JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

High Temperature during Grain Fill Alters the Morphology of Protein and Starch Deposits in the Starchy Endosperm Cells of Developing Wheat (*Triticum aestivum* L.) Grain

William J. Hurkman* and Delilah F. Wood

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710, United States

ABSTRACT: High temperature during grain fill reduces wheat yield and alters flour quality. Starchy endosperm cell morphology was investigated in wheat (*Triticum aestivum* L. 'Butte 86') grain produced under a 24/17 or 37/28 °C day/night regimen imposed from anthesis to maturity to identify changes in cell structure related to the functional properties of flour. The duration of grain fill decreased substantially under the 37/28 °C regimen, but, like the 24/17 °C regimen, endosperm cells in the mature grain were packed with starch and protein. However, A-type starch granules increased in number, decreased in size, and exhibited pitting; B-type granules decreased in both number and size; and the protein matrix was proportionally greater in endosperm cells of grain produced under the 37/28 °C regimen. Such changes in starch granule number, size, and structure and in protein amount are known to contribute to variations in wheat flour quality.

KEYWORDS: endosperm, grain fill, high temperature, starch granules, protein bodies, *Triticum aestivum* L., wheat

INTRODUCTION

High temperature during wheat grain fill is a significant environmental factor that negatively affects yield and quality.[1] Starch and gluten proteins deposited in the starchy endosperm cells (henceforth "endosperm") are major determinants of these important agronomic traits. Starch accounts for 65−75% and gluten protein for 10−15% of the dry weight of the mature wheat grain. These provide carbohydrates and amino acids that are essential for germination and early seedling growth and are primary sources of nutrition for humans and livestock. Wheat starch is composed of 25−30% amylose and 70−75% amylopectin. Both are polymers of D-glucose, but amylose is linear and lightly branched, whereas amylopectin is much larger and highly branched. Starch is utilized as an ingredient in foods and in industrial products such as paper, adhesives, cosmetics, bioplastics, and biofuel. The gluten proteins, a mixture of monomeric gliadins and polymeric glutenins, provide the extensibility and elasticity properties unique to wheat flour dough that make possible the production of a wide variety of baked goods. To develop a comprehensive picture of the effect of high temperature on the wheat grain, our group is defining the effects of a 37/28 °C day/night regimen on starch and protein composition in grain produced by 'Butte 86', a hard red spring wheat. The 37/28 °C regimen is more severe than might be encountered under field conditions, but was selected to accentuate high-temperature responses. This temperature regimen shortens the duration of grain fill,[2] reduces starch and protein amount per grain,[3,4] decreases levels of enzymes involved in starch biosynthesis,[4] and alters the relative amounts of specific gliadins, high molecular weight glutenin subunits (HMW-GS), and low molecular weight glutenin subunits (LMW-GS).[5]

In this study, we investigate the effect of high temperature on starch granule formation and protein deposition in the endosperm cells of the developing wheat grain. Previous studies[6−14] have described the ontogeny of starch granules, protein bodies, and matrix protein in wheat endosperm cells. Although high temperature during grain fill causes morphological and cellular damage to the mature grain,[15] systematic structural studies on the effect of high temperature on starch and protein accumulation during grain development are lacking. Preliminary scanning electron microscopy (SEM) observations on the effect of high temperature on endosperm cell structure in a previous paper[16] are extended in this study. Because protein bodies and starch granules are difficult to distinguish in scanning electron micrographs, light microscopy and centrifugation were combined with SEM to assist in the identification of these structures. Starch and protein accumulation were compared in developing endosperm cells in grain produced under moderate-temperature (24/17 °C) and high-temperature (37/28 °C) regimens to identify distinctive differences that may be related to the potential for variations in flour quality.

MATERIALS AND METHODS

Plant Material. *Triticum aestivum* L. 'Butte 86' was grown in matching climate-controlled greenhouses under moderate- and high-temperature regimens. For the moderate-temperature regimen, plants were grown at a maximum daytime temperature of 24 °C and a minimum nighttime temperature of 17 °C (24/17 °C). For the high-temperature regimen, plants were transferred at anthesis to a second greenhouse and grown at a maximum daytime temperature of 37 °C and a minimum nighttime temperature of 28 °C (37/28 °C). Equivalent water and fertilizer (300 mg/day of Plantex 20−20−20, Plant Products Co. Ltd., Brampton, ON, Canada) levels were applied by drip irrigation

Received: July 30, 2010
Revised: February 14, 2011
Accepted: February 22, 2011
Published: March 21, 2011

under both temperature regimens. Natural light was supplemented with 100 W high-pressure sodium lamps to maintain a day length of 16 h. Grain produced from two experiments was collected for analysis by light and scanning electron microscopy. Heads were harvested at 4 day intervals beginning at 6 days postanthesis (DPA) from plants grown under the 24/17 °C regimen. Because high temperature substantially shortened the duration of grain fill, heads were harvested at 2 day intervals beginning at 6 DPA from plants grown under the 37/28 °C regimen. Grain was collected, weighed, placed directly into fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.9), and stored at 4 °C. For starch granule isolation, heads were harvested at 10 day intervals from 10 to 40 DPA from plants grown under the 24/17 °C regimen and at 4 day intervals from 6 to 18 DPA from plants grown under the 37/28 °C regimen.

Starch Granule Isolation. Starch granules were isolated from freshly harvested grain using a modification of the amyloplast isolation procedure of Balmer et al.[17] At each time point, grain was removed from one head and the region containing the embryo excised with a razor blade. Endosperm was squeezed through the resultant opening in the pericarp/testsa and placed in a beaker containing 20 mL of ice-cold (4 °C) buffer (50 mM HEPES−NaOH, pH 7.5). The buffer was poured off and endosperm transferred to a plastic Petri dish containing 6 mL of plasmolysis buffer [50 mM HEPES, pH 7.5, and 1 mM EDTA supplemented with a protease inhibitor cocktail (Complete Mini, Roche Applied Science, Indianapolis, IN)]. Following incubation on ice for 1 h, endosperm was chopped for 5 min using an electric knife with the blades replaced by holders fitted with single-edge razor blades. The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) moistened with plasmolysis buffer and gently pipetted into a 15 mL conical tube containing 4 mL of 2% Nycodenz (Nycomed, Oslo, Norway) in plasmolysis buffer layered over a 2 mL, 1% agar pad. Following centrifugation for 8 min at 30*g* and 4 °C (centrifuge 5810 R, Eppendorf, Westbury, NY), the supernatant was removed by aspiration and discarded. The pellet was suspended in plasmolysis buffer and centrifuged through Nycodenz once more, and the resulting pellet was rinsed three times with 5 mL of ice-cold deionized water (Milli-Q UF Plus, Millipore Corp., Billerica, MA). The final pellet was suspended in 1 mL of fixative and stored at 4 °C.

Light Microscopy. Fixed grain was rinsed three times with 0.1 M sodium cacodylate buffer, pH 6.9, and dehydrated in a graded series of ethanol−butanol (30% ethanol, 40% ethanol/10% butanol, 50% ethanol/20% butanol, 50% ethanol/35% butanol, 25% ethanol/75% butanol, 100% butanol) with at least 30 min per exchange. Samples were infiltrated and embedded in glycol methacrylate resin according to the manufacturer's protocol (Technovit 7100, Heraeus Kulzer GmbH, Germany, imported by Electron Microscopy Sciences, Hatfield, PA). The blocks were sectioned with a rotary microtome (Leica RM 2265, Leica Microsystems GmbH, Wetzlar, Germany), and 2 μm thick sections were collected on microscope slides. The sections were stained for protein with 0.01% acid fuchsin in 1% acetic acid for approximately 1 min. The sections were dried, mounted in immersion oil, and photographed using a fluorescence compound microscope (Zeiss Research Microscope, Carl Zeiss MicroImaging, Inc., Thornwood, NY) with a blue filter set (excitation, 450−490 nm; barrier, long pass 515 nm). The areas occupied by protein matrix (red fluorescence) and starch granules plus cell walls (black silhouettes) were determined in endosperm cells of 10 images of cross sections of mature grain produced under the two temperature regimens using Progenesis (Nonlinear Dynamics Limited, Newcastle upon Tyne, U.K.). Starch granule preparations were assessed for purity by acid fuchsin staining and birefringence of granules under polarized light. Aliquots of starch granule preparations were spread onto slides and allowed to air-dry. Identical fields of each starch preparation were viewed and photographed using a microscope fitted with Hoffman modulation contrast optics (http://www.olympusmicro.com/primer/techniques/hoffman.html) and crossed polarizers. Digital images were collected using a QImaging Retiga 2000R FAST color camera (Surrey, BC, Canada).

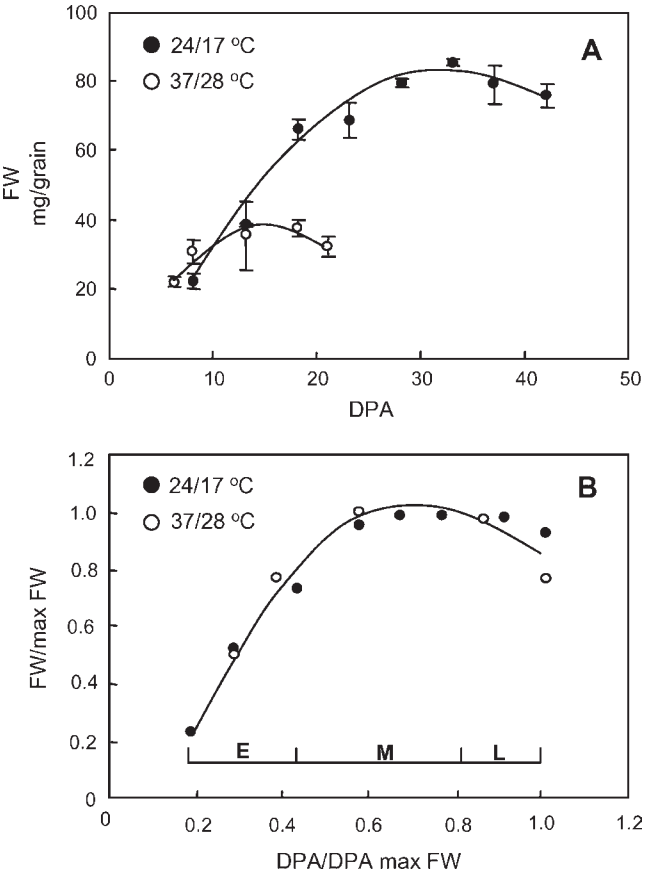

Figure 1. Effect of high temperature on wheat grain fresh weight. Plants were grown under two day/night temperature regimens: 24/17 °C from germination to grain maturity or 24/17 °C from germination to anthesis and 37/28 °C to grain maturity; (A) fresh weight (FW) plotted as a function of grain developmental age (DPA, days postanthesis); (B) FW and DPA normalized for the two temperature regimens to show the relationship of the early (E), middle (M), and late (L) stages of grain development between the two temperature regimens.

Scanning Electron Microscopy. Fixed grain was rinsed three times in 0.1 M sodium cacodylate buffer, pH 6.9, and dehydrated in a graded series of ethanol (30, 50, 70, 95, and 3 × 100%) with 20 min per exchange. The grain was fractured in liquid nitrogen using a prechilled razor blade held in locking pliers. The pieces were collected with a chilled tweezers, placed in 100% ethanol, and dried in a critical point dryer (Tousimis Autosamdri-815, Rockville, MD). The pieces were mounted onto aluminum specimen stubs using a mixture of carbon graphite (Conductive Graphite Adhesive, water-based, Electron Microscopy Sciences) and two-component epoxy (Loctite Extra Time, Henkel, Düsseldorf, Germany) at approximately 1:1:1. The mounting medium was allowed to dry and outgas, and the samples were coated with gold−paladium in sputter coating unit (Denton Desk II, Denton Vacuum, Inc., Moorestown, NJ). The coated samples were viewed and photographed in a field emission scanning electron microscope (S4700, Hitachi High-Technologies Corp., Tokyo, Japan).

RESULTS

Fresh Weight and Equivalent Developmental Stages. The 37/28 °C regimen substantially shortened the duration of grain

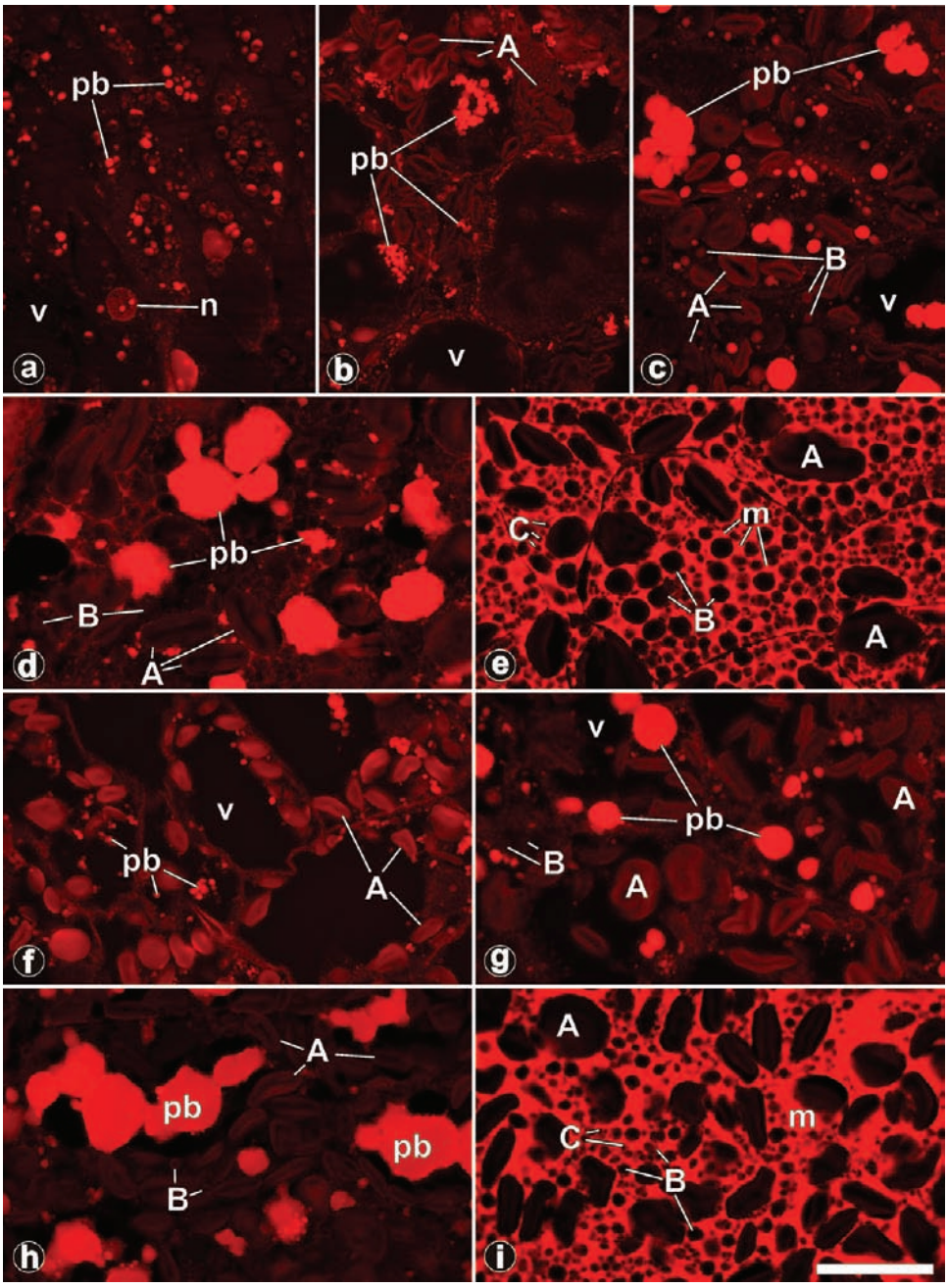

Figure 2. Fluorescence micrographs of protein accumulation in developing wheat endosperm cells under moderate- and high-temperature regimens. Grain cross sections were stained for protein with acid fuchsin. Grain was collected 6 (a), 10 (b), 14 (c), 26 (d), and 42 (e) DPA under the 24/17 °C regimen. Grain was collected at 6 (f), 8 (g), 10 (h), and 24 (i) DPA under the 37/28 °C regimen. A, A-type starch granule; B, B-type starch granule; C, C-type starch granule; m, matrix protein; n, nucleus; pb, protein body; v, vacuole. Scale bar = 50 μm for a−i.

fill and decreased fresh weight/grain (Figure 1A). Grain matured by 21 DPA under the 37/28 °C regimen compared to 42 DPA under the 24/17 °C regimen. Although mature grain produced under the 37/28 °C regimen was much smaller, the germination rate was 100% (unpublished observation). Maximal fresh weight/grain was 38 mg and final fresh weight/grain was 32 mg under the 37/28 °C regimen compared to 85 and 75 mg, respectively, under the 24/17 °C regimen. To distinguish developmental changes from the effects of high temperature, equivalent developmental stages for the two temperature regimens were determined using fresh weight.[18,19] The early stage of grain fill begins at anthesis and ends when the grain attains half-maximum fresh weight, the middle stage ends when the grain attains maximum fresh weight, and the late stage ends at grain maturity. These stages correspond to late milk (start of grain fill), soft dough (maximum fresh weight), and hard kernel (ripening) stages of grain development.[20] Because the duration of grain fill was reduced by high temperature, fresh weight was normalized with respect to maximum fresh weight (fresh weight/maximum fresh weight) and DPA with respect to maximum DPA (DPA/maximum DPA) and plotted (Figure 1B). This analysis showed that 6−18, 22−34, and 38−42 DPA under the 24/17 °C

regimen corresponded to 6−8, 10−14, and 16−24 DPA under the 37/28 °C regimen. Comparison of light and scanning electron micrographs (Figures 2, 4, and 5) also showed that endosperm cells were structurally similar for these early, middle, and late developmental stages under the two temperature regimens.

Fluorescence Microscopy: 24/17 °C. Protein accumulation in central endosperm tissue sections was examined using fluorescence microscopy (Figure 2). When sections were stained with acid fuchsin, a protein-specific stain, protein deposits fluoresced brightly and were easily distinguishable from other endosperm cell components. At 6 DPA under the 24/17 °C regimen, organelles and cytoplasm were located at the cell periphery, surrounding large central vacuoles (Figure 2a). Nuclei were the largest organelles in the endosperm cells and contained brightly fluorescing nucleoli. Nonfluorescing amyloplasts, although difficult to discern in the figures, contained large lenticular A-type starch granules that occupied the majority of the cell interior. Numerous highly fluorescent protein bodies approximately 1.2−3.6 μm in diameter were present in both the cytoplasm and vacuoles. Endosperm cell structure was similar at 10 DPA, except that a large proportion of protein bodies were located in clusters within the vacuoles (Figure 2b). At 14 DPA, vacuoles were smaller as a consequence of substantial increases in protein bodies and deformation by intrusion of starch granules. A second population of spherical B-type starch granules, nonfluorescing and difficult to discern in the figures, was evident at this developmental stage. The granules were approximately 2.5−5 μm in diameter and, in keeping with their origin within amyloplasts, were located in close proximity to the A-type starch granules (Figure 2c). Protein bodies, some as large as 19 μm in diameter, were located within the vacuoles. From 18 to 34 DPA, vacuolar protein bodies continued to increase in size and fuse to form large, irregularly shaped aggregates (Figure 2d). Smaller protein bodies were also present in the cytoplasm, forming a protein network around the starch granules. From 36 to 42 DPA, protein accumulation was rapid, filling endosperm cells with a continuous matrix that completely surrounded the starch granules (Figure 2e). Individual protein bodies were not detected at this stage of development, indicating that the protein matrix formed by coalescence of the protein deposits scattered throughout the cell. A- and B-type starch granules as well as C-type starch granules, which form late in development, and cell walls were conspicuous as black silhouettes against the red fluorescence of the protein matrix. B-type starch granules were approximately 6.3−11.3 μm in diameter, and C-type starch granules were approximately 5 μm or less in diameter. Computer analysis of micrographs of endosperm cross sections at this stage of development revealed that starch granules and cell walls comprised 89.4 ± 0.6% and the protein matrix 10.6 ± 0.6% of cell area.

Fluorescence Microscopy: 37/28 °C. Although grain matured much more quickly under the 37/28 °C regimen, protein accumulation followed a pattern similar to that described for the 24/17 °C regimen. At 6 DPA, endosperm cells contained large central vacuoles, nonfluorescing A- and B-type starch granules, and small fluorescing protein bodies present singly or in clusters (Figure 2f). At 8 DPA, the protein bodies within the vacuoles and A-type starch granules were substantially larger (Figure 2g). At 10 DPA, vacuolar protein bodies had fused together to form large, irregularly shaped aggregates (Figure 2h). At 24 DPA, endosperm cells consisted primarily of A-, B-, and C-type starch granules embedded within a continuous protein matrix (Figure 2i). Starch granule populations were different from those in the endosperm of grain produced under the 24/17 °C regimen. The A-type starch granules were smaller and more numerous, and the B-type granules were also smaller, but less numerous (compare Figures 2e,i). Computer analysis of micrographs of endosperm cells at this stage of development showed that starch granules and cell walls comprised 79.8 ± 1.2% of endosperm cell area and the protein matrix 20.02 ± 1.3%. Compared to the analysis for the 24/17 °C regimen, the protein matrix was nearly 10% greater in cross sections of endosperm cells in grain produced under the 37/28 °C regimen.

Identification of Components in Scanning Electron Micrographs of Endosperm Cells. Grain was fixed in formaldehyde and glutaraldehyde, reagents that cross-link proteins and stabilize fine structure. Cell walls, cytoplasm, vacuoles, nuclei, A- and B-type starch granules, and protein bodies were identifiable in fluorescence micrographs of endosperm cell cross sections (Figure 3a). Nuclear membranes appeared to be preserved, whereas amyloplast, vacuolar, and RER membranes were not. This is expected because aldehydes do not react strongly with lipids, resulting in loss of cellular membranes during the dehydration and embedding steps of sample preparation. Fluorescence micrographs of endosperm cells stained with acid fuchsin also revealed that small protein bodies in the cytoplasm appeared to be associated with protein-staining strands. Resolution was not sufficient to unequivocally identify these structures as matrix protein originating in the RER, the site of synthesis of the proteins that form protein bodies.

SEM images not only provide higher resolution of cell components but reveal three-dimensional structure and surface topology. The identities of endosperm cell structures in SEM images emerged when light and scanning electron micrographs were compared. Like the fluorescence micrographs, SEM images of endosperm cells contained an extensive protein network interspersed with vacuoles and protein bodies (Figure 3b). However, resolution was not sufficient to determine whether this protein network was of cytoplasmic or RER origin (Figure 3c). Consistent with fluorescence micrographs, SEM images revealed that large protein bodies in the vacuoles were composed of aggregates of various sized protein bodies (Figure 3d).

Starch granules were isolated from developing grain and examined by light and scanning electron microscopy to verify their identities. The purity of the preparations was assessed by fluorescence and polarized light microscopy. None of the particles fluoresced with acid fuchsin staining (Figure 3e), indicating the absence of protein bodies in these preparations, and all exhibited the cross-like birefringence under polarized light that is a characteristic of the crystalline structure of starch granules (Figure 3f). Scanning electron micrographs of endosperm cells showed that the A-type starch granules were the most abundant and largest structures in endosperm cells. The unique topology of these granules (flattened oblate spheroids with prominent equatorial grooves and, often, semispherical surface protrusions) also contributed to their identification (e.g., Figures 4a−d). B-type starch granules were spherical in shape and readily distinguishable from A-type starch granules. Starch granules isolated from grain during late development under the 24/17 °C regimen showed that many of the B-type granules had angular surfaces (Figure 3g), which allowed them to be identified in endosperm cells at 26−33 DPA (Figures 4f,g). Irregularly shaped starch

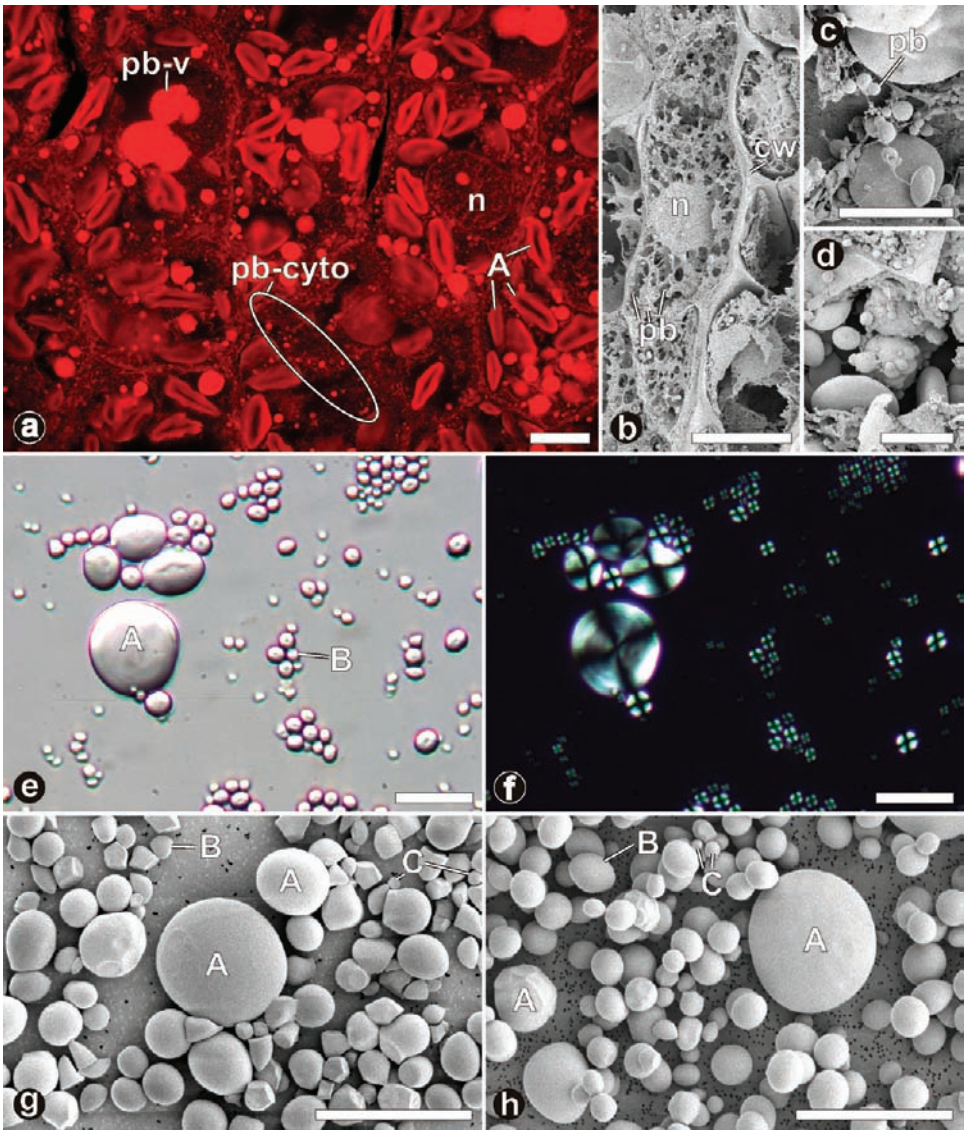

Figure 3. Identification of protein bodies and starch granules in wheat endosperm cells. (a) Fluorescence micrograph of an endosperm tissue cross section from grain collected at 14 DPA under the 24/17 °C regimen; section was stained for protein with acid fuchsin. (b) Scanning electron micrograph of an endosperm cell from grain collected at 6 DPA under the 37/28 °C regimen. (c) Scanning electron micrograph of protein bodies in an endosperm cell from grain collected at 14 DPA under the 24/17 °C regimen. (d) Scanning electron micrograph of protein body clusters within a vacuole in an endosperm cell from grain collected at 10 DPA under the 37/28 °C regimen. (e) Light micrograph of starch granules isolated from grain collected at 14 DPA under the 37/28 °C regimen, stained with acid fuchsin, and then visualized by Hoffman Modulation Contrast optics. (f) Light micrograph of sample in e, but visualized by polarized light microscopy. (g) Scanning electron micrograph of starch granules isolated from grain collected at 29 DPA under the 24/17 °C regimen. (h) Scanning electron micrograph of starch granules isolated from grain collected at 14 DPA under the 37/28 °C regimen. A, A-type starch granule; B, B-type starch granule; cw, cell wall, cyto, cytoplasm; n, nucleus; pb, protein body; pb-cyto, protein body in cytoplasm; pb-v, protein body within vacuole. All scale bars = 20 μm.

granules were not common in starch preparations from grain produced under the 37/28 °C regimen (Figure 3h). Thus, B-type starch granules were distinguishable from the smaller protein bodies in endosperm cells at 12−14 DPA (Figure 5d,e), but not later in development, especially for protein bodies that were similar in size to the B-type starch granules (Figures 4i and 5h). The small C-type starch granules, which form later in development than the A- and B-type starch granules, could not be distinguished from small protein bodies in scanning electron micrographs of endosperm cells. Their presence was confirmed in starch granule preparations (Figure 3g,h) and fluorescence micrographs (Figure 2e,i).

Early in grain development, protein bodies were smaller than B-type starch granules (Figure 4b), closely associated with the protein network (Figures 3c and 4d), and organized in strands or clusters (Figures 4a−d). During middle development, protein bodies formed large vacuolar aggregates, which were composed of various sized protein bodies that appeared to be fusing together (Figure 3d). These aggregates were difficult to detect late in development, because they were masked by increasing levels of starch granules and by fusion of protein bodies to form increasingly large protein masses.

SEM: 24/17 °C. At 6 DPA under the 24/17 °C regimen, A-type starch granules of various sizes were the predominant

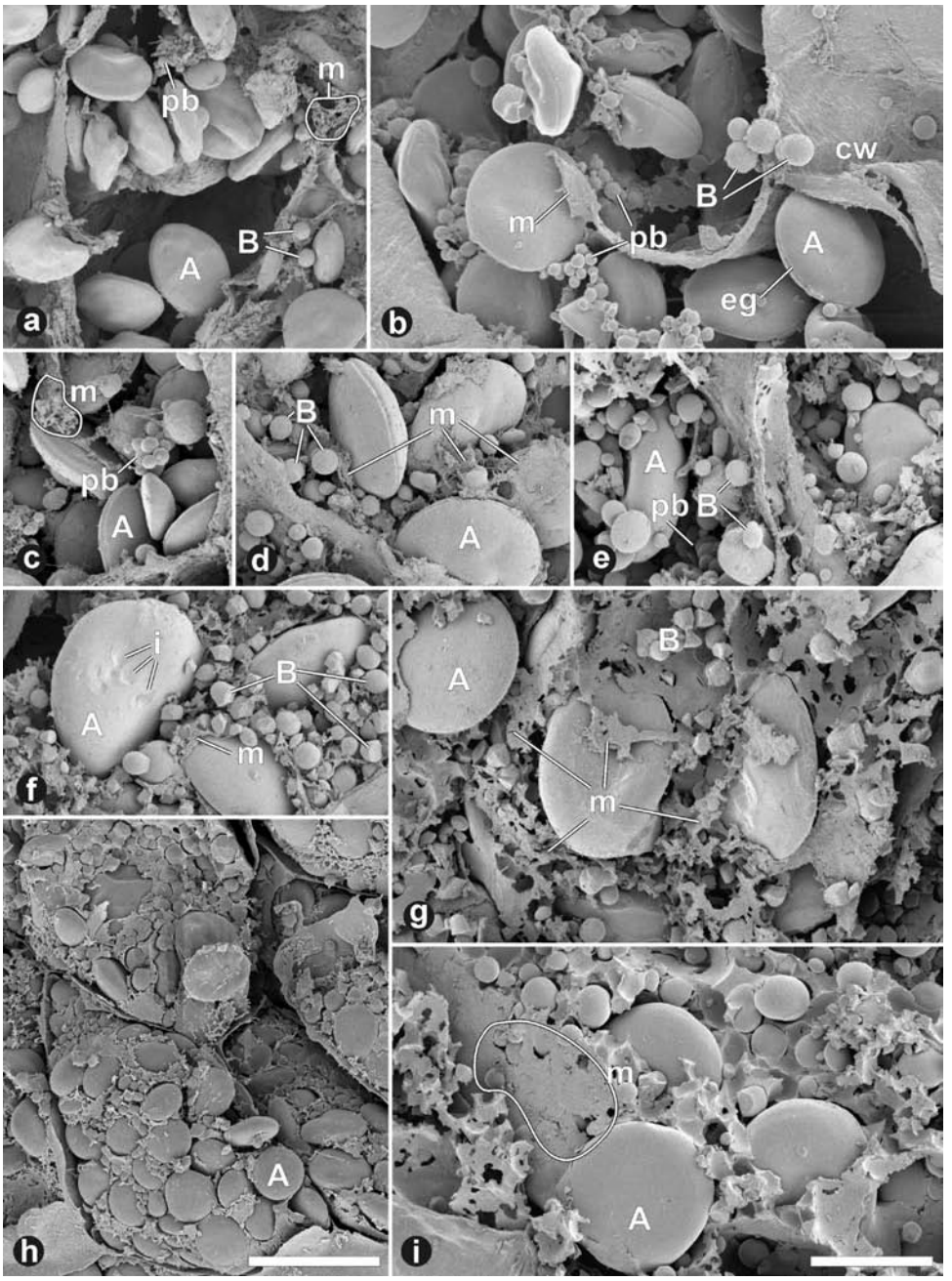

Figure 4. Scanning electron micrographs of developing endosperm cells in wheat grain produced under the 24/17 °C regimen. Images depict structures in endosperm cells in grain collected at 6 (a), 11 (b), 14 (c), 18 (d); 22 (e), 26 (f), 33 (g), and 40 (h, i) DPA. A, A-type starch granule; B, B-type starch granule; cw, cell wall; eg, equatorial groove; i, indentation; m, matrix protein; pb, protein body. Scale bar = 50 μm for h. Scale bar = 20 μm for a−g, i.

component in developing endosperm cells, B-type starch granules were present in low numbers, and protein strands were distributed throughout the cells (Figure 4a). By 11 DPA, protein bodies had increased in number and were organized in bead-like chains (Figure 4b). The protein bodies were 1.4−3.0 μm in diameter and smaller than B-type starch granules, which were 3.7−6.5 μm in diameter. At 14 DPA, protein body aggregates were present in vacuolar regions of the cells (Figure 4c). At 18 DPA, A-type starch granules were larger, B-type starch granules more numerous, and protein strands were more abundant (Figure 4d). From 22 to 26 DPA, B-type starch granules increased in number, and many were irregularly shaped (Figure 4e,f). During this developmental stage, the surface protrusions typical of A-type starch granules were largely absent, but surfaces often had indentations (Figure 4f). Together with the angular surfaces of the B-type starch granules, they suggest that A- and B-type starch granules were tightly packed within the endosperm cells. Protein body aggregates were not easily identifiable during later stages of development due to the similarity in size of the protein bodies to B-type starch granules and their concealment by the increasing numbers of starch granules. The protein strands were more abundant from 18 to 26 DPA (Figure 4d−f) and by 33 DPA formed an extensive, porous network that encompassed the starch granules (Figure 4g). This

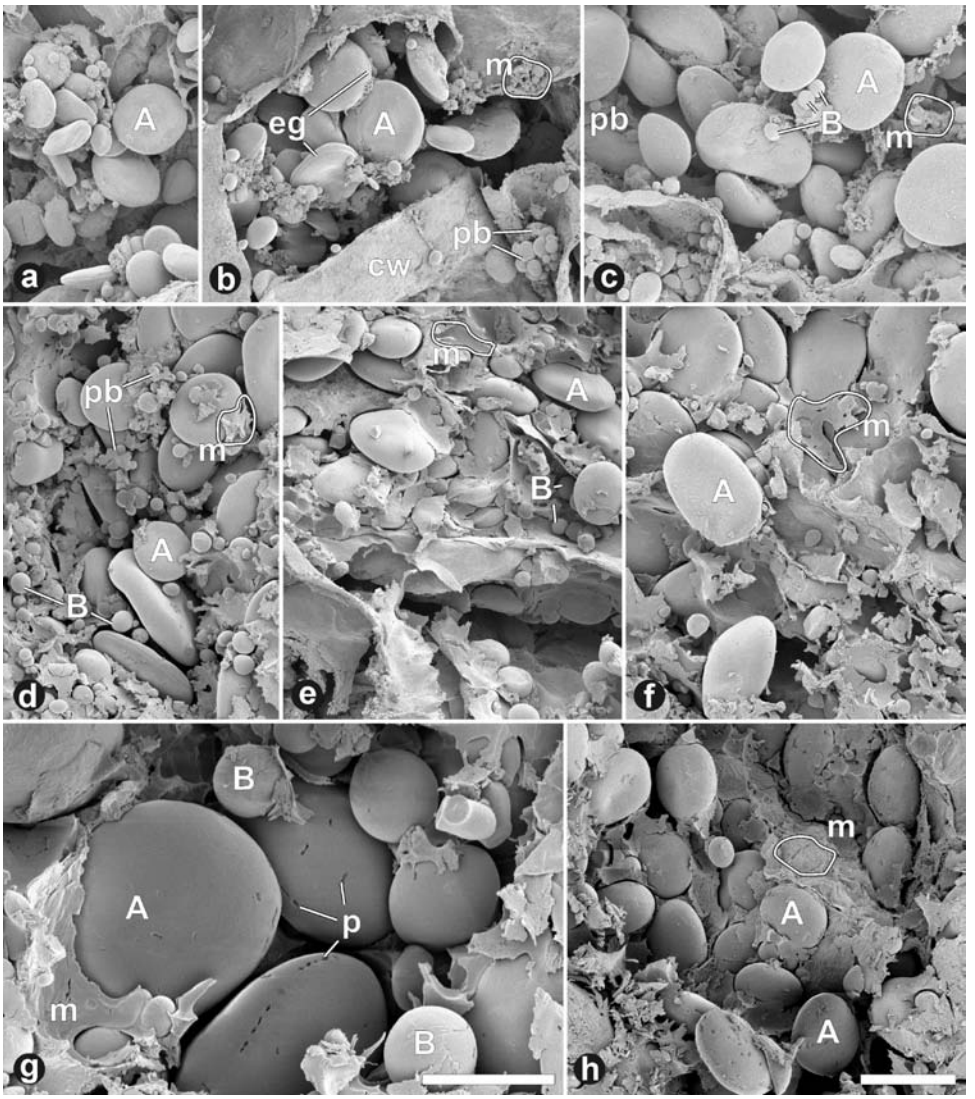

Figure 5. Scanning electron micrographs of developing endosperm cells in wheat grain produced under the 37/28 °C regimen. Images depict endosperm cells in grain collected at 6 (a), 8 (b), 10 (c), 12 (d), 14 (e), 16 (f, g), and 18 (h) DPA. A, A-type starch granule; B, B-type starch granule; cw, cell wall; eg, equatorial groove; i, indentation; m, matrix protein; p, pit; pb, protein body. Scale bar = 10 μm for g. Scale bar = 20 μm for a−f, h.

network continued to enlarge and expand to form swollen protein "sheets" that, by day 40, engulfed the starch granules (Figure 4h,i). On the basis of our fluorescence (Figure 2d) and SEM (Figure 4i) micrographs and the TEM images of Bechtel et al.,[8] we interpret this network to be composed of coalescing protein bodies (e.g., Figure 2i).

SEM: 37/28 °C. Endosperm cell development under the 37/28 °C regimen was similar to that for the 24/17 °C regimen. Starch granules increased in size and abundance, and the protein matrix increased in complexity and amount (Figure 5a−h). However, starch granule size, structure, and population distributions were altered in endosperm cells of mature grain produced under the 37/28 °C regimen. A-type starch granules were more numerous but smaller, and the B-type starch granules were less numerous and smaller (compare Figures 4I and 5h), observations in agreement with the fluorescence micrograph images (Figure 2e,i). In addition, the A-type starch granules were sometimes pitted, often at the equatorial groove (Figure 5g, h), a phenomenon not observed in starch granules formed under the 24/17 °C regimen. In keeping with the increase in relative amount of protein matrix in grain produced under the 37/28 °C regimen that was observed by fluorescence microscopy, protein bodies and starch granules were not as tightly packed together in the endosperm cells of mature grain. A-type starch granules were not marred by surface depressions under the 37/28 °C regimen, and the irregularly shaped B-type granules that formed under the 24/17 °C regimen were also absent under this temperature regimen.

DISCUSSION

Grain maturity under the 37/28 °C regimen was accelerated and accompanied by a substantial decrease in grain fresh weight. Earlier studies showed that dry weight and protein and starch contents per grain were also reduced by this high-temperature regimen.[2,4,5,21] The relationship between grain development and fresh weight made it possible to compare changes in endosperm cell morphology at equivalent stages for the two temperature

regimens. Despite the shortened duration of grain fill, starch and protein accumulation under the 37/28 °C regimen followed a pattern similar to that under the 24/17 °C regimen. A-and B-type starch granules formed early in development and increased in size during middle to late development, whereas C-type starch granules formed during middle and late development. Small protein bodies accumulated early in development and coalesced to form large irregularly shaped deposits within the vacuoles during middle to late development. Early in development, a network of protein strands formed throughout the endosperm cells. During middle to late development, this network increased in amount to form a continuous protein matrix that together with the starch granules became the principal components in the endosperm cells of mature grain.

Within this developmental program, starch granule number and size were altered by the 37/28 °C regimen. A-type starch granules were greater in number and smaller in size, and B-type starch granules were smaller in both number and size in the mature grain, findings in agreement with a previous study.[4] These changes were accompanied by an increase in total amylose content under the 37/28 °C regimen.[4] Variations in starch granule composition and ratios of A- to B-type starch granules are significantly correlated with changes in starch pasting viscosity,[22] dough mixing properties,[23] and bread crumb structure.[24] Under the high-temperature regimen, A-type starch granules were sometimes pitted, often at the equatorial groove. The reduction in wheat starch granule size and appearance of surface pitting was also observed when developing wheat grain was exposed to a 2 day, 36/31 °C regimen initiated 3 days after anthesis.[15] The pitting was reproduced when starch granules isolated from wheat[25] were incubated with α-amylase, supporting the hypothesis that pitting is due to the action of starch-degrading enzymes. Pitting of starch granules is common in endosperm cells of shriveled[26] or germinating[27] grain, processes that are accompanied by large increases in α-amylase activity. Another consequence of the 37/28 °C regimen is the increase in relative amount of protein matrix, which is consistent with the increase in protein content in flour milled from grain produced under this regimen.[5] With respect to breadmaking, flour milled from grain produced under the 37/28 °C regimen produced dough with decreased mixing tolerance and bread with increased loaf volume.[5]

The similarity in protein and starch deposition under the two temperature regimens suggests that the many genes involved in the coordinate regulation of these biosynthetic processes are also regulated by a mechanism that responds to high temperature. This suggestion is supported by proteome[18] and transcriptome[19,21] profiling studies in which the sequence of protein and transcript accumulation was maintained but accelerated by high temperature. Numerous biochemical and ultrastructural studies indicate that many genes are involved in protein and starch deposition. Ultrastructural studies show that protein body and matrix formation utilize two mechanisms for transport of storage protein aggregates from site of synthesis to site of deposition. Storage proteins are synthesized within the RER and transported to vacuoles via vesicles derived from the RER or via passage from the RER vesicles to Golgi apparatus vesicles.[6−14,28] Before reaching the vacuole, RER vesicles can fuse with one another or with Golgi apparatus vesicles to form larger protein bodies. The protein bodies enter vacuoles by autophagy, a process by which the protein body membrane fuses with the vacuolar membrane to deposit proteins into the vacuole interior. Protein bodies in the vacuoles fuse together to form irregularly shaped homogeneous protein masses scattered throughout the cell that ultimately merge to form a continuous matrix.[6,8] Whether protein is transported via the Golgi apparatus or the RER pathway depends on the developmental stage of the grain. Levanony et al.[11] suggested that transport via the Golgi apparatus was prominent early in development and that transport via the RER also occurred at this time, but was more prominent during middle development when protein deposition is maximal. This relationship between protein accumulation pathway and developmental stage was also reported by Loussert et al.[12] and Tosi et al.[28] In contrast to protein bodies, starch granules are synthesized within amyloplasts, specialized organelles that function in the synthesis and storage of starch. Amyloplasts are bound by a double-membrane envelope enclosing the stroma, which contains soluble proteins and starch granules. The A-type starch granules form in the stroma of the main amyloplast compartment, whereas B- and C-type starch granules form in tubular protrusions that emanate from the amyloplast into the cytoplasm.[9,29]

This study provides three-dimensional images of protein and starch deposition in developing wheat endosperm cells and new insights into the effects of high temperature on these complex processes. The known variations in flour quality associated with the morphological changes brought about by high temperature reinforce the need to develop wheat lines that maintain grain and flour quality regardless of environmental conditions during grain fill. An increased understanding of mechanisms that control the duration of grain fill; grain size; synthesis, size, and composition of A- and B-type starch granules; α-amylase biosynthesis; and gliadin and glutenin biosynthesis and composition is required to provide breeders with the necessary tools to develop wheat varieties with consistent end use quality.

AUTHOR INFORMATION

Corresponding Author

*Phone: 510-559-5720. Fax: 510-559-5818. E-mail: william.hurkman@ars.usda.gov.

DISCLOSURE

Mention of a specific product name by the U.S. Department of Agriculture does not imply recommendation over other suitable products.

ACKNOWLEDGMENT

We thank Tina G. Williams for processing the samples and capturing the SEM images.

ABBREVIATIONS USED

DPA, days postanthesis; HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits.

REFERENCES

(1) Skylas, D. J.; Cordwell, S. J.; Hains, P. G.; Larsen, M. R.; Basseal, D. J.; Walsh, B. J.; Blumenthal, C.; Rathmell, W.; Copland, L.; Wrigley, C. W. Heat shock of wheat during grain filling: proteins associated with heat-intolerance. *J. Cereal Sci.* **2002**, *35*, 175–188.

(2) Altenbach, S. B.; DuPont, F. M.; Kothari, K. M.; Chan, R.; Johnson, E. L.; Lieu, D. Temperature, water and fertilizer influence the timing of key events during grain development in a U.S. Spring Wheat. *J. Cereal Sci.* **2003**, *37*, 9–20.

(3) DuPont, F. M.; Hurkman, W. J.; Vensel, W. H.; Chan, R.; Lopez, R.; Tanaka, C. K.; Altenbach, S. B. Differential accumulation of sulfur-rich and sulfur-poor wheat flour proteins is affected by temperature and mineral nutrition during grain development. *Cereal Chem.* **2006**, *44*, 101–112.

(4) Hurkman, W. J.; McCue, K. F.; Altenbach, S. B.; Korn, A.; Tanaka, C. K.; Kothari, K. M.; Johnson, E. L.; Bechtel, D. B.; Wilson, J. D.; Anderson, O. D.; DuPont, F. M. Effect of temperature on expression of genes encoding enzymes for starch biosynthesis in developing wheat endosperm. *Plant Sci.* **2003**, *164*, 873–881.

(5) DuPont, F. M.; Hurkman, W. J.; Vensel, W. H.; Tanaka, C. K.; Kothare, K. M.; Chung, O. K.; Altenbach, S. B. Protein accumulation and composition in wheat grains: effects of mineral nutrients and high temperature. *Eur. J. Agron.* **2006**, *25*, 96–107.

(6) Bechtel, D. B.; Barnett, B. D. A freeze-fracture study of storage protein accumulation in unfixed wheat starchy endosperm. *Cereal Chem.* **1986**, *63*, 232–240.

(7) Bechtel, D. B.; Gaines, R. L.; Pomeranz, Y. Early stages in wheat endosperm formation and protein body formation. *Ann. Bot.* **1982**, *50*, 507–518.

(8) Bechtel, D. B.; Gaines, R. L.; Pomeranz, Y. Protein secretion in wheat endosperm − formation of the matrix protein. *Cereal Chem.* **1982**, *59*, 336–343.

(9) Bechtel, D. B.; Wilson, J. D. Amyloplast formation and starch granule development in hard red winter wheat. *Cereal Chem.* **2003**, *80*, 175–183.

(10) Bechtel, D. B.; Wilson, J. D. Endosperm structural changes in wheat during drying of maturing caryopses. *Cereal Chem.* **2005**, *82*, 385–389.

(11) Levanony, H.; Rubin, R.; Altschuler, Y.; Galili, G. Evidence for a novel route of wheat storage proteins to vacuoles. *J. Cell Biol.* **1992**, *119*, 1117–1128.

(12) Loussert, C.; Popineau, Y.; Mangavel, C. Protein bodies ontogeny and localization of prolamin components in the developing endosperm of wheat caryopses. *Cereal Sci.* **2008**, *47*, 445–456.

(13) Parker, M. L. Storage protein deposition in developing wheat endosperm. *Micron* **1981**, *12*, 187–188.

(14) Parker, M. L.; Hawes, C. R. The Golgi apparatus in developing endosperm of wheat (*Triticum aestivum* L.). *Planta* **1982**, *154*, 277–283.

(15) Tashiro, T.; Wardlaw, I. F. The response to high temperature shock and humidity changes prior to and during the early stages of grain development in wheat. *Aust. J. Plant Physiol.* **1990**, *17*, 551–561.

(16) Hurkman, W. J., Wood, D. Starch granule formation and protein deposition in wheat (*Triticum aestivum* L.) starchy endosperm cells is altered by high temperature during grain fill. *Proc. SPIE−Int. Soc. Opt. Eng.* **2010**, *7729*, 58 (doi: 10.1117/12.853183).

(17) Balmer, Y.; Vensel, W. H.; DuPont, F. M.; Buchanan, B. B.; Hurkman, W. J. Proteome of amyloplasts isolated from developing wheat endosperm presents evidence of broad metabolic capability. *J. Exp. Bot.* **2006**, *57*, 1591–1602.

(18) Hurkman, W. J.; Vensel, W. H.; Tanaka, C. K.; Whitehand, L.; Altenbach, S. B. Effect of high temperature on albumin and globulin accumulation in the endosperm proteome of the developing wheat grain. *J. Cereal Sci.* **2009**, *49*, 12–23.

(19) Wan, Y.; Poole, R. L.; Huttly, A. K.; Toscano-Underwood, C.; Feeney, K.; Welham, S.; Gooding, M. J.; Mills, C.; Edwards, K. J.; Shewry, P. R.; Mitchell, R. A. C. Transcriptome analysis of grain development in hexaploid wheat. *BMC Genomics* **2008**, *9*, 121.

(20) Zadoks, J. C.; Chang, T. T.; Konzak, C. F. A decimal code for the growth stages of cereals. *Weed Res.* **1974**, *14*, 415–421.

(21) Altenbach, S. B.; Kothari, K. M.; Lieu, D. Environmental conditions during wheat grain development alter temporal regulation of major gluten protein genes. *Cereal Chem.* **2002**, *79*, 279–285.

(22) Zeng, M.; Morris, C. F.; Batey, I. L.; Wrigley, C. W. Sources of variation for starch gelatinization, pasting, and gelation properties of wheat. *Cereal Chem.* **1997**, *74*, 63–71.

(23) Peterson, D. D.; Fulcher, R. G. Variation in Minnesota HRS wheats: starch granule size distribution. *Food Res. Int.* **2001**, *34*, 357–363.

(24) Park, S.-H.; Wilson, J. D.; Seabourn, B. W. Starch granule size distribution of hard red winter and hard red spring wheat: its effects on mixing and breadmaking quality. *J. Cereal Sci.* **2009**, *49*, 98–105.

(25) Evers, A. D.; McDermott, E. E. Scanning electron microscopy of wheat starch. *Staerke* **1970**, *22*, 23–26.

(26) Heneen, W. K.; Brismar, K. Scanning electron microscopy of mature grains of rye, wheat, and triticale with emphasis on grain shriveling. *Hereditas* **1987**, *107*, 147–162.

(27) Dronzek, B. L.; Hwang, P.; Bushuk, W. Scannning electron microscopy of starch from sprouting wheat. *Cereal Chem.* **1972**, *49*, 232–239.

(28) Tosi, P.; Parker, M.; Gritsch, C. S.; Carzaniga, R.; Martin, B.; Shewry, P. R. Trafficking of storage proteins in developing grain of wheat. *J. Exp. Bot.* **2009**, *60*, 979–991.

(29) Langeveld, S. M. J.; van Wijk, R.; Stuurman, N.; Kijne, J. W.; de Pater, S. B-type granule containing protrusions and interconnections between amyloplasts in developing wheat endosperm revealed by transmission electron microscopy and GFP expression. *J. Exp. Bot.* **2000**, *51*, 1357–1361.