The Development of Endosperm in Grasses[1]

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The grass seed or caryopsis originates from a monocarpellary ovary with a single ovule and contains the main storage tissue, the endosperm. For most grass crop species (i.e. cereals), the value of the crop is largely determined by the endosperm, both in quantitative and qualitative terms.

The endosperm is the result of the fertilization of two polar nuclei in the central cell of the embryo sac by one sperm cell nucleus, which generates a triploid (3n, 3C) nucleus, whereas the diploid (2n, 2C) embryo originates from fertilization of the egg cell by the second sperm cell nucleus. The main function of the endosperm is to provide nutrients to the developing and, later, germinating embryo. In contrast to many species, including Arabidopsis (*Arabidopsis thaliana*), the grass endosperm is a persistent seed structure. It is the foremost source of calories for human and livestock nutrition and provides the raw material for countless manufactured foods, goods, and biofuels.

In spite of the importance of the grass endosperm, its development has not been thoroughly investigated in many crop species, much less in noncrop species. There is considerable uniformity in the development of the endosperm among grasses, especially during its early stages (Weatherwax, 1930; Bennett et al., 1975). And although deviations are known, these generally involve secondary aspects of development. It is generally true that the endosperm of most grasses is starchy and dry at maturity, which of course is a valuable trait, but there are exceptions. For example, in a survey of 169 grass genera (over 25% of total genera in the family), 30 were found to possess species with liquid or soft endosperms at maturity, and the viscous state of the endosperm can be retained for several decades. Nine additional genera were found to have semisolid endosperms (Terrell, 1971).

Among grasses, endosperm development is by far best characterized in maize (*Zea mays*) for historical, economic, anatomical, and genetic reasons; therefore, we will primarily refer to knowledge obtained from this cereal as a paradigm for grass endosperm development. Wherever appropriate and possible, differences between maize and other grass species will be highlighted and discussed. Here, we provide an overview of the phases of endosperm development, including the unique features of genetic, molecular, and cell regulatory mechanisms. The reader interested in an in-depth discussion of different aspects of endosperm development in grasses is referred to several previous works (Kiesselbach, 1949; Bennett et al., 1975; Kowles and Phillips, 1988; Lopes and Larkins, 1993; Olsen et al., 1999; Becraft, 2001; Larkins et al., 2001; Olsen, 2001, 2004, 2007; Sabelli et al., 2005b, 2007). The analysis of mutants has provided substantial knowledge of the regulation of endosperm development, and the relevant literature is extensive. Rather than reviewing every mutation, we will discuss selected mutations that in our opinion provide crucial insight. There are a number of publications that contain a wealth of information about mutations affecting endosperm development in cereals (Jarvi and Eslick, 1975; Nelson, 1980; Neuffer and Sheridan, 1980; Satoh and Omura, 1981; Bosnes et al., 1987; Kowles et al., 1992; Scanlon et al., 1994; Kurata et al., 2005; Dolfini et al., 2007).

GRASS ENDOSPERM DEVELOPMENT: MAJOR EVENTS

Development of the endosperm in grasses has several distinct phases that can overlap considerably (Fig. 1). These are distinguished as follows: early development, comprising double fertilization, syncytium formation, and cellularization; differentiation, which includes the formation of the main cell types (transfer cells, aleurone, starchy endosperm, and embryo-surrounding cells), the periods of mitosis and endoreduplication, and the accumulation of storage compounds; and maturation, which includes programmed cell death (PCD), dormancy, and desiccation.

EARLY DEVELOPMENT

Double Fertilization

In many species, including maize (Kiesselbach, 1949), wheat (*Triticum aestivum*; Bennett et al., 1975), Job's tears (*Coix lacryma-jobi*; Weatherwax, 1930), Koda millet (*Paspalum scrobiculatum*; Narayanaswami, 1954), and Chinese lovegrass (*Eragrostis unioloides*; Deshpande, 1976), syngamy (the fusion of one sperm nucleus with the egg cell nucleus) and fusion between a sperm nucleus and two polar nuclei to create the endosperm

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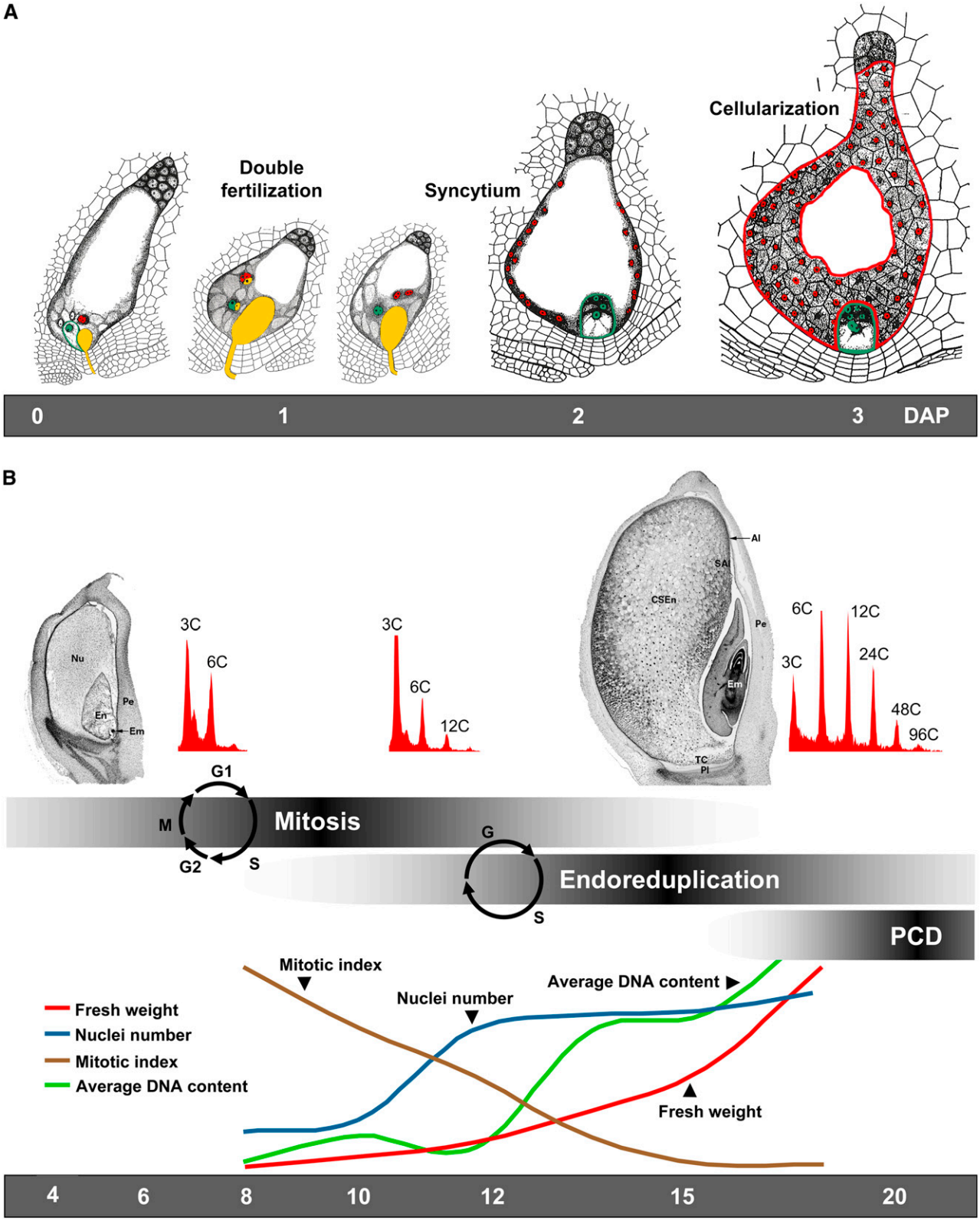

Figure 1. Phases in endosperm development. Although this figure refers to maize, it is a good example of endosperm development in other grasses as well. A, Double fertilization, syncytium formation, and cellularization of the endosperm occur within 3 to 4 DAP. The pollen tube and sperm nuclei are shown in yellow, polar nuclei in the central cell of the female gametophyte and endosperm nuclei are shown in red, and the egg cell nucleus and embryo nuclei are shown in green. Outlines

are simultaneous. It is intriguing that the mitotic activity of the triploid primary endosperm nucleus is very fast, whereas this process in the zygote undergoes a noticeable hiatus. Recent findings in Arabidopsis indicate that proliferation of the central cell requires a positive signal from the fertilized egg cell (Nowack et al., 2006), implying that the polar nuclei are "primed" for faster cell cycle activity. Whether a similar mechanism operates in the grass family is not known. The presence of supernumerary polar nuclei prior to fertilization has been documented in Koda millet and sugarcane (*Saccharum officinarum*; Narayanaswami, 1954) and in the *indeterminate gametophyte* maize mutant (Lin, 1978), suggesting a latent pathway with the potential to lead to early cell cycle activity and premature endosperm proliferation, which are otherwise normally repressed.

Syncytium Formation

Grass endosperm follows a frequently encountered mode of endosperm development, the nuclear (or coenocytic) type (Lopes and Larkins, 1993; Olsen, 2004), in which the initial triploid nucleus undergoes several rounds of often synchronous division in the absence of cell wall formation and cytokinesis, resulting in the formation of a syncytium. Fertilization of the polar nuclei results in the primary endosperm nucleus, which within hours begins to rapidly divide. As previously noted, cell division in the zygote is invariably slower. For example, by the time the zygote divides for the first time there are four to eight endosperm nuclei in maize (Randolph, 1936), up to 24 endosperm nuclei in Koda millet (Narayanaswami, 1954), and a large number of endosperm nuclei in Chinese lovegrass (Deshpande, 1976). When the embryonic cells do start to proliferate, they divide at a slower rate than the endosperm nuclei (comparable to the rate of cell division of meristematic cells), most likely because proliferation of the endosperm nuclei does not involve the synthesis of cytoplasm, cell membranes, and cell walls. The observation that a decline in the rate of endosperm proliferation (to approximately equal that of embryonic cells) is concomitant with cellularization of the syncytium lends support to this interpretation (Bennett et al., 1975). Indeed, the cell cycle of the coenocytic endosperm typically lacks the formation of interzonal phragmoplasts between daughter nuclei, reinforcing the view that the program responsible for creating some parts of the cytoskeletal apparatus found in somatic cells is suppressed. Thus, coenocytic endosperm development could be viewed as an evolutionary strategy, through repression of the program controlling cytokinesis and cell wall formation, to rapidly populate the large, preformed cytoplasm of the central cell and attain a greater basal cell number to support the growth of this tissue and prepare it to nurture growth of the embryo, especially during the period in which specific cells dedicated to nutrient uptake by the endosperm have not yet differentiated. The maternally derived nucellus and antipodal cells probably support coenocytic endosperm growth with amino acids, nucleotides, and carbohydrates (Bennett et al., 1975; Radchuk et al., 2006).

For about 1 d after pollination (DAP), all endosperm nuclei appear synchronous with respect to cell cycle stages. Subsequently, developmental gradients are formed in which neighboring nuclei proliferate synchronously. These developmental gradients, from the domain adjacent to the embryo to the chalazal region, appear to invert between 1 and 2 DAP in Triticeae (Bennett et al., 1975). In maize, nuclear proliferation in the syncytium generates up to 512 nuclei, usually during the first 3 DAP. In several *Triticum* and *Hordeum* species, in excess of 2,000 syncytial nuclei have been counted (Bennett et al., 1975). These nuclei migrate toward the chalazal region of the embryo sac and, as a result of enlargement of the central vacuole, become distributed at the periphery of the primary endosperm cell. Interestingly, in barley (*Hordeum vulgare*), there is a hiatus for about 2 d after the initial period of intense proliferation, which correlates with a dramatic rearrangement of the cytoskeleton to prepare for the ensuing cellularization of the first layer of nuclei. It is not clear if this interruption of mitotic activity is conserved in other grasses, such as maize and wheat, in which cellularization proceeds more rapidly (Olsen, 2001). Clonal analysis of maize endosperm development using *Activator*-induced mutations at the *Waxy* locus showed that the first division of the primary endosperm nucleus establishes two endosperm halves and suggested a conical pattern of cell proliferation from the center to the periphery of the tissue (McClintock, 1978). Analysis of wheat-rye (*Secale cereale*) chromosome addition lines revealed that the main genetic factors

Figure 1. (*Continued.*)
of the multicellular endosperm and embryo are drawn in red and green, respectively. Modified from Kiesselbach (1999) with permission. B, From about 4 to 20 DAP, the endosperm undergoes a phase of mitotic cell proliferation, followed (from around 8–10 DAP) by endoreduplication, as shown by flow-cytometric profiles obtained from 7-, 11-, and 19-DAP endosperms (red), and by PCD (starting around 16 DAP). The endoreduplication phase and part of the cell division phase coincide with a dramatic growth of the endosperm and the accumulation of storage compounds. The dynamics of key parameters during mid endosperm development, such as fresh weight (red line), nuclei number (blue line), mitotic index (brown line), and average DNA content (C value; green line), are shown at bottom. They are loosely based on the work of Engelen-Eigles et al. (2001) and only illustrate trends. Al, Aleurone; CSEn, central starchy endosperm; Em, embryo; En, endosperm; Nu, nucellus; Pe, pericarp; Pl, placentochalaza; SAl, subaleurone layer; TC, transfer cells. This panel is reproduced in part from Larkins et al. (2001) and Sabelli et al. (2005b), with permission.

controlling the rate of coenocytic endosperm development can be identified on specific chromosomes (Bennett et al., 1975). The population of haploid antipodal cells of the embryo sac proliferates by mitotic division during growth of the endosperm and appears to persist at late endosperm developmental stages (Randolph, 1936; Kowles and Phillips, 1988).

Cellularization

In cereals, the coenocytic endosperm undergoes cellularization by the formation of internuclear radial microtubule systems and an open-ended alveolation process that proceeds from the periphery of the endosperm toward the central vacuole (Brown et al., 1994; Olsen et al., 1999; Olsen, 2004). Initially, nuclear-cytoplasmic domains are defined by microtubules that radiate from the nuclear surface, resulting in approximately equally distanced nuclei in one layer lining the central cell wall. Cell wall formation is initiated anticlinally through the repolarization of microtubules at sites of microtubule intersection and deposition of an adventitious phragmoplast. This is followed by centripetal extension of the cell walls, resulting in alveoli, which are open tubular structures lacking an inner periclinal cell wall that surround each nucleus. After centripetal extension of the cell walls, the nuclei divide synchronously and periclinally, which is immediately followed by cytokinesis. Thus, the layer of alveoli is displaced farther inward toward the central cavity, along with an overlying layer of residual syncytial cytoplasm. The process of cellularization proceeds centripetally until the central cell cavity is completely filled with cells, which in cereals is completed around 3 to 6 DAP. Curiously, this process occurs without the formation of a preprophase band, a cytoskeletal structure that typically marks the future position of the cell wall during the somatic cell cycle (Olsen, 2001).

ENDOSPERM DIFFERENTIATION

Among grasses, endosperm development is best understood in cereals. Four major cell types constitute the cereal endosperm: transfer cells, aleurone cells, starchy endosperm cells, and embryo-surrounding region (ESR) cells.

Transfer Cells

Several cell layers of the cereal endosperm, near the placenta, stop dividing and differentiate early, sometimes before cellularization is completed, into transfer cells. These cells have extensive cell wall invaginations and increased plasma membrane surface, which facilitate nutrient (primarily Suc and amino acids) uptake by the endosperm. Transfer cells have been described in some detail in several grass species (references cited in Charlton et al., 1995). While these cells are frequently found at the base of the endosperm, their position within the caryopsis varies among species (Rost et al., 1984). In barley and wheat, transfer cells are located over the nucellar projection, and in maize, they are located over the chalazal pad. They allow rapid solute transport at symplastic/apoplastic bottlenecks, such as at the interface between maternal vascular tissue and the endosperm. By analogy with other systems, the plasma membrane of these cells probably has a high density of various solute transporters (Offler et al., 2003). Transfer cells typically have a dense cytoplasm that is rich in small, spherical mitochondria. High metabolic rates are required during differentiation of the transfer cells; mutation of the maize *EMPTY PERICARP4* gene, encoding a mitochondrion-targeted pentatricopeptide repeat protein that regulates mitochondrion gene expression, results in a defective transfer cell layer and endosperm (Gutierrez-Marcos et al., 2007).

The *END1* gene has been linked to transfer cell fate specification in barley, and its pattern of transcript accumulation has been interpreted as a marker for gene expression in a specific domain of the coenocytic endosperm that will differentiate transfer cells (Doan et al., 1996). Transfer cell fate specification apparently occurs during a narrow temporal window of syncytial development, as shown by the phenotype of the maize *globby-1* mutant, which has an abnormal basal layer of transfer cells (Costa et al., 2003). Patterning events in the central cell of the maize embryo sac are also important for patterning of the transfer cell layer (Gutierrez-Marcos et al., 2006a). Three groups of maize genes are preferentially expressed in transfer cell layers, *BETL*, *BAP*, and *EBE* (Magnard et al., 2003). These gene products resemble antimicrobial proteins, suggesting a role in protecting the kernel from potential pathogenic invaders. *BETL1* and *BAP2* expression appears to be transactivated by a *MYB*-related gene, *ZmMRP-1*, which is expressed before the *BETL* genes in the basal area of the coenocytic endosperm (Gomez et al., 2002). Recent in vitro experiments with cultured maize endosperm have reinforced previous views that development of the basal transfer cell layers requires a contribution from maternal sporophytic tissue (Gruis et al., 2006).

Aleurone

Aleurone cells form a sheet generally comprising one (maize, wheat, and rice [*Oryza sativa*]) to three (barley) or several (rice) layers of cells that surround the endosperm except in the transfer cell region. In maize, the aleurone differentiates between 6 and 10 DAP from the outer layers of endosperm cells, which noticeably tend to accumulate spherosomes and protein bodies and become cuboidal. Because aleurone cells have preprophase bands and other cytoskeletal structures typical of meristematic cells, their fate is believed to be specified soon after alveolation and the first periclinal division of the cellularized endosperm (Brown et al., 1994). However, the first discernible events in aleurone cell differ-

entiation differ among cereals and may relate to the number of aleurone layers in different species. For example, in barley, the first evidence of aleurone cell differentiation is the accumulation of small vacuoles and dense cytoplasm (around 8 DAP). In maize, instead, it follows a period of periclinal cell divisions emanating radially from the cellularized endosperm and is marked by redistribution of cell division planes from random to mostly anticlinal, which results in a sheet of cuboidal cells surrounding most of the inner starchy endosperm. Although aleurone formation involves both periclinal and anticlinal cell divisions, only the latter contribute to its expansion after 20 DAP (Kiesselbach, 1949). Endosperm cells adjacent to the aleurone layer are usually smaller and mitotically more active than the inner starchy endosperm cells and are sometimes referred to as subaleurone cells. Surface growth of the aleurone is thought to affect overall endosperm growth (Olsen, 2004). The *disorgal1-2* mutants in maize, with disorganized cell division planes in the aleurone and reduced development of starchy endosperm, support this view (Lid et al., 2004). Aleurone cells are normally triploid, but in barley they undergo endoreduplication and are polyploid (Olsen, 2001). Molecular markers of aleurone include *Ltp1-2*, *B22E*, *pZE40*, *ole-1-2*, *per-1*, and *chi33* in barley and *C1* (Olsen, 2004) and *Vpp1* (Wisniewski and Rogowsky, 2004) in maize.

Differentiation of aleurone cells seems to be independent from that of transfer cells, as shown by the maize *dek1* mutant, which lacks aleurone but displays a normal layer of transfer cells (Becraft et al., 2002). The cytoplasm of aleurone cells is dense and granular, because of numerous small vacuoles with inclusion bodies termed aleurone grains (Olsen, 2004). Mature aleurone cells contain anthocyanins, which impart a familiar range of colors to the maize kernel. The aleurone is the only "live" tissue at endosperm maturity, having a specific developmental program that protects it from desiccation (Hoecker et al., 1995). Upon seed imbibition, aleurone cells, in response to gibberellic acid stimulation from the embryo, activate a gene expression program that results in the synthesis of a suite of proteolytic and hydrolytic enzymes, which cause digestion of endosperm cell walls and mobilization of starch and proteins stored in the endosperm for uptake by the growing embryo.

In several mutants, such as *crinkly4* (*cr4*), *dek1*, and *sal1*, the aleurone layer is defective, absent, and supernumerary, respectively (for review, see Olsen, 2004). Although the corresponding genes have been identified (Becraft et al., 1996; Lid et al., 2002; Shen et al., 2003), understanding the hierarchical regulation of these gene networks has not been straightforward. Recent results suggest a key role for plasma membrane-anchored factors. Accordingly, some aleurone-specifying positional signal would first be perceived or transmitted by Dek1 (a calpein-like proteinase) at the cell membrane and relayed by CR4 (a protein receptor-like kinase) to aleurone precursor cells, while the proper concentration of both factors on the cell membrane is maintained by Sal1 (a class E vacuolar sorting protein) through endosome-mediated recycling or degradation (Tian et al., 2007). The same study showed that the fate of aleurone cells is strictly based on positional cues and also that the fate of starchy endosperm cells and aleurone cells is not fixed, as the two cell types can interchange during development, which reinforces earlier conclusions based on elegant genetic approaches (Becraft and Asuncion-Crabb, 2000). The analysis of *dap* mutants indicates that aleurone cell fate and cell differentiation are two genetically separable processes (Gavazzi et al., 1997). Based on the expression patterns of several aleurone markers in different aleurone mutants, a stepwise model for aleurone cell fate was proposed (Wisniewski and Rogowsky, 2004). Additional mutants affecting the aleurone have been isolated in both maize and barley, but molecular information is currently lacking (Olsen, 2001).

Starchy Endosperm and the Accumulation of Storage Compounds

Cereal seeds are one of the most important sources of food calories worldwide, because they contain about 70% starch in terms of dry weight. Starch is made of two α-glucan polymers, amylose and amylopectin, that are packed into semicrystalline granules in amyloplasts (Fig. 2; Smith, 1999; James et al., 2003). Starch is synthesized from Suc after the latter is converted to ADP-Glc. Starch biosynthesis is the result of the concerted action of four distinct enzymatic activities: ADP-Glc pyrophosphorylase (AGPase), starch synthase (SS), and starch-branching (BE) and starch-debranching (DBE) enzymes (Hannah, 2007). Whereas SS, BE, and DBE are found within amyloplasts, AGPase activity, which represents the rate-limiting step in starch biosynthesis, is almost confined exclusively to the cytosol in cereal endosperm cells. The cytosolic localization of AGPase, which appears to be unique to the Poaceae (Beckles et al., 2001), may facilitate starch biosynthesis in the presence of plentiful Suc (James et al., 2003).

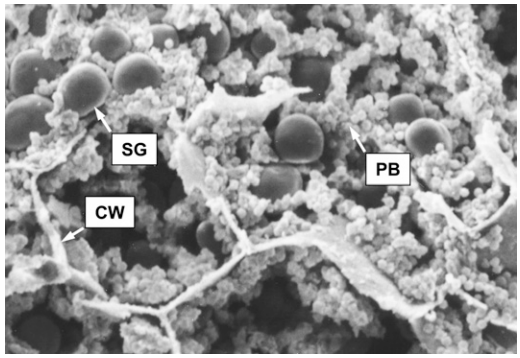

Figure 2. Electron scanning microscopy image of developing maize endosperm illustrating cell walls (CW), starch granules (SG), and protein bodies (PB).

Starch grain accumulation starts soon after cellularization in the Triticeae, whereas in maize it begins around 10 DAP (Bennett et al., 1975; Charlton et al., 1995; Borisjuk et al., 2004). Several studies in maize and wheat have shown that the rate and potential of grain filling and seed weight correlate with the number of starch granules in the endosperm. In turn, starch granule number depends on the number of cells (Brocklehurst, 1977; Chojecki et al., 1986a, 1986b; Jones et al., 1996). These observations imply that the extent of the cell division phase (i.e. the time of its initiation, its duration, and its rate) plays a key role in endosperm development and grain yield (Reddy and Daynard, 1983; Ober et al., 1991; Commuri and Jones, 1999). Starch granule morphology plays an important role in grain digestibility and industrial applications. Within Poaceae, the compound granule is the most common type and is found, for example, in rice and oat (*Avena sativa*). Compound granules develop as "multigranules" of starch within one amyloplast and are typically small and polyhedral. In simple granules, in contrast, only one large granule develops in one amyloplast; this is typical of most Panicoideae species. The Triticeae appear to be unique in having a so-called bimodal form of starch accumulation, with both large A-type and small B-type granules (Shapter et al., 2008).

Because the cereal endosperm is such a phenomenal energy sink, an important aspect of its development concerns the role of carbon metabolism, sugar partitioning and signaling, nutrient fluxes, and the regulation of energy states (Wobus and Weber, 1999b; Borisjuk et al., 2004). In cereals, cell size, cell differentiation, endoreduplication, starch accumulation, and starch granule size are associated with high levels of ATP and high energy states, suggesting that the cell expansion and starch accumulation phase is associated with high metabolic activity and is energy limited. However, starchy endosperm cells appear to experience severe hypoxia, suggesting that cereal endosperm cells are adapted to carry out starch synthesis at extremely low levels of oxygen. Induction of Suc synthase gene expression in response to low oxygen concentrations could be part of such an adaptive mechanism, helping to ensure high starch accumulation under hypoxic conditions (Rolletschek et al., 2005).

The transition from the cell division phase into the storage phase of endosperm development is accompanied by extensive reprogramming of gene expression patterns (Sreenivasulu et al., 2004; Drea et al., 2005; Laudencia-Chingcuanco et al., 2007; Wan et al., 2008) and appears to be regulated by Suc (Giroux et al., 1994) and the induction of Suc synthase (Borisjuk et al., 2004). However, invertase activity appears to be more important during the early formative phase, coincident with cell proliferation, as shown by the analysis of cell wall invertase in maize (Vilhar et al., 2002) and sorghum (*Sorghum bicolor*; Jain et al., 2008a). Thus, a high Glc-Suc ratio in the caryopsis is associated with endosperm cell proliferation, whereas a spike in Suc correlates with the transition into the starch accumulation phase.

A likely candidate for the integration of sugar and abscisic acid (ABA) signaling and the onset of starch biosynthesis appears to be *SnRK1*, a gene encoding a protein kinase closely related to yeast SNF1 (Suc nonfermenting 1) and AMPK (AMP-activated protein kinase) in mammals. SnRK1 was originally cloned from rye endosperm and functionally complements yeast *snf1* mutants that otherwise would not grow on substrates lacking Glc (Alderson et al., 1991), because SNF1 is essential for the expression of genes that are repressed by Glc, including invertase, following Glc deprivation. SnRK1 may be induced in response to Suc and could affect starch biosynthesis by regulating both Suc synthase gene expression and the activation of AGPase (Halford and Paul, 2003). The SnRK1b subfamily is particularly interesting, because it appears to be specific to cereals and is highly expressed in the caryopsis. In rice, sorghum, and maize, SnRK1b expression is associated with the development of sink tissue capacity and the transfer cell region (Kanegae et al., 2005; Jain et al., 2008b) and may play an important role in linking ABA and Suc signaling with the transition into the storage phase (Sreenivasulu et al., 2006) and/or by inhibiting cell division, similar to yeast SNF1.

It has been estimated that cereals are the main source of protein in livestock feed worldwide and are the principal food protein source in certain regions (Shewry, 2000). Endosperm storage proteins are responsible for the cohesive and viscoelastic properties of the dough made from endosperm flour, which are essential for bread and pasta making (wheat) and the functional properties of other baked goods. The principle storage proteins in cereals are prolamins (highly hydrophobic and soluble in alcoholic solutions or denaturing solvents) and globulins (soluble in saline solutions), although additional minor proteins also accumulate (Coleman and Larkins, 1999; Shewry and Halford, 2002). Two basic prolamin types are (1) those found in Triticeae, which are closely related and comprise monomeric gliadins and polymeric glutenins in wheat, hordein in barley, and secalins in rye (Kreis et al., 1985), and (2) those found in Panicoideae, which includes maize zeins and the related proteins in sorghum (kafirins), millet, and *Coix* (Coleman and Larkins, 1999; Leite et al., 1999).

Prolamins are rich in Pro and Gln and are generally deficient in charged amino acids, in particular the essential amino acids Lys and Trp. They derive their peculiar amino acid composition from the reiteration of Pro- and Gln-rich repeats in their sequences. Prolamins represent 50% to 60% of total endosperm proteins in the genera *Hordeum* (barley), *Pennisetum* (millet), *Secale* (rye), *Sorghum* (sorghum), *Triticum* (wheat), and *Zea* (maize) but account for only 5% to 10% of endosperm proteins in *Oryza* (rice) and *Avena* (oat), in which most storage proteins consist of 11S globulin. In contrast, *Brachypodium distachyon* (purple false brome) endosperm primarily accumulates storage proteins that resemble maize 7S and oat 12S globulins (Laudencia-Chingcuanco and Vensel, 2008).

Both prolamins and globulins form insoluble accretions called protein bodies in the lumen of the rough endoplasmic reticulum (RER; Fig. 2). In wheat and related grasses, these accretions are trafficked to large protein storage vacuoles. In maize and other panicoid cereals, as well as rice, the prolamin-containing protein bodies are retained within the RER through an unknown mechanism (Herman and Larkins, 1999; Holding and Larkins, 2006). The prolamin and globulin storage proteins in maize and rice are stored in different types of protein bodies (Larkins and Hurkman, 1978; Krishnan et al., 1986; Yamagata and Tanaka, 1986; Woo et al., 2001), and RNA trafficking results in differential distribution of mRNAs on the RER (cisternal ER versus protein body ER; Okita and Choi, 2002; Washida et al., 2004). The organization of prolamins within the protein body appears directed by specific interactions between these proteins, and mutations that alter prolamin structure disrupt the organization of protein bodies and lead to the unfolded protein response (Coleman et al., 1997; Hunter et al., 2002; Kim et al., 2004; Holding et al., 2007).

Although there are exceptions, generally prolamin genes are organized into multigenic loci (Wilson and Larkins, 1984; Okita et al., 1985; Sabelli and Shewry, 1991; Shewry et al., 2003; Xu and Messing, 2008). Accumulation of prolamins in the endosperm, however, is controlled primarily at the transcriptional level, and the proteins accumulate generally during middle and late periods of endosperm development, according to specific spatial/temporal patterns (Woo et al., 2001; Shewry et al., 2003; Halford and Shewry, 2007; Xu and Messing, 2008). A critical regulatory sequence for prolamin gene expression was first identified in barley and termed the −300 element; later, related sequences were found in wheat, rye, and maize prolamin promoters (Forde et al., 1985; Ueda et al., 1994). This highly conserved region typically contains two motifs: the prolamin box and a GCN4-like sequence (Halford and Shewry, 2007; Marzabal et al., 2008). The former interacts with a Dof-type transcription factor termed the prolamin box-binding factor (PBF; Vicente-Carbajosa et al., 1997), whereas the latter is bound by basic Leu zipper transcription factors, such as Opaque2 in maize (Schmidt et al., 1992), which plays a major role in the expression of maize 22-kD α-zein genes (Schmidt et al., 1990). The importance of PBF in modern cereals is underscored by the fact that allelic selection at the *Pbf* locus played a major role in the domestication of maize from teosinte (Jaenicke-Despres et al., 2003). Additional cis-regulatory sequences in prolamin promoters include the 5′-AACA-3′ motif, which binds MYB-related transcription factors in rice and barley (Marzabal et al., 2008), and the wheat high molecular weight prolamin enhancer, which has only limited similarity with the prolamin box (Halford et al., 1989).

ESR

Among the cereals, the ESR has been best characterized in maize (Cossegal et al., 2007). It comprises several cell layers that completely envelop the young embryo (i.e. at around 4 DAP). As the embryo grows, the ESR progressively shrinks, and by early to mid endosperm development (i.e. around 12 DAP), there are only vestigial remnants of the ESR at the base of the endosperm. ESR cells differentiate upon completion of the endosperm cellularization phase (Kiesselbach, 1949; Kiesselbach and Walker, 1952) and are cytoplasmically dense, rich in small vacuoles, and with a complex membrane system. Based on several cytological characteristics, ESR cells are believed to be metabolically highly active and involved in supplying the embryo with sugars, primarily through an apoplastic route (Cossegal et al., 2007). Indeed, an invertase inhibitor is expressed specifically in the maize ESR, which may prevent deleterious Suc cleavage in the apoplast (Bate et al., 2004). Additional potential roles for the ESR include defense from pathogens and signaling at the embryo-endosperm interface. Evidence of the former comes from at least two genes expressed in the ESR, *ZmAE3* and *ZmEsr6*, which have broad-range antimicrobial activities (Balandin et al., 2005). Support for a role of ESR in mediating signaling between embryo and endosperm comes from the *ZmEsr1-3* gene family, which potentially encodes receptor ligands similar to Arabidopsis CLV3 (Cock and McCormick, 2001; Bonello et al., 2002). The ESR may also play an important role in establishing the so-called embryonic cavern in maize (Cossegal et al., 2007). Cytological analyses revealed that cells with characteristics similar to maize ESR are also present in wheat and barley, although functional data on these are lacking. The rice genome lacks homologs of maize ESR-specific genes (Cossegal et al., 2007).

CELL CYCLE REGULATION DURING ENDOSPERM DEVELOPMENT

Three different types of cell cycles occur during endosperm development: one is acytokinetic mitosis, which results in a syncytium; the second is mitosis coupled to cell division, which produces most cells comprising the mature endosperm; and the third is endoreduplication, which entails reiterated rounds of DNA replication without chromatin condensation, sister chromatid segregation, or cytokinesis, resulting in endopolyploid cells. As discussed above, information about the regulation of syncytial nuclear proliferation and the ensuing cellularization is scarce and primarily descriptive, whereas the latter two types of cell cycles have been characterized in some detail in maize.

The Mitotic Cell Division Phase

A phase of mitotic cell division occurs after cellularization of the endosperm and is largely responsible for generating the final population of endosperm cells. This period lasts until 8 to 12 DAP in the central

endosperm but continues until approximately 20 to 25 DAP in the aleurone and subaleurone layers (Kowles and Phillips, 1988). Cell division patterns appear to be conserved in the cereal endosperm. Cell divisions typically occur in a wave-like pattern, stopping first at the base of the endosperm and then in the central region. Similar spatial/temporal patterns have also been observed with regard to the increase in size of the nuclei, starch granules, and cells and are consistent with cell differentiation gradients that follow cell division (Kowles and Phillips, 1988). The mitotic index peaks around 8 to 10 DAP and then declines sharply. During the period from 8 to 12 DAP, the endosperm grows rapidly to fill the entire seed cavity. This growth appears to be correlated with cell division and enlargement as well as endoreduplication (Fig. 1), since the mean volume of centrally located nuclei increases roughly 10-fold (Kowles and Phillips, 1988).

The Endoreduplication Phase

From approximately 8 to 10 DAP, maize endosperm cells gradually and asynchronously switch from a mitotic to an endoreduplication cell cycle, in which seemingly complete and reiterated rounds of DNA synthesis take place without chromatin condensation, sister chromatid segregation, and cytokinesis (Kowles and Phillips, 1985; Larkins et al., 2001; Sabelli and Larkins, 2008; Fig. 1).

Because of the spatial/temporal pattern of the mitosis/endoreduplication switch mentioned above, a gradient in nuclear size is observed in tissue sections, with the smallest nuclei (3C and 6C) located at the periphery of the endosperm and increasingly larger nuclei in the inner central region. DNA content, nuclear size, and cell size are clearly correlated (Kowles and Phillips, 1988; Vilhar et al., 2002).

The endoreduplication cycle results in loosely polytenic chromosomes (Kowles and Phillips, 1988), which are, however, tightly associated at the heterochromatic centromeric and knob regions (Bauer and Birchler, 2006). Chromatin structure in endoreduplicating cells is likely to play an important role in the biology of the grass endosperm, as shown by the analysis of interploidy crosses, in which dramatic alterations in chromatin organization seem correlated with perturbed development of the caryopsis (Bauer and Birchler, 2006). Although the chromatin of endoreduplicating endosperm nuclei is believed to be permanently decondensed in most species, in durum wheat (*Triticum durum*) it appears to become highly condensed, which could result in the repression of gene expression (Polizzi et al., 1998).

Endoreduplication during endosperm development appears ubiquitous in cereals (Chojecki et al., 1986a; Ramachandran and Raghavan, 1989; Giese, 1992; Kladnik et al., 2006) and is correlated with nuclear and cell size, the rapid growth of the caryopsis, and the synthesis and accumulation of storage compounds such as starch and storage proteins (Fig. 1).

Although several possible functions have been proposed for endoreduplication in the endosperm, including (1) a mechanism to provide more gene templates to support high transcription rates, (2) driving cell expansion and tissue growth without cell division, and (3) enhancing the pool of nucleotides utilized by the embryo during germination (Sabelli and Larkins, 2008), unequivocal experimental evidence supporting any one of these remains elusive.

Factors Affecting the Cell Cycle during Endosperm Development

The roles of different cell cycle genes have been intensely investigated, such as those of cyclin-dependent kinases (CDKs) and their cyclin partners, CDK inhibitors, and retinoblastoma-related (RBR) proteins, all of which play crucial but distinct roles in cell cycle regulation (Larkins et al., 2001; Sabelli et al., 2005b, 2007; Inze and De Veylder, 2006). CDKs can be broadly classified as S-phase or M-phase CDKs, and their respective activities are important for the G1/S and G2/M transitions. A peak in CDK activity occurs at 10 to 12 DAP in maize, concomitant with the onset of endoreduplication, and convincing evidence supports the view that the switch from the mitotic to the endoreduplication cell cycle entails simultaneous down-regulation of mitotic CDKs and up-regulation of S-phase CDKs (Grafi and Larkins, 1995; Sun et al., 1999a, 1999b; Leiva-Neto et al., 2004; Coelho et al., 2005; Barroco et al., 2006). Thus, modulation of CDK activity appears to be important for the transition from a mitotic to an endoreduplication cell cycle during endosperm development.

Increasing evidence also implicates RBRs in endosperm development. RBRs are a conserved family of proteins that primarily prevent cells from entering S phase by inhibiting E2F transcription factors, the activity of which is required for the expression of many S-phase genes. Grasses may be unique in that their genomes encode at least two distinct RBR genes, in maize termed *RBR1* and *RBR3* (Sabelli et al., 2005a; Sabelli and Larkins, 2006). However, the roles of *RBR1* and *RBR3* in endosperm development are not clear. Although early investigation suggested that RBR1 becomes hyperphosphorylated (and, by analogy with other systems, inhibited) in endoreduplicating cells (Grafi et al., 1996), recent analyses have shown that the relative expression of RBR1 increases during the endoreduplication phase of endosperm development, suggesting that at least some RBR1 activity might be present (Sabelli et al., 2005a). RBR3 expression is repressed by RBR1, suggesting a compensatory interplay between *RBR1* and *RBR3*, and its expression is more tightly associated with mitotic activity than endoreduplication, which suggests functional differences between these two genes (Sabelli et al., 2005a; Sabelli and Larkins, 2006). RBR3 down-regulation during endoreduplication supports the view that RBR1 activity is retained during this phase of development. Forward

genetics experiments that modulate the expression of RBR1 and RBR3 should help elucidate their precise roles.

Besides the activity of key cell cycle regulators, both the cell cycle and the development of the endosperm depend significantly on hormonal and environmental factors, which have been reviewed elsewhere (Sabelli et al., 2005b, 2007).

MATURATION: CELL DEATH, DORMANCY, AND DESICCATION

PCD plays an important role in cereal endosperm development, and it is thought to facilitate nutrient hydrolysis and uptake by the embryo at germination (Nguyen et al., 2007). PCD in maize starchy endosperm starts at around 16 DAP in two separate regions, the central starchy endosperm cells and apical cells near the silk scar. PCD spreads from these two regions, which eventually merge, so that by 28 DAP approximately the top half of the endosperm is dead (Young and Gallie, 2000b). In wheat, a similar process occurs, although more random in its spatial pattern, and culminates with all endosperm cells but the aleurone having undergone PCD by roughly 30 DAP (Young and Gallie, 2000a). Although PCD in plants involves some of the typical hallmarks of PCD in animals, such as DNA fragmentation, chromatin condensation, and nuclear membrane disassembly, the effectors (which in animals are caspases) have not clearly been identified. However, there is circumstantial evidence for a role by a range of proteases with caspase-like activity (Hatsugai et al., 2004; Nguyen et al., 2007). Convincing evidence implicates hormones in the onset and progression of PCD in the endosperm. Ethylene levels, both in the unperturbed caryopsis and upon specific manipulation, are positively correlated with PCD (Young et al., 1997). In addition, ABA biosynthesis affects PCD indirectly, via ethylene biosynthesis, as shown by increased ethylene levels and PCD in maize *viviparous* mutants, in which the ABA biosynthetic pathway is altered (Young and Gallie, 2000b). Differently from starchy endosperm, PCD in aleurone cells is promoted by gibberellic acid rather than ethylene. In both starchy endosperm and aleurone cells, ABA appears to inhibit or delay PCD (Nguyen et al., 2007). Transcriptome analysis supports the involvement of proteases as well as the ethylene and ABA pathways in endosperm PCD and maturation during barley seed development (Sreenivasulu et al., 2006).

A great deal is known about how seed maturation, dormancy, and desiccation are regulated in dicots and the critical role played by ABA signaling and gene regulation networks (Wobus and Weber, 1999a; Vicente-Carbajosa and Carbonero, 2005; Gutierrez et al., 2007). However, most information concerns the role of the embryo and aleurone, rather than the starchy endosperm. ABA and gene expression are clearly implicated in these processes in cereals (Chono et al., 2006; Cao et al., 2007; Sreenivasulu et al., 2008), but understanding is far from complete. Study of the mechanisms implicated in the suppression of premature germination in cereals has highlighted the important role played by the viviparous class of genes (McCarty et al., 1989), as shown by the repression of α-amylase expression in the aleurone by the *VIVIPAROUS1* gene (Hoecker et al., 1999).

PARENT-OF-ORIGIN EFFECTS AND EPIGENETICS

Deviation from the normal 2:1 maternal:paternal genome dosage is deleterious for endosperm and seed development (Cooper, 1951; Lin, 1984). Indeed, recent analysis indicates that proper genome dosage in the endosperm is important to coordinate cell proliferation with endoreduplication and cell differentiation (Leblanc et al., 2002; Pennington et al., 2008). In addition, an unbalanced genomic ratio often results in abnormal or suppressed development of the transfer cell domain (Charlton et al., 1995). Because of the 2:1 ratio of genome complements in endosperm cells, development of the endosperm is expected to be largely under maternal genetic control; indeed, there is ample evidence that this is the case (Jones et al., 1996; Kowles et al., 1997; Dilkes et al., 2002). However, endosperm development is also the result of complex genetic and epigenetic interactions, which are only beginning to be understood in cereals. The activity of alleles derived from the two parents is finely regulated in the endosperm by imprinting; in fact, this is the only tissue in angiosperms in which imprinting is known to take place. In maize, several genes are imprinted, such as *R1* (Kermicle, 1970), *DZR1* (Chaudhuri and Messing, 1994), α-tubulin (Lund et al., 1995b), zein (Lund et al., 1995a), *EBE1* (Magnard et al., 2003), *FIE1* and *FIE2* (Danilevskaya et al., 2003; Gutierrez-Marcos et al., 2003), *MEG1* (Gutierrez-Marcos et al., 2004), and *NRP1* (Guo et al., 2003). *FIE1* and *FIE2* are particularly interesting because they encode Polycomb group proteins, which are part of large complexes regulating imprinting through epigenetic modifications, such as cytosine methylation and histone modifications (Huh et al., 2008). Mutation of *FIE* (and other interacting Polycomb group genes) in Arabidopsis leads to autonomous development of the endosperm without fertilization, but no maize *fie* mutant has been described. Expression of *FIE1* and *FIE2* is differently regulated in maize, suggesting diversification of function during endosperm development (Gutierrez-Marcos et al., 2006b; Hermon et al., 2007). Although understanding the regulation of imprinting in grasses is in its infancy, evidence of cross talk between cell cycle regulation and endosperm imprinting and development is emerging in Arabidopsis (Jullien et al., 2008), and similar pathways may operate in grasses as well.

CONCLUSION

Although substantial progress has been made in unraveling developmental patterns, cell proliferation,

and differentiation patterns, the molecular factors that control these processes, as well as key aspects such as polarity, cell division, cell shape, endoreduplication, and the accumulation of storage compounds, remain unknown. Certain developmental transitions are dramatic and abrupt, such as cellularization of the syncytium. This suggests that gene expression patterns become globally and rapidly reprogrammed, possibly as a result of the activation of feedback regulatory loops and/or extensive chromatin modifications. The onset of cellularization in many syncytial nuclear domains occurs synchronously, suggesting homogenously distributed molecular signals that exceed a critical threshold and/or cross talk among the nuclear domains of many cells to coordinate the whole process.

Several important questions remain to be answered. What signals trigger the major endosperm developmental transitions? How do cells know when they need to stop dividing and engage in endoreduplication and cell expansion? What signals coordinate peripheral/surface growth of the endosperm with its inner expansion? Does endoreduplication precede the biosynthesis and accumulation of storage compounds in individual cells, and is it necessary for these processes? How are sugar metabolism and signaling coordinated with cell proliferation, cell expansion, and the accumulation of storage compounds? How is endosperm development in grasses controlled by epigenetic pathways? Many questions ultimately relate to the mechanisms that ensure the coordination of different pathways and events. This reflects the fact that the endosperm is far from an "amorphous" and simple tissue stocked with starch and proteins. On the contrary, endosperm is a sophisticated tissue with highly specialized cell types, and it undergoes many of the canonical steps encountered during the development of more complex tissues and organs, including cell proliferation, cell fate specification, patterning, differentiation, and senescence. The ability of grasses to reproduce depends on the successful execution of these processes.

The few grasses that have been domesticated and cultivated were essential for the development of human civilization. Likewise, they will sustain the current and future world population and its standard of living. Understanding the factors responsible for converting the insignificant (from a human consumption perspective) endosperm of wild grasses into the remarkable energy sinks of modern cereals, which occurred through domestication and breeding, is important and could help us enhance the current pool of cereal species to provide enough food for the future.

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