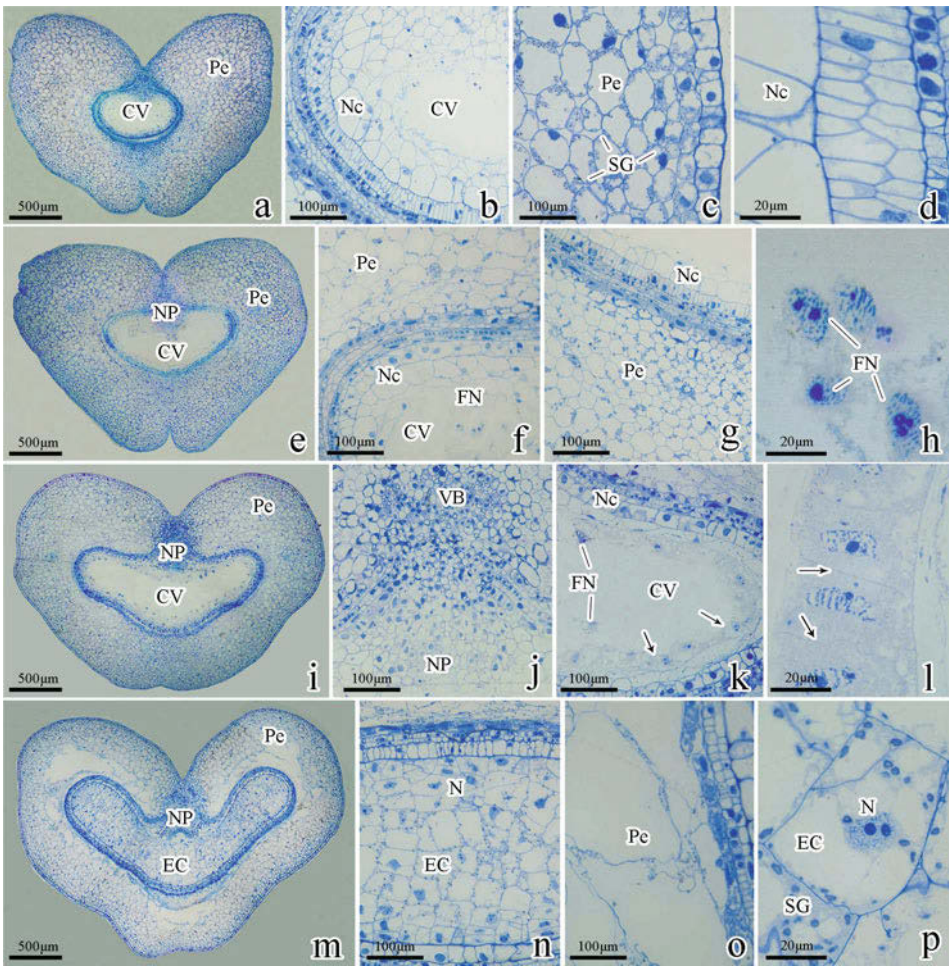

Figure 1. The morphogenetic processes of endosperm

Notes: (a–d) 1 DAP. (a) The central transverse-cut of the caryopsis. (b) The central vacuole and nuclei during the free nucleus stage. The central vacuole has occupied most of the embryo sac. (c) The pericarp. (d) The epidermal cell of the nucellus. (e–h) 2 DAP. (e) The central transverse cut of the caryopsis. (f) The central vacuole and nuclei showing that the free nuclei are located between the nucellus and central vacuole. (g) The pericarp and the nucellus. (h) Free nuclei without cell walls. (i–l) 3 DAP. (i) The central transverse-cut of the caryopsis. (j) The nucellar projection and vascular bundle. (k) The endosperm tissue at the cellularization stage. The arrow indicates open-ended cells. (l) The open-ended cells. The arrows indicate that the freely growing wall grew between the free nuclei and that open-ended cells formed without periclinal walls. (m–p) 4 DAP. (m) The central transverse cut of the caryopsis. (n) Endosperm cell. The endosperm cell filled the whole embryo sac after the cellularization stage had finished. (o) The pericarp tissue near the endosperm caused programmed cell death, and (p) starch granules appeared in the endosperm cell. CV: central vacuole, EC: endosperm cell, FN: free nucleus, N: nucleus, Nc: nucellus, NP: nucellar projection, Pe: pericarp, SG: starch granule, VB: vascular bundle. 500 μm (a, e, i and m), 100 μm (b, c, f, g, j, k, n and o) and 20 μm (d, h, l and p)

The cellularization stage occurred at 3–4 DAP (Figs 1i–1p). The anticlinal wall was initiated and was joined to the surrounding embryo sac wall. It grew towards the interior and became inserted between the free nuclei, in which an open-ended cell formed without a periclinal wall (Fig. 1l). Periclinal division followed and resulted in a double cell layer. The periphery cell completed cellularization and the interior daughter cell re-entered the above growth pattern and remained an open-ended cell (Fig. 1k). The cellularization process advanced from outside to inside until the endosperm cells completely filled the embryo sac. Eventually the central vacuole disappeared (Fig. 1m).

Development of SECs

The wheat SEC was irregularly shaped and contained two kinds of starch granules: the large A-type starch granules and the small B-type starch granules. SEC size increased as the number of development days increased. The growth rate of SECs at 16–24 DAP was faster than during other periods. The long axis, short axis, area and perimeter changes for SECs produced an S-curve over the complete period of endosperm development (Fig. 2).

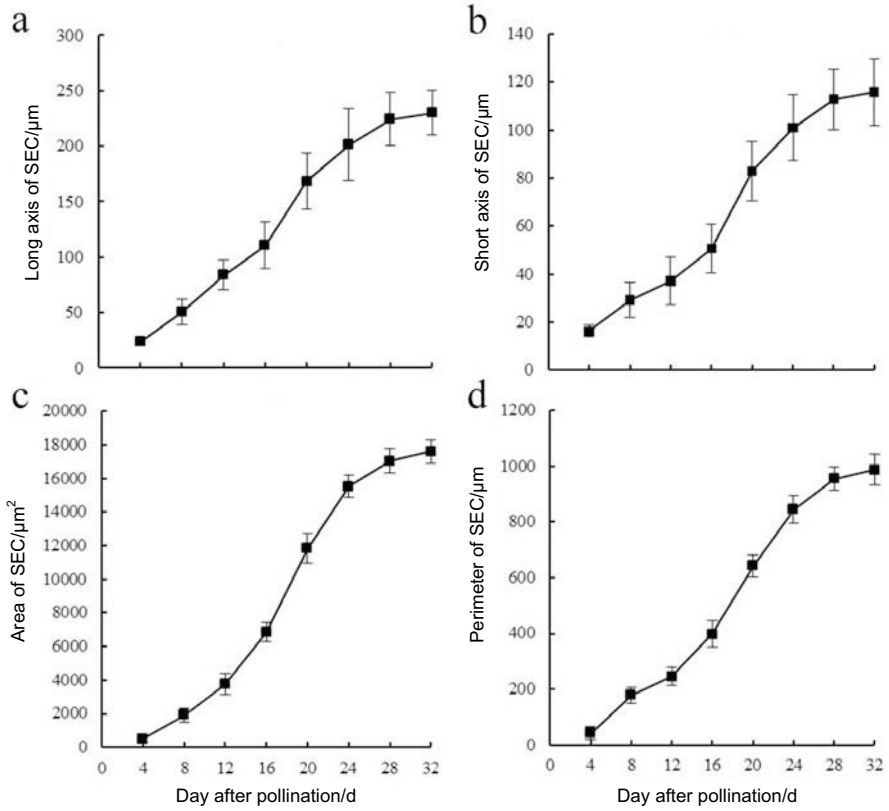

Figure 2. Growth curves of SECs in WanMai33

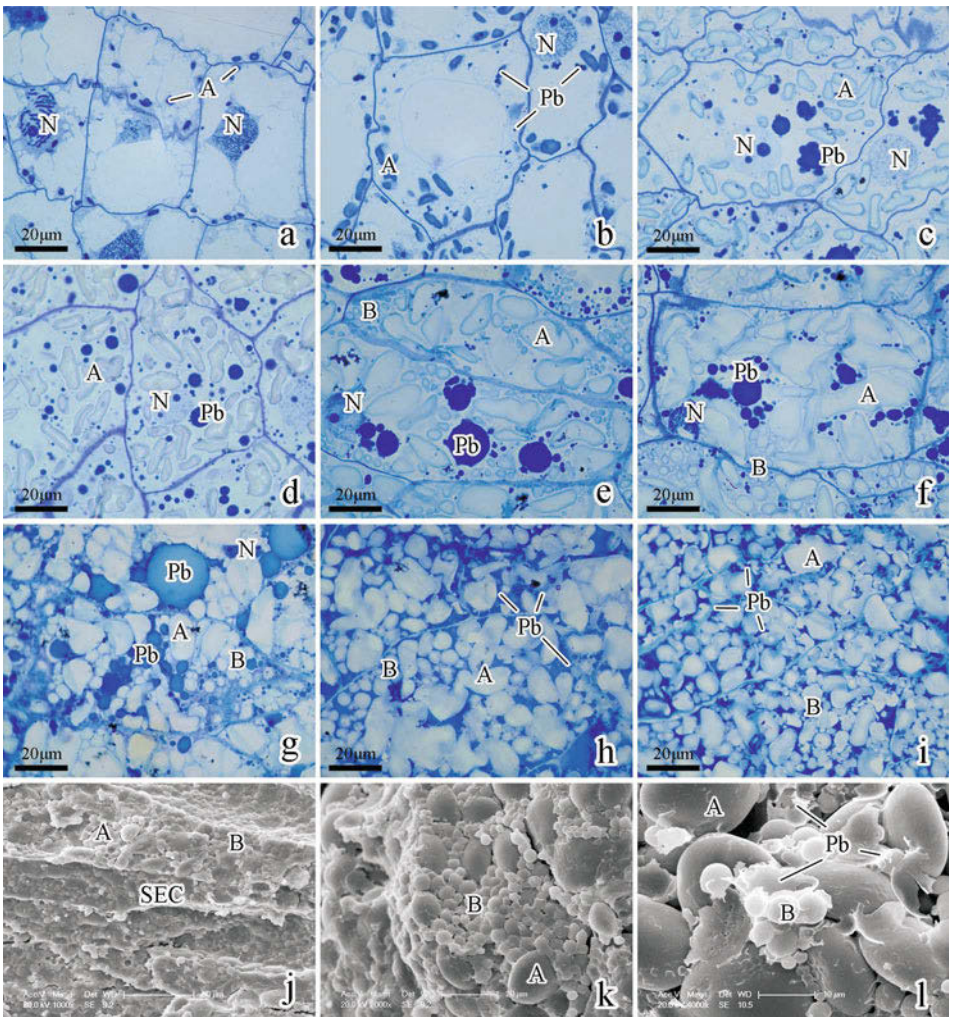

Figure 3. Development of SECs in WanMai33

Notes: (a–i) Resin semi-thin sections of SECs at 4, 8, 12, 14, 16, 20, 24, 28 and 32 DAP, respectively; (j–l) the SEM of the mature caryopsis. (j) The SECs. (k) The starch granules. The A-type starch granules were oval-shaped and the B-type starch granules were spherical. (l) The protein bodies remained on the surface of the starch granules or between the starch granules. A: A-type starch granule, B: B-type starch granule, N: nucleus. Pb: protein body, SEC: starch endosperm cell. 50 μm (j), 20 μm (a–i, k), 10 μm (l)

As the SECs grew, nutrients, such as starch and protein, were gradually synthesized and accumulated within the SECs. The starch was stored in the starch granules and the protein was stored in the protein bodies. Very few starch granules had developed by 4 DAP (Fig. 3a). The protein bodies appeared at 8 DAP and could be stained dark blue by TBO (Fig. 3b). The newly formed small protein bodies mixed together and generated

large protein bodies (Figs 3c–3g). The protein bodies had changed shape, but remained in the gap between the starch granules because of extrusion by the starch granules (Figs 3h and 3i). The SECs were fully filled by starch granules and protein bodies by the mature stage. The B-type starch granules were distributed in the gap between the A-type starch granules and were spherical-shaped, whereas the A-type starch granules were oval-shaped. The modified protein bodies were distributed on the surface or in the gaps between the starch granules (Figs 3h–3l).

The SEC nuclei were deformed at 16 DAP and gradually degraded as the growth and proliferation of starch granules increased (Figs 3e–3i). SEC metabolism was gradually reduced and then underwent Programmed Cell Death (PCD). Eventually, the nuclei disappeared and the SECs became dead storage cells (Figs 3i and 4).

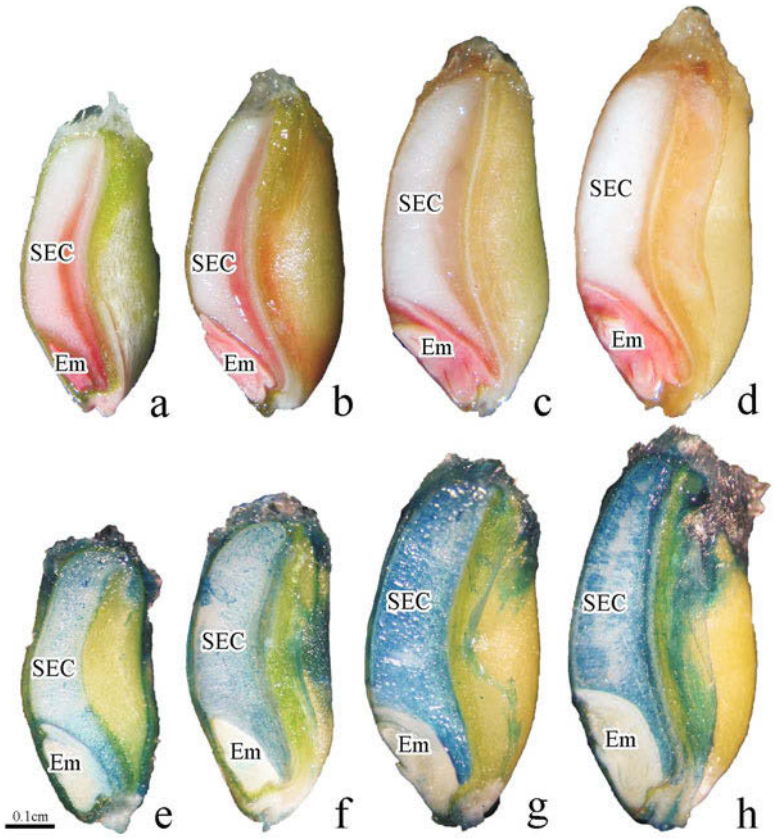

Figure 4. Histochemical assays of WanMai33 caryopses

Notes: (a–d) The caryopses at 16, 20, 28 and 32 DAP, respectively, were cut longitudinally from the center and stained with TTC. The dehydrogenase activity in SECs decreased as the number of developmental days increased. (e–h) The caryopses at 16, 20, 28 and 32 DAP, respectively, were cut longitudinally from the center and stained with Evans blue. 0.1 cm (a–h)

The two kinds of starch granules changed considerably during SEC development. The A-type starch granules were synthesized at 4 DAP and quickly proliferated between 4 and 16 DAP (Figs 3a–3e and 5a). The proliferation rate changed slowly, but the starch granules continued to grow after 16 DAP, and the diameter of the A-type starch granules reached 30 μm or above during the later stages (Figs 5a and 5b). The B-type starch granules appeared at 16 DAP and quickly increased in numbers (Figs 3e–3i and 5c). The B-type starch granules accounted for more than 90% of total number of starch granules in SECs after 28 DAP (Fig. 5d). The proliferation and growth changes in A-type and B-type starch granules showed an S-curve during endosperm development (Fig. 5).

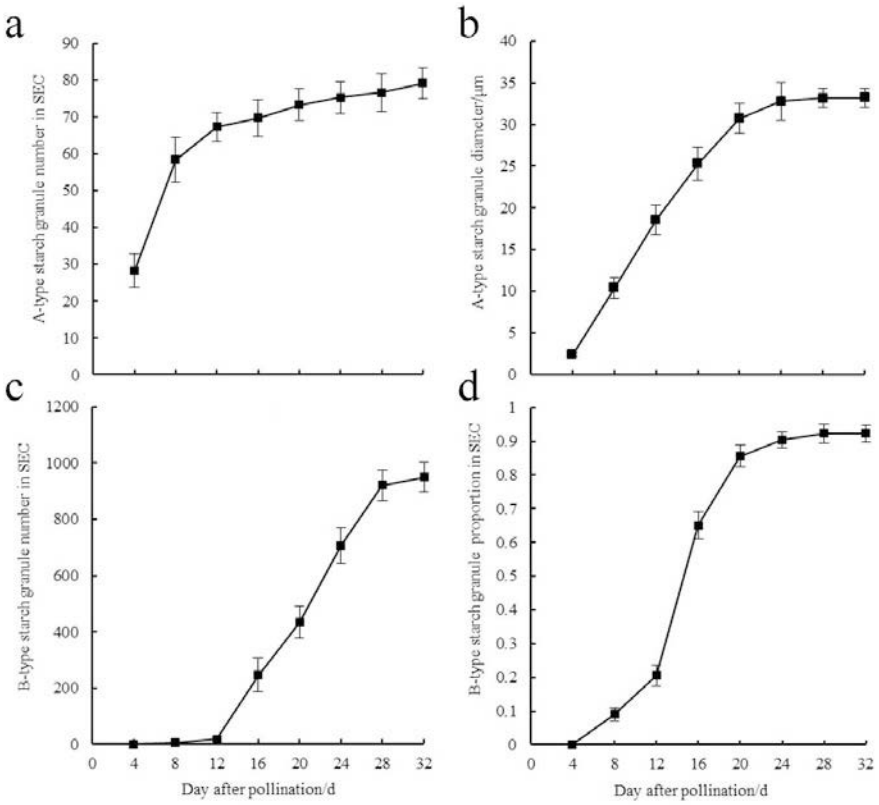

Figure 5. The proliferation and growth curve for starch granules in WanMai33

Discussion

The relationship between endosperm development and caryopsis development

The development of the caryopsis is divided into the formation stage, the milk stage, the ripening stage and the full ripeness stage (Hong et al. 2011). The free nuclear stage and

cellularization stage are collectively referred to as the formation stage of endosperm development, which corresponds to the formation stage of caryopsis development. The free nuclear stage occurred at 0–2 DAP and at this point the proliferated free nuclei were dispersed in the cytoplasm of the central cell and there was no cell wall. The endosperm cells relied on anticlinal division and periclinal division to complete cellularization. The differentiation stage of endosperm development corresponds to the milk stage of caryopsis development. During this period, the endosperm cells proliferated and grew. Nutrients, such as starch and protein, were also synthesized and accumulated in the endosperm cells. The maturation stage of endosperm development is equal to the ripening stage and full ripeness stage of caryopsis development. During the maturation stage, the SECs were completely filled by starch granules and protein bodies, the SEC nuclei disappeared and the SECs became dead storage cells.

Wheat caryopsis size gradually increased as development progressed and the SEC numbers and sizes determined the caryopsis. The three types of cell cycles: acytokinetic mitosis, mitosis coupled to cell division and endo-reduplication, all affected SEC development (Sabelli and Larkins 2009a) and cell division determined the number of SECs. Wang et al. (2003) showed that wheat endosperm cells rapidly proliferated after formation and that cell numbers stabilized at 15 DAP. They also showed that cell growth without division and nutrient accumulation were the main cell activities in endosperm after 15 DAP. In this study, we observed that SECs continued to grow after formation and the growth rate increased rapidly between 16 and 24 DAP. We suggest that the proliferation and growth of SECs were the main reason for the increased caryopsis size during the early and middle stages of caryopsis development. However, the numbers of SECs stabilized during the later stages and at that point, only the growth of SECs determined the caryopsis size. Endo-reduplication is thought to be correlated with the rapid growth of SECs and allows large cell sizes by circumventing the need for cytokinesis and the accompanying synthesis of membranes, cell walls and other cellular components (Sabelli and Larkins 2009a; Becraft and Gutierrez-Marcos 2012). Sabelli et al. (2009b, 2013) suggested that the retinoblastoma-related genes controlled and regulated gene expression of endo-reduplication and it regulated SEC growth in maize. However, it was not known whether the retinoblastoma-related genes regulate the growth of SECs in wheat.

The programmed cell death (PCD) of SECs

PCD is critical in plant tissue development. Generally, the vacuoles of cells ruptured and released hydrolytic enzymes that degraded various organelles and the inclusions evacuated from cells during the PCD process. The SEC nuclei degraded during endosperm development in cereals, whereas synthesis metabolism in the SECs increased. This phenomenon has been reported as a special PCD process (Young and Gallie 1999, 2000). The PCD in wheat SECs involved nuclear degeneration and subsequent de-nucleated cell development. The process of nucleus degeneration showed deformation, chromatin condensation, nuclear envelope disruption, and nuclear residue formation from the degenerative nucleus, during the PCD process. However, starch synthesis enzyme in SECs showed high activity and the starch granules and protein bodies could still proliferate and grow (Chen

et al. 2012). In this study, we have shown that SEC nuclei began to degrade at 16 DAP and disappeared during the mature stage, whereas the starch granules and the protein bodies completely filled the SECs. As a result the extruding force between the starch granules and protein bodies in the SECs increased as their numbers rose. We suggest that the extruding force increase in the SECs may have caused the nuclei to deform and eventually disappear, which might have acted as a signal to cell metabolism and caused PCD in SECs.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant no. 3107134, 31270228, 31171482), the National Natural Science Foundation of Jiangsu province (grant no. BK2011445), Jiangsu Graduate Innovation Project (CXLX12-0910), Priority Academic Program Development from Jiangsu Government and the Research Fund for the Doctoral Program of Higher Education of China (grant no. 2009320004).

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