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Cereal Endosperms: Development and Storage Product Accumulation

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Keywords

aleurone and starchy endosperm differentiation, sugar loading, grain filling, storage product accumulation, imprinted gene expression, programmed cell death

Abstract

The persistent triploid endosperms of cereal crops are the most important source of human food and animal feed. The development of cereal endosperms progresses through coenocytic nuclear division, cellularization, aleurone and starchy endosperm differentiation, and storage product accumulation. In the past few decades, the cell biological processes involved in endosperm formation in most cereals have been described. Molecular genetic studies performed in recent years led to the identification of the genes underlying endosperm differentiation, regulatory network governing storage product accumulation, and epigenetic mechanism underlying imprinted gene expression. In this article, we outline recent progress in this area and propose hypothetical models to illustrate machineries that control aleurone and starchy endosperm differentiation, sugar loading, and storage product accumulations. A future challenge in this area is to decipher the molecular mechanisms underlying coenocytic nuclear division, endosperm cellularization, and programmed cell death.

Contents

1. INTRODUCTION

Endosperm: a triploid tissue derived from fertilized central nuclei, which may nourish the embryo during embryogenesis and seed germination

Aleurone: the outermost layer of the cereal endosperm that stores proteins, lipids, vitamins, and micronutrients

Starchy endosperm: the inner and main body of the cereal endosperm that stores starch and some storage proteins

Coenocytic endosperm: an early stage endosperm in which nuclear divisions are not followed by cytokinesis, leading to the formation of a multinucleated endosperm

The endosperms of cereal crops such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), millet (*Setaria italic* L.), oat (*Avena sativa* L.), and sorghum (*Sorghum bicolor* L.) contribute the largest percentage of human food and animal feed, and also provide a substantial amount of raw material for manufactured goods and biofuels (74). From a developmental point of view, the early stages in the development of persistent cereal endosperms are similar to those in *Arabidopsis thaliana*, starting from coenocytic nuclear divisions of the triploid central nucleus in the embryo sac after fertilization, and continuing with cellularization to form cell walls between individual nuclei (109). The differences begin later, with the differentiation of various tissue types, including aleurone and starchy endosperm, and the rapid accumulation of storage products in cereal endosperms (11). Thus, storage products accumulated in the cereal endosperm nourish the embryo during not only embryogenesis but also seed germination, unlike in species such as *Arabidopsis* that have an exalbuminous seed, in which the endosperm supports embryogenesis alone. This article provides an overview of the progress made in recent decades in understanding the development of cereal endosperms and the accumulation of storage products, with primary attention given to studies performed in rice and maize.

2. MORPHOGENESIS OF CEREAL ENDOSPERMS

As illustrated in **Figure 1** (with rice as an example), all cereal plants have a nuclear-type endosperm, and the first phase of endosperm development is coenocytic, with synchronized nuclear divisions but no intervening cytokinesis. In rice, this process takes about two days. The nuclei produced, together with their surrounding cytoplasm, are localized to the periphery, while a large vacuole occupies the central region of the embryo sac (131). Cellularization of the coenocytic endosperm starts with the formation of a radial microtubular network from the nuclear envelopes, and then cell walls form at the periphery of the embryo sac, but without phragmoplast formation (18), in a process reminiscent of cell cleavage observed in non-vascular plant species such as *Chlamydomonas* (33). Further cytokinesis of the endosperm proceeds with the formation of phragmoplasts in interzones between opposing radial microtubular systems, providing guidance for the deposition of cell wall materials (17). The cellularization continues through repeated cycles of nuclear division

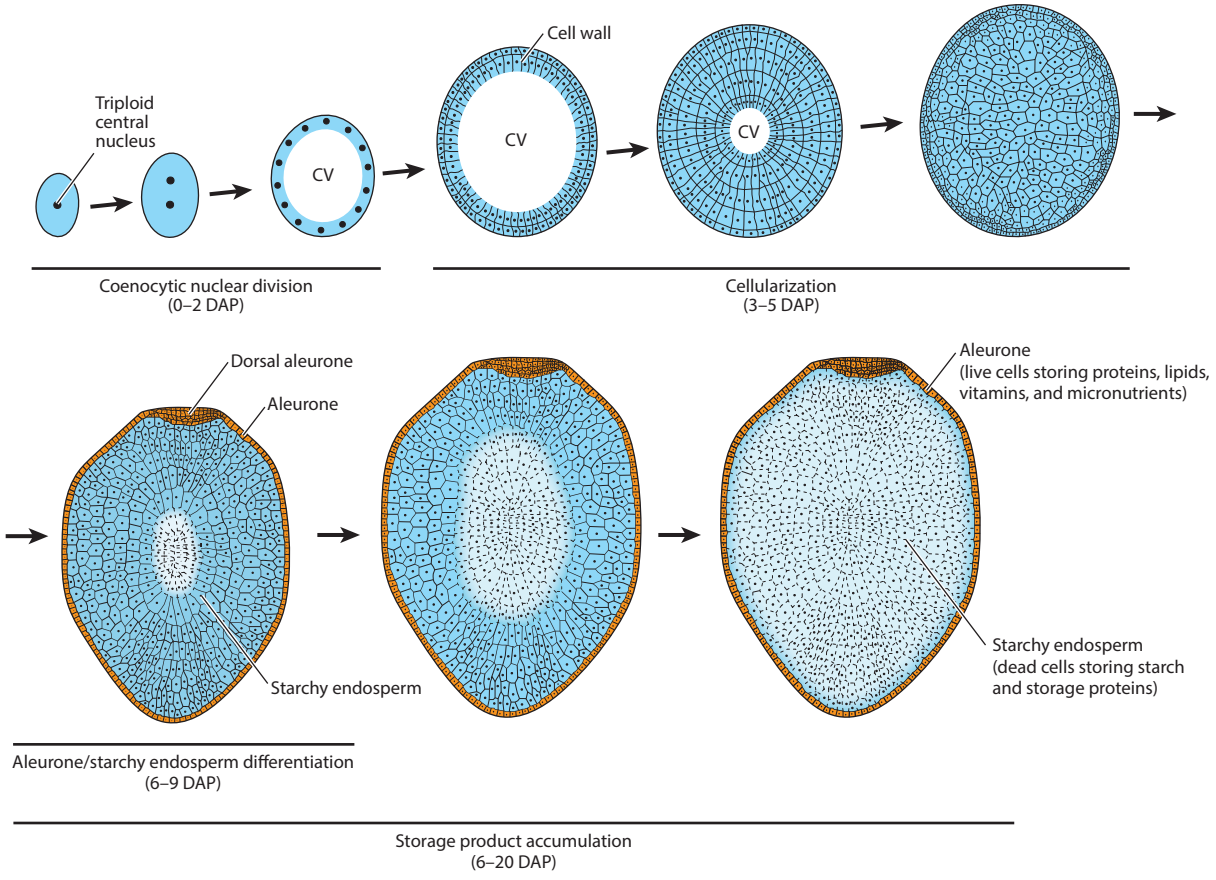

Figure 1

Endosperm development in cereals (using rice as an example). The main stages of rice endosperm development include coenocytic nuclear division (0–2 DAP), cellularization (3–5 DAP), aleurone/starchy endosperm differentiation (6–9 DAP), and storage product accumulation (6–20 DAP). Aleurone/starchy endosperm differentiation overlaps with the storage product accumulation stage. Most regions of the aleurone, except the dorsal aleurone (3–5 cell layers) located near the dorsal vascular bundle, are made of a single layer of cells. The starchy endosperm, consisting of dead cells in the mature endosperm, starts to accumulate starch from the center (*light blue*), whereas aleurone (*orange*) cells remain alive and accumulate proteins, lipids, vitamins, and micronutrients. Diagrams are not to scale. Abbreviations: CV, central vacuole; DAP, days after pollination.

and cell wall formation, and eventually invades the entire central vacuole. In rice, this process takes about three days (184).

Differentiation of the cereal endosperm starts after the completion of cellularization, with the establishment of two major tissues: the outer aleurone layer and the inner starchy endosperm (**Figure 1**). Due to their distinct cell division patterns, cell sizes, and storage product accumulations, these two tissues are easily distinguishable (184). During the grain-filling stage, the starchy endosperm occupies the central region of the endosperm and accumulates mainly starch, along with a small amount of storage proteins. In most cereal crops, the starchy endosperm accounts for over 90% of the total grain weight. In rice, the accumulation of starch grains usually starts from the central region of the starchy endosperm and progresses toward the peripheral subaleurone layer that forms the starchy endosperm in mature grains (184). The aleurone layer accumulates lipids, proteins, vitamins, and minerals in most cereals but lacks visible starch grains throughout

Grain filling: a process in endosperms whereby sugars delivered from leaves are used to synthesize starch and other storage products

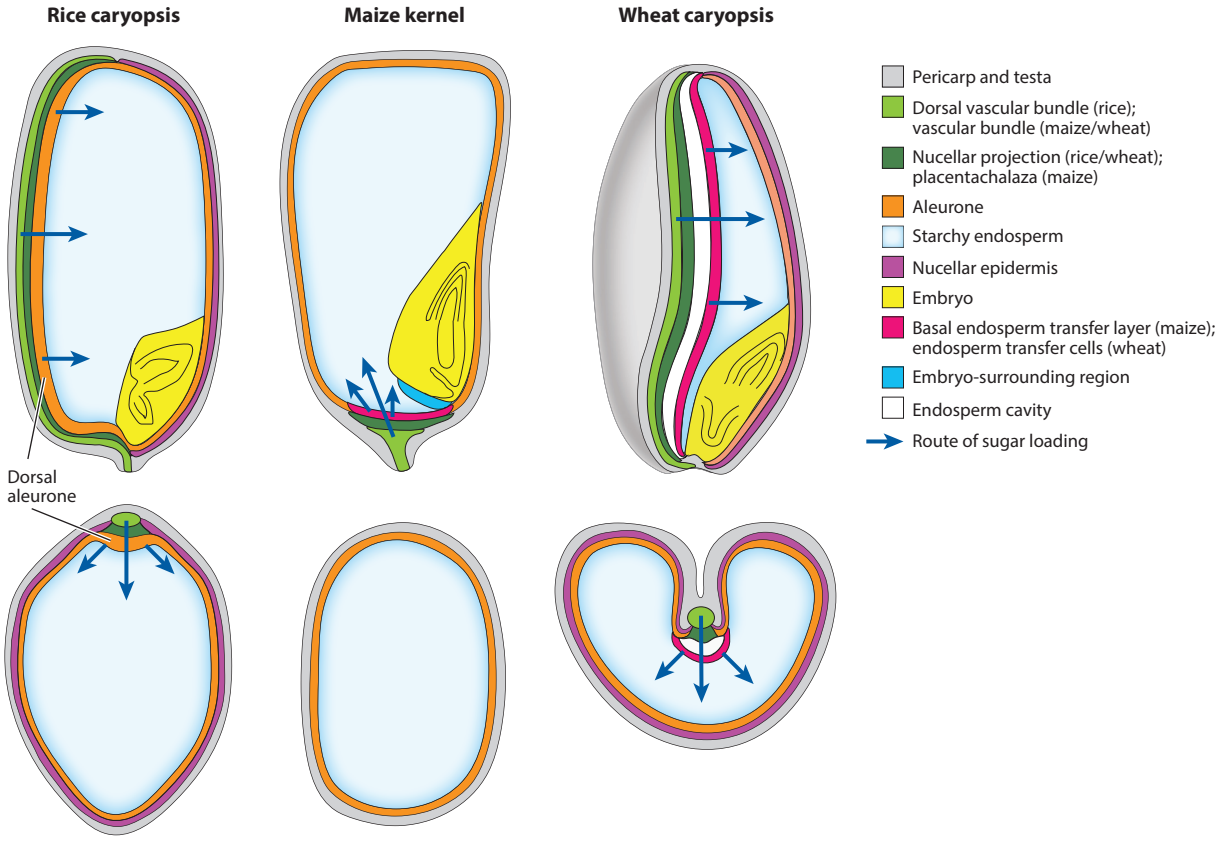

Figure 2

Schematic comparison of caryopses in rice, maize and wheat at the longitudinal (*upper*) and transversal sections (*lower*), with attention to embryo (*yellow*), starchy endosperm (*light blue*), aleurone (*orange*), and sugar-loading vascular bundle (*light green*). Routes of sugar loading into starchy endosperm are represented by blue arrows. Diagrams are not to scale.

development. The aleurone in rice, maize, and wheat comprises mostly a single layer of cells, with occasional two-cell layers. In rice, the dorsal aleurone located near the dorsal vascular bundle (the main vasculature in rice caryopsis) is composed of three or five cell layers (184). Barley is the only known cereal crop with a two- to three-cell-layer aleurone (66).

Although the aleurone and starchy endosperm share the same developmental origin, they have very different cell fates. In mature cereal grains, the aleurone and embryo are live tissues, while the starchy endosperm and all maternal tissues including the pericarp and testa are dead (183, 184, 201). Programmed cell death (PCD) in the starchy endosperm has been reported in several cereal species. During this process, cytoplasmic membranes gradually lose their integrity (184, 200, 201), although DNA fragmentation as a common mark for PCD has only been detected in a few cases (200, 201).

Other endosperm tissues such as the basal endosperm transfer layer (BETL) and embryo-surrounding region (ESR) have also been identified in species such as maize (**Figure 2**); these have proposed roles in sugar loading and embryo–endosperm interaction, respectively (8, 30, 107). In other species, such as rice, these tissues are not always recognizable.

3. REGULATION OF ENDOSPERM DEVELOPMENT IN CEREALS

3.1. Cell Cycle Regulation in Cereal Endosperms

Endoreduplication: DNA replication that is not followed by mitosis, leading to the formation of polyploid nuclei with increased DNA content

Cereal endosperms display three distinct types of cell cycle regulation: (*a*) coenocytic mitosis, in which mitosis is not followed by cytokinesis, resulting in the formation of a multinucleated coenocyte in a shared cytoplasm; (*b*) normal mitosis, leading to the proliferation of the cellular endosperm; and (*c*) endoreduplication, during which DNA synthesis is not followed by mitosis, leading to increased genome copies per cell. Coenocytic mitosis takes place in endosperms of most angiosperm species and resembles the early embryonic development of the fruit fly (*Drosophila melanogaster*). For some cereals, such as *Coix lacryma-jobi* (Job's tears), the coenocytic stage is very short, lasting only a few cell cycles (188), whereas for many other species such as rice and maize the coenocytic stage is quite long, producing several thousand nuclei (131, 184). In maize, endoreduplication first occurs in the central region of the starchy endosperm and gradually progresses toward the outer regions, and the ploidy levels may reach as high as 384C (C represents the DNA content of the haploid genome per nucleus), corresponding to seven rounds of endoreduplications (10, 136). However, in rice a higher ploidy level has been observed in the intermediate region between the central and peripheral regions (76). The existence of these types of cell cycle regulation at different stages of endosperm development suggests that the highly specialized checkpoint controls regulate the progression from DNA replication to mitosis and from mitosis to cytokinesis in endosperms.

3.1.1. Major cell cycle regulators in cereal endosperms. Multiple key cell cycle regulators are involved in coordinating endosperm development, including the retinoblastoma-related (RBR) protein, the cyclin-dependent kinase (CDK)/cyclin complex, CDK-specific inhibitors, and the anaphase-promoting complex/cyclosome (APC/C) (35). Downregulation of *RBR1* through RNA interference (RNAi) in maize enhances both mitosis and endoreduplication, leading to increased cell numbers and DNA contents (132). Further, cell cycle progression in cereal endosperms may involve differential expression and/or activation of specific cyclins and CDKs, while the endoreduplication of endosperm nuclei is associated with reduced proteolytic degradation of cyclin (36). In maize endosperm, inhibition of the M-phase CDK activity and activation of the S-phase CDK activity lead to nuclear endoreduplication (50). Conversely, overexpression of a dominant-negative form of A-type CDK in maize endosperm blocks endoreduplication but does not affect cell size, causing a slightly reduced accumulation of storage products (82).

Another type of cell cycle regulator acting in cereal endosperms is the Kip-related proteins (KRPs), which are CDK inhibitors that bind to a complex formed by A-type CDKs and D-type cyclins (35). *OsKRP1* and *OsKRP2* are predominantly expressed in developing rice grains, and overexpression of *OsKRP1* in rice affects grain filling and results in smaller grains with compromised germination, probably due to an inhibition of endosperm cell proliferation (2, 6). Unexpectedly, a similar phenotype has also been observed in the *oskrp2* single mutant, as well as in the *oskrp1 oskrp2* double mutant, suggesting that steady levels of OsKRP1 and/or OsKRP2 are critical for endosperm development (2). These KRPs may regulate cell cycle progression in a stage-specific manner through interactions with CDKs (103). Further, mutations in the APC activators *CELL CYCLE SWITCH 52A* (*CCS52A*) and *CCS52B* in rice led to reduced grain sizes. CCS52A may regulate the transition from mitosis to endoreduplication (151), whereas CCS52B may regulate cell expansion in the endosperm (150).

3.1.2. Other cell cycle regulators in cereal endosperms. Additional cell cycle–related proteins are also important for cereal endosperm development (**Table 1**). *ENDOSPERMLESS 1*

Table 1 Genes critical for cereal endosperm development and storage product accumulation

Section	Species	Gene name	Functional molecule	Accession number	Reference(s)
Cell cycle regulation in cereal endosperms	Rice	*Kip-related protein 1* (*KRP1*)	Cyclin-dependent kinase (CDK) inhibitor	Os02g0762400	2, 6
		KRP2	CDK inhibitor	Os06g0213700	2
		CELL CYCLE SWITCH 52A (*CCS52A*)	Anaphase-promoting complex (APC) activator	Os03g0123300	151
		CCS52B	APC activator	Os01g0972900	150
		ENDOSPERMLESS 1 (*ENL1*)	Sucrose nonfermenting 2 (SNF2)-type DNA helicase	Os04g0692750	54
		Helicase family protein (*HeFP*)	Helicase family protein	Os03g0586900	213
		MIS-SHAPEN ENDOSPERM (*MISSEN*)	Long noncoding RNA (lncRNA)	XLOC_057324	213
	Maize	*RBR1*	Retinoblastoma-related (RBR) protein	Zm00001d007407	132
		DEFECTIVE KERNEL 15 (*DEK15*)	Cohesion-loading complex subunit, SISTER CHROMATID COHESION PROTEIN 4	Zm00001d052197	56
		VARIED KERNEL SIZE 1 (*VKS1*)	Kinesin-14 motor protein	Zm00001d018624	63
		ZmATR	Ataxia-telangiectasia mutated (ATM)- and RAD3-related (ATR) kinase	Zm00001d014813	117
Differentiation of aleurone and starchy endosperm	Rice	*OsCR4*	Receptor-like kinase, ortholog of maize CRINKLY4 (CR4)	Os03g0637800	118
		ADAXIALIZED LEAF1 (*ADL1*)/*OsDEK1*	Calpain-like cysteine proteinase, ortholog of maize DEK1	Os02g0709400	58
		OsGCD1	Mitochondria-targeted protein, ortholog of *Arabidopsis* GAMETE CELL DEFECTIVE 1 (GCD1)	Os01g0801700	62
		THICK ALEURONE 2 (*TA2*)/*OsROS1*	DNA demethylase, ortholog of *Arabidopsis* REPRESSOR OF SILENCING 1 (ROS1)	Os01g0218032	93
		OsmtSSB1	Mitochondria-targeted single-stranded DNA-binding protein (mtSSB)	Os05g0509700	84

(*Continued*)

Table 1 (*Continued*)

Section	Species	Gene name	Functional molecule	Accession number	Reference(s)
	Maize	*CR4*	Receptor-like kinase	Zm00001d023425	12, 157
		DEK1	Calpain-like cysteine proteinase	Zm00001d028818	89, 157
		SUPERNUMERARY ALEURONE LAYER 1 (*SAL1*)	Class E vacuolar sorting protein	Zm00001d046599	138, 157
		THICK ALEURONE 1 (*THK1*)	NEGATIVE ON TATA-LESS1 (NOT1) subunit of the CCR4-NOT complex	Zm00001d027278	179, 197
		NAKED ENDOSPERM 1 (*NKD1*)	INDETERMINATE-domain (IDD) transcription factor (TF)	Zm00001d002654	49, 198
		NKD2	IDD TF	Zm00001d026113	49, 198
		YELLOW STRIPE-LIKE 2 (*ZmYSL2*)	Plasma membrane–localized iron transporter	Zm00001d017427	57
		ZmDOF3	DNA-binding with one finger (DOF) TF	Zm00001d035651	121
Roles of hormones in cereal endosperm development	Rice	*YUCCA9* (*OsYUC9*)	Flavin-containing monooxygenases	Os01g0273800	1, 191
		OsYUC11	Flavin-containing monooxygenases	Os12g0189500	1, 191
		OsTAR1	Tryptophan aminotransferase, ortholog of *Arabidopsis* TRYPTOPHAN AMINOTRANSFERASE RELATED1 (TAR1)	Os05g0169300	1, 191
		THOUSAND-GRAIN WEIGHT 6 (*TGW6*)	Indole-3-acetic acid (IAA)-glucose hydrolase	Os06g0623700	65
		BIG GRAIN 1 (*BG1*)	Cytoplasmic membrane–associated protein	Os03g0175800	94
		DEFECTIVE GRAIN-FILLING 1 (*DG1*)	Multidrug and toxic compound extrusion (MATE) efflux family protein, abscisic acid (ABA) efflux transporter	Os03g0229500	124
		DWARF4	Cytochrome P450	Os03g0227700	208
	Maize	*Defective endosperm 18* (*De18*)/*ZmYUC1*	Flavin-containing monooxygenase	Zm00001d023718	15, 41
		ZmEHD1	Eps15 homology domain (EHD) protein	Zm00001d053858	171
		ZmPIN1a	Auxin transport PIN-FORMED 1 (PIN1)	Zm00001d044812	41
		ZmPIN1b	PIN1	Zm00001d018024	41
		ZmPIN1c	PIN1	Zm00001d052269	41

(*Continued*)

Table 1 (*Continued*)

Section	Species	Gene name	Functional molecule	Accession number	Reference(s)
Epigenetic regulation in cereal endosperms	Rice	*OsFIE1*	WD40-containing component of Polycomb Repressive Complex 2 (PRC2), ortholog of *Arabidopsis* FERTILIZATION-INDEPENDENT ENDOSPERM (FIE)	Os08g0137250	25, 204
		OsFIE2	WD40-containing component of PRC2, ortholog of *Arabidopsis* FIE	Os08g0137100	25, 86
		OsEMF2a	Zinc-finger-containing component of PRC2, ortholog of *Arabidopsis* EMBRYONIC FLOWER2 (EMF2)	Os04g0162100	26, 159
	Maize	*ZmFIE1*	WD40-containing component Of PRC2, ortholog of *Arabidopsis* FIE	Zm00001d049608	34, 52
		ZmFIE2	WD40-containing component of PRC2, ortholog of *Arabidopsis* FIE	Zm00001d024698	34, 52
		MATERNALLY EXPRESSED GENE1 (*MEG1*)	Small cysteine-rich peptide	Zm00001d019030	31
Coordinated actions of maternal and filial tissues in grain filling	Rice	*CELL WALL INVERTASE 2* (*CIN2*)/*GRAIN INCOMPLETE FILLING 1* (*GIF1*)	Cell wall invertase	Os04g0413500	164
		OsMADS29	MADS-box TF	Os02g0170300	106, 199
		OsSUT1	Sucrose transport (SUT)	Os03g0170900	43, 137
		NF-YB1	Nuclear factor-Y TF subunit B (NF-YB)	Os02g0725900	4, 14
		OsSWEET4	Sugar transporter, ortholog of maize SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER 4c (SWEET4c)	Os02g0301100	144
		OsSWEET11	Sugar transporter	Os08g0535200	99, 193
		OsSWEET15	Sugar transporter	Os02g0513100	193
		OsDOF11	DOF TF	Os02g0707200	186
	Maize	*MINIATURE1* (*Mn1*)	Cell wall invertase	Zm00001d003776	24, 69, 81
		ZmSUT1	Sucrose transporter	Zm00001d027854	139
		SWEET4c	Sugar transporter	Zm00001d015912	144
		ZmSWEET11	Sugar transporter	Zm00001d031647	139

(*Continued*)

Table 1 (*Continued*)

Section	Species	Gene name	Functional molecule	Accession number	Reference(s)
Starch biosynthesis in the starchy endosperm	Rice	*OsGBSSI/Waxy*	Granule-bound starch synthase I (GBSSI)	Os06g0133000	133
		STARCH REGULATOR1 (*RSR1*)	APETALA2(AP2)/EREBP TF	Os05g0121600	42
		SALT-RESPONSIVE ERF1 (*SERF1*)	Dehydration Response Element-Binding (DREB) TF	Os05g0420300	135
		LncRNA_1267	LncRNA	Not available (NA)	212
		LncRNA_1631	LncRNA	NA	212
		LncRNA_2308	LncRNA	NA	212
		BINDING PROTEIN-5 (*OsBP-5*)	MYC TF	Os03g0639300	216
		OsEBP-89	Ethylene-responsive element binding protein (EREBP) TF	Os03g0182800	216
		NF-YC12	Nuclear factor-Y TF subunit C (NF-YC)	Os10g0191900	14
		bHLH144	Basic helix-loop-helix (bHLH) TF	Os04g0429400	14
		RADICLELESS 1 (*RL1*)	Pentatricopeptide repeat (PPR) protein	Os08g0525500	182
		FLOURY ENDOSPERM10 (*FLO10*)	PPR protein	Os03g0168400	181
		SMALL KERNEL 1 (*OsSMK1*)	PPR protein	Os11g0213500	88
		OPAQUE AND GROWTH RETARDATION 1 (*OGR1*)	PPR protein	Os12g0270200	75
		NADH DEHYDROGENASE 1 ALPHA SUBCOMPLEX SUBUNIT 9 (*OsNDUFA9*)	Mitochondrial complex I subunit	Os02g0816800	61
	Maize	*ZmGBSSI/Waxy*	Granule-bound starch synthase I	Zm00001d045462	185
		ZmMYB14	MYB TF	Zm00001d021537	189
		ZmDOF36	DOF TF	Zm00001d029512	180
		SMALL KERNEL 3 (*SMK3*)	Mitochondrial transcription termination factor	Zm00001d041537	114
	Wheat	*TaRSR1*	AP2/EREBP TF, ortholog of rice RSR1	JX473823	92
		TabZIP28	Basic leucine zipper (bZIP) TF	MN022880 MN022881 MN022882	143
	Barley	*HvSUSIBA2*	WRKY TF	AY323206	148, 149

(*Continued*)

Table 1 (*Continued*)

Section	Species	Gene name	Functional molecule	Accession number	Reference(s)
Storage protein accumulation in cereal endosperms	Rice	*OsMYB5*	MYB TF	Os05g0490600	152
		OsbZIP58/RICE BASIC LEUCINE ZIPPER 1 (RISBZ1)	bZIP TF	Os07g0182000	73, 167
		RICE PROLAMIN-BOX BINDING FACTOR (RPBF)	DOF TF	Os02g0252400	73
		OsNAC20	NAM/ATAF/CUC (NAC) TF	Os01g0104500	166
		OsNAC26	NAC TF	Os01g0393100	166
		RBP-L	RNA-binding protein (RBP)	Os04g0625800	156
		RBP-P	RBP	Os01g0265800	155
		OsTudor-SN	Cytoskeletal-associated RBP	Os02g0523500	29, 163
		VPE1/GLUTELIN PRECURSOR 3 (GLUP3)	Vacuolar processing enzyme (VPE)	Os04g0537900	79
		PDIL1-1/ENDOSPERM STORAGE PROTEIN MUTANT 2 (ESP2)	Protein disulfide-isomerase-like 1 (PDIL1)	Os11g0199200	153
		Rab5a/GLUTELIN PRECURSOR OVERACCUMULATION 1 (GPA1)	Small GTPase	Os12g0631100	91, 172
		VACUOLAR PROTEIN SORTING 9A (VPS9A)/GPA2	Guanine exchange factor	Os03g0262900	91
		GPA3	Kelch-repeat-containing protein	Os03g0835800	127, 172
		GOLGI TRANSPORT1B (GOT1B)/GPA4	Membrane protein	Os03g0209400	170
		GPA5	Plant-unique phox-homology domain-containing protein	Os06g0643000	128

(*Continued*)

Table 1 (*Continued*)

Section	Species	Gene name	Functional molecule	Accession number	Reference(s)
	Maize	*OPAQUE2* (*O2*)	bZIP TF	Zm00001d018971	177, 211
		PROLAMIN-BOX BINDING FACTOR (*PBF*)	DOF TF	Zm00001d005100	83, 211
		O2 HETERODIMERIZING PROTEIN 1 (*OHP1*)	bZIP TF	Zm00001d034457	210
		OHP2	bZIP TF	Zm00001d013074	210
		ZmbZIP22	bZIP TF	Zm00001d021191	83
		ZmMADS47	MADS-box TF	Zm00001d027957	123
		ZmNAC128	NAC TF	Zm00001d040189	209
		ZmNAC130	NAC TF	Zm00001d008403	209
		O11	bHLH TF	Zm00001d003677	40
		ABSCISIC ACID INSENSITIVE 19 (*ZmABI19*)	B3 domain TF	Zm00001d011712	194
		FLOURY1 (*FL1*)	Endoplasmic reticulum protein	Zm00001d003398	59
		O1	Myosin XI protein	Zm00001d052110	165
		O10	Cereal-specific protein body protein	Zm00001d033654	196
	Wheat	*STORAGE PROTEIN ACTIVATOR* (*SPA*)	bZIP TF	CAA70216	3
		SPA HETERODIMERIZING PROTEIN (*SHP*)	bZIP TF	TraesCS5A02G440400	16
		TaNAC019	NAC TF	TraesCS3A02G077900 TraesCS3B02G092800 TraesCS3D02G078500	44
	Barley	*BARLEY LEUCINE ZIPPER 1* (*BLZ1*)	bZIP TF	X80068	162
		BLZ2	bZIP TF	CAA71795	110
		HvGAMYB	MYB TF	X87690	38

(*ENL1*) in rice encodes a sucrose nonfermenting 2 (SNF2)-type DNA helicase, which is homologous to the human Polo-like kinase 1 (Plk1)-interacting checkpoint helicase. ENL1 localizes to the cytoplasm during interphase and to the chromosomes during mitosis, and an *enl1* mutant showed enlarged nuclei in endosperms at the coenocytic stage, due to abnormal chromosome segregation, and aborted endosperms at a later stage (54). Another helicase family protein (HeFP) is also important for cytoskeleton polymerization during mitosis and cellularization in the endosperm (213). A maternally expressed long noncoding RNA (lncRNA), *MIS-SHAPEN ENDOSPERM* (*MISSEN*), can sequester HeFP to inhibit the interaction between HeFP and tubulin, leading to abnormal cytoskeleton polymerization during endosperm development. Suppressing *MISSEN* expression promoted nuclear division and cellularization during early stage endosperm development, resulting in slightly enlarged seeds, whereas overexpression of *MISSEN* inhibited endosperm development, leading to prominent dents and bulges in the seed (213).

The maize gene *DEFECTIVE KERNEL 15* (*DEK15*) encodes a cohesin-loading complex subunit, SISTER CHROMATID COHESION PROTEIN 4, which holds sister chromatids together during chromosome segregation. Mutation in *DEK15* causes precocious and abnormal sister chromatid separation, interrupting mitosis and DNA endoreduplication, and thus producing grains with a defective endosperm (56). In addition, the maize kinesin-14 protein VARIED KERNEL SIZE 1 (VKS1) localizes to both microtubules and nuclei and is critical for mitosis and cytokinesis in the early endosperm. Mutations of *VKS1* led to abnormalities in spindle assembly, sister chromatid separation, and phragmoplast formation, resulting in smaller kernels with reduced cell proliferation (63). An ataxia-telangiectasia mutated (ATM) and RAD3-related (ATR) kinase in maize, ZmATR, plays important roles in DNA repair and cell cycle checkpoint activation and is required to repress DNA endoreduplication and PCD in the endosperm. Loss of ZmATR results in a reduced kernel size, decreased protein and starch contents, and accelerated PCD in the endosperm (117). These results together suggest that distinct cell cycle checkpoint controls for DNA replication, mitosis, and cytokinesis, as well as related events such as chromosomal segregation and DNA repair, are critical for cereal endosperm development and, consequently, for grain size. The developmental and evolutionary advantages of the unusual cell cycle regulation in endosperms of most angiosperm species, whereby mitosis and cytokinesis are separated into two phases, as well as how these processes are regulated, remain largely unknown and need to be elucidated.

3.2. Differentiation of Aleurone and Starchy Endosperm

The aleurone and starchy endosperm in cereal crops have distinct cell fates and storage product reserves. The former is rich in proteins, lipids, vitamins, and micronutrients, whereas the latter is rich in starch. The differentiation of cereal endosperms starts after cellularization is completed and forms the outer aleurone layer and the inner starchy endosperm (184). Genes regulating aleurone and starchy endosperm differentiation have been identified in maize and rice (**Figure 3**; **Table 1**). *CRINKLY4* (*CR4*) encodes a receptor-like kinase, while *DEK1* encodes a membrane-localized protein with an extracellular loop and a calpain-like Cys proteinase domain. Mutations of either *CR4* or *DEK1* in maize, and silencing of the *OsCR4* or *DEK1* ortholog *ADAXIALIZED LEAF1* in rice, cause a partial loss of the aleurone cell layer in the endosperm (12, 58, 89, 118), suggesting that CR4 and DEK1 are positive regulators of aleurone differentiation. A similar phenotype has been observed recently in a maize mutant defective in YELLOW STRIPE-LIKE 2 (ZmYSL2), a plasma membrane–localized iron transporter (57), suggesting that proper iron distribution is important for the differentiation of the aleurone layer (**Figure 3**). By contrast, mutations in either *SUPERNUMERARY ALEURONE LAYER 1* (*SAL1*) or *THICK ALEURONE 1* (*THK1*) in

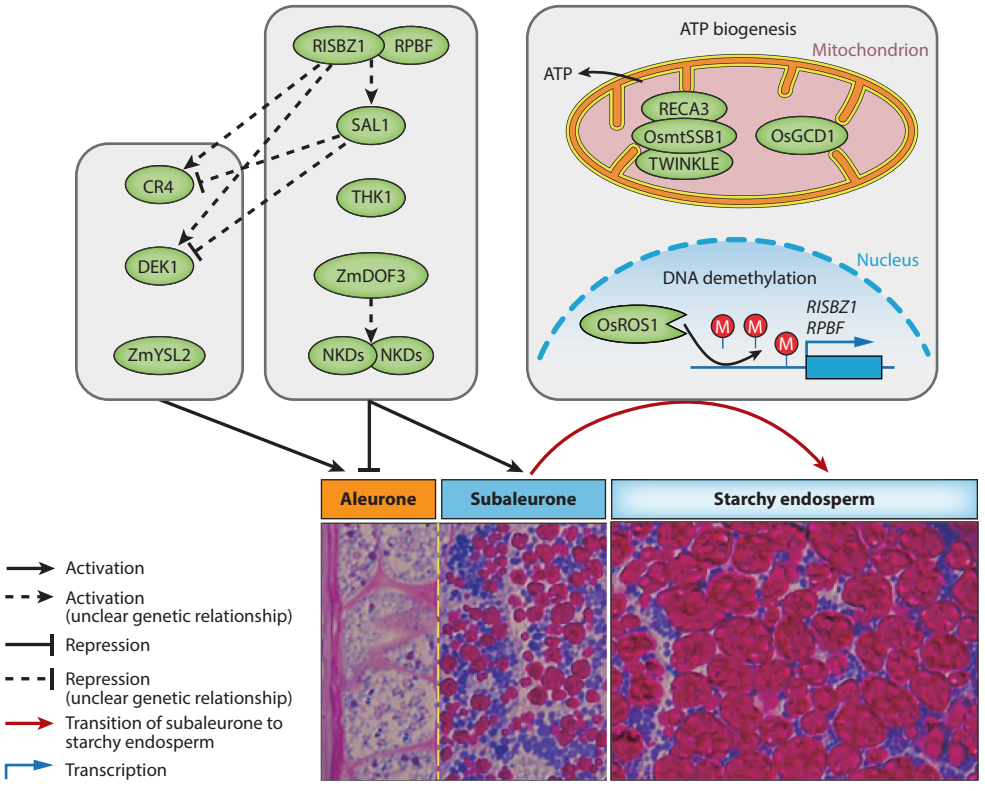

Figure 3

Genetic networks regulating aleurone and starchy endosperm differentiation in cereal endosperms. Note that regulators promoting starchy endosperm cell fate (restricting aleurone cell fate; RISBZ1, RPBF, SAL1, THK1, ZmDOF3, and NKDs) and aleurone cell fate (CR4, DEK1, and ZmYSL2) may establish a feedback regulation network to maintain the balance between aleurone and starchy endosperm. Energy homeostasis (regulated by the RECA3-OsmtSSB1-TWINKLE complex and OsGCD1) and DNA demethylation (regulated by OsROS1) as fundamental cell biological processes in endosperms promote the transition of subaleurone to starchy endosperm. Abbreviations: ATP, adenosine triphosphate; CR4, CRINKLY4; DEK1, DEFECTIVE KERNEL 1; M, 5-methylcytosine DNA methylation; NKD, NAKED ENDOSPERM; OsGCD1, *Oryza sativa* ortholog of GAMETE CELL DEFECTIVE 1; OsmtSSB1, *Oryza sativa* mitochondrially targeted single-stranded DNA-binding protein 1; OsROS1, *Oryza sativa* REPRESSOR OF SILENCING 1; RECA3, recombinase A3; RISBZ1, RICE BASIC LEUCINE ZIPPER 1; RPBF, RICE PROLAMIN-BOX-BINDING FACTOR; SAL1, SUPERNUMERARY ALEURONE LAYER 1; THK1, THICK ALEURONE 1; ZmDOF3, *Zea mays* DNA-binding with one finger 3; ZmYSL2, *Zea mays* YELLOW STRIPE-LIKE 2.

maize as well as *THICK ALEURONE 2* (*TA2*) in rice resulted in endosperms with increased numbers of aleurone cell layers (93, 138, 179, 197). *SAL1* encodes a class E vacuolar sorting protein that colocalizes with CR4 and DEK1 in endosomes of the endosperm (13, 157), suggesting that SAL1 may target to, and interfere with, CR4 and DEK1 cycling in membranes. *THK1* encodes a homolog of NEGATIVE ON TATA-LESS1 (NOT1), and a mutation in *THK1* led to the production of grains with multiple aleurone layers and aborted embryos (179, 197). *TA2* encodes an ortholog of the DNA demethylase REPRESSOR OF SILENCING 1 (ROS1) in *Arabidopsis* (48). Grains produced in weak mutant alleles of *ta2* exhibited an increased number of aleurone cell layers and improved nutritional profile (93), although a null mutant of *OsROS1* is lethal (111). DNA

methylation analyses revealed that the *ta2* mutant had an increased level of genome-wide DNA methylation in the endosperm (93), suggesting that DNA demethylation plays a critical role in restricting the number of aleurone cell layers (**Figure 3**).

Several transcription factors (TFs) are also important in restricting the number of aleurone cell layers in maize and rice endosperms (**Figure 3**). Two duplicate genes, *NAKED ENDOSPERM 1* (*NKD1*) and *NKD2*, encode INDETERMINATE-domain (IDD)-containing TFs in maize, and the *nkd1 nkd2* double mutant exhibited an aleurone with multiple cell layers and an opaque starchy endosperm (198). These two TFs may form a dimeric complex via their IDDs to restrict the number of aleurone cell layers (49). RNAi-mediated silencing of an endosperm-specifically expressed DNA-binding with one finger (DOF) family TF of *ZmDOF3* led to defective kernels with reduced starch content and multiple layers of aleurone cells at local positions of the endosperm, and *NKD1* and *NKD2* expression was also reduced in endosperms of these plants, raising the possibility that *ZmDOF3* may act upstream of *NKD1* and *NKD2* (121). In rice, two TFs, RICE BASIC LEUCINE ZIPPER 1 (RISBZ1) and RICE PROLAMIN-BOX BINDING FACTOR (RPBF), are involved in restricting the aleurone cell layers in the endosperm, as RNAi-mediated silencing of both *RISBZ1* and *RPBF* led to the formation of endosperms with multiple aleurone cell layers (73). The silencing also led to reduced expressions of *OsCR4*, *OsDEK1*, and *OsSAL1*, suggesting that RISBZ1 and RPBF may act upstream of those three genes (73). Interestingly, in the *ta2* mutant mentioned above, expression levels of *RISBZ1* and *RPBF* were decreased in the endosperm, and the levels of DNA methylation in promoter regions of *RISBZ1* and *RPBF* were increased, suggesting that OsROS1 may restrict the aleurone cell layers through DNA-demethylation-mediated activation of *RISBZ1* and *RPBF* (93).

Energy supply may play an important role in the determination of aleurone cell fate (**Figure 3**). OsGCD1, the rice ortholog of the *Arabidopsis* GAMETE CELL DEFECTIVE 1, is a mitochondria-targeted protein that is required for both embryo and endosperm development. Endosperms in the loss-of-function mutant of *OsGCD1* are characterized by aleurone with variable cell layers and disorganized cell patterns (62). The recently reported *TA1* in rice encodes a mitochondria-targeted single-stranded DNA-binding protein, OsmtSSB1, and interacts with mitochondrial DNA recombinase A3 (RECA3) and DNA helicase TWINKLE. Mutations in *TA1* resulted in increased sugar content and decreased adenosine triphosphate (ATP) content in endosperms, along with a switch of subaleurone cells toward aleurone (84).

As a hypothetical model, supported by genetic evidence collected so far, we propose that the aleurone is the default and primitive state of the endosperm, whereas the starchy endosperm is an advanced feature (**Figure 3**). The model predicts that loss of function or mutation of genes involved in promoting starchy endosperm differentiation (for example, *RISBZ1*, *RPBF*, *SAL1*, *THK1*, *ZmDOF3*, and *NKD*s, which at the same time restrict aleurone differentiation) will allow more cells to stay in the aleurone state, resulting in an increased number of aleurone cell layers. Other genes such as *CR4*, *DEK1*, and *ZmYSL2* are involved in maintaining and promoting the integrity of the aleurone layer, and mutation of any of these genes leads to the formation of patchy and partially formed aleurone. These two sets of genes may build a feedback regulation loop that maintains the balance between these two cell types (**Figure 3**). Further, energy homeostasis and DNA demethylation (as shown by functional analyses of *TA1*/*OsmtSSB1* and *TA2*/*OsROS1* in rice) as fundamental cell biological processes play critical roles in executing starchy endosperm differentiation, consequently promoting the transition of the intermediate subaleurone cells toward starchy endosperm (84, 93) (**Figure 3**).

Mutants such as *ta1* and *ta2*, with increased aleurone cell layers, showed improved nutrition profiles for vitamins and micronutrients, which could potentially be used in developing nutrient-rich cereal crops if grain filling is not compromised (84, 93). A nutrient-rich black rice variety of

Zhongzi-1^{ta1} has recently been developed by crossing *ta1* with a black rice variety (84) since black rice is traditionally consumed as whole grains in Asian countries (207).

3.3. Roles of Hormones in Cereal Endosperm Development

3.3.1. Auxin. Auxin homeostasis, maintained by the combined action of indole-3-acetic acid (IAA) biosynthesis, conjugation, oxidation, and transport, seems to play indispensable roles in cereal endosperm development (7). Active IAA biosynthesis has been detected in rice and maize grains after fertilization. In rice, the expression levels of the IAA biosynthetic genes *YUCCA1* (*OsYUC1*), *OsYUC9* and *OsYUC11* (both encoding flavin-containing monooxygenases), and *TRYPTOPHAN AMINOTRANSFERASE RELATED1* (*OsTAR1*) (encoding a tryptophan aminotransferase) increase gradually during endosperm development, correlating with an increased IAA level in grains (1, 208). Mutations of *OsYUC9*, *OsYUC11*, or *OsTAR1* led to defective grain filling and chalky endosperm (191). In maize, *Defective endosperm 18* (*De18*) encodes an endosperm-specific OsYUC1 ortholog ZmYUC1, and the *de18* mutant exhibited a reduced IAA content, abnormal BETL differentiation, and small kernels (15, 41). The expression of *ZmYUC1* is induced by sugars, and a mutant of the cell wall invertase *MINIATURE1* (*Mn1*) exhibited decreased *ZmYUC1* expression levels, IAA content, and kernel weight (81), suggesting that auxin biosynthesis and sugar loading in endosperms are closely linked. Further, a major quantitative trait locus for thousand-grain weight of rice, *THOUSAND-GRAIN WEIGHT 6* (*TGW6*), encodes a novel IAA-glucose hydrolase, and a near-isogenic line carrying a loss-of-function *tgw6* allele produces larger grains with significantly decreased IAA content (65). TGW6 may function in timing the transition from the coenocytic stage to cellularization by controlling the IAA supply and consequently limiting the cell number and grain length. Maize ZmEHD1, an Eps15-homology-domain-containing protein, regulates auxin homeostasis indirectly by modulating clathrin-mediated endocytosis, and a mutation in *ZmEHD1* led to reduced IAA accumulation in grains, resulting in shrunken grains with fewer starch granules (171).

Auxin polar transport is essential to establish auxin maxima and gradients in plants. Within the maize endosperm, cells in the aleurone, BETL, and ESR accumulate higher levels of auxin than the inner starchy endosperm, which seem to correlate with auxin polar transport mediated by PIN-FORMED 1 (ZmPIN1) (41). Maize grains treated with the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA) showed aleurone with multiple cell layers, suggesting that auxin polar transport is involved in restricting the number of aleurone cell layers (41). The dominant rice mutation *big grain 1* (*bg1-D*) results in increased grain size, and at the physiological level the *bg1-D* mutant is hypersensitive to auxin and auxin transport inhibitors, showing increased basipetal auxin transport and altered auxin distribution. The *BG1* gene encodes a cytoplasmic membrane-associated protein, and its expression is induced by auxin treatment, suggesting that *BG1* acts as a positive regulator in auxin response during grain development (94). Whether the BG1 protein exerts its role throughout the entire grain, or specifically in the endosperm, remains to be investigated.

3.3.2. Cytokinin. Cytokinin is another important hormone regulating cereal endosperm development (67). Cytokinin content is positively correlated with endosperm cell division (208), rising dramatically early in endosperm development and declining afterwards (20, 208). The expression patterns of cytokinin biosynthetic genes, including *ISOPENTENYL TRANSFERASE* (*IPT*) and *CYTOKININ OXIDASE* (*CKX*), coincide with cytokinin distribution in endosperm (19, 20, 208). Exogenous application of cytokinin promotes endosperm cell division and grain filling, along with elevated expression of several cell cycle–related genes such as *CDK*s and *Cyclin*s (115). An elevated cytokinin level upon overexpression of *IPT* genes led to a partial loss of aleurone in

maize endosperm, suggesting that a proper cytokinin level is critical in maintaining the aleurone cell fate (47).

Imprinted gene expression: monoallelic gene expression in which only the gene derived from the father or from the mother is expressed

3.3.3. Abscisic acid and brassinosteroids. Abscisic acid (ABA) is implicated in the cellularization of cereal endosperms. In barley, the *shrunken endosperm genetic 8* (*seg8*) mutant, with a reduced ABA level in grains, displayed abnormal endosperm cellularization and shrunken endosperm (146). In rice, genes related to ABA biosynthesis and signaling are upregulated at 3 days after pollination (DAP), the time point at which endosperm cellularization begins (208). *DEFECTIVE GRAIN-FILLING 1* (*DG1*) encodes a multidrug extrusion protein that functions as an efflux carrier loading ABA from leaf to caryopsis (124). After arriving at the caryopsis, ABA may activate the expression of genes for starch synthesis, storage protein accumulation, and grain filling, as *dg1* caryopses have a reduced ABA content and incompletely filled and floury endosperm (124).

Brassinosteroids (BRs) may also play a role in endosperm development. In rice, expression levels of BR biosynthesis and signaling genes in endosperm peak at 2–3 DAP and then decrease by 6 DAP. Rice *DWARF4* encodes a cytochrome P450 and is involved in BR biosynthesis. Ectopic expression of *DWARF4* in rice starchy endosperm led to increased grain length and width and reduced chalkiness (208).

3.4. Epigenetic Regulation in Cereal Endosperms

DNA methylation and histone modifications mediate epigenetic regulations of gene expressions in cereal endosperm development (37, 203).

3.4.1. DNA methylation. DNA methylation in plants generally occurs on the cytosine residues of DNA strands (CG, CHG, and CHH methylation, with H = A, C, or T), whereby one cytosine (asymmetric methylation) or two cytosines (symmetric methylation) within the CG or CHG sequences are modified (203). The most striking feature of endosperm genomes is their hypomethylation as compared with that of embryo and vegetative tissues (60, 202, 205). Localized hypomethylation occurs primarily on the maternal genome, especially in regions of high DNA accessibility (129, 168), suggesting a potential role in regulation of imprinted gene expressions. Imprinted genes show parent-of-origin-specific expression, in which either the maternal or the paternal copy of a gene is expressed depending on the differences in epigenetics between the maternal and paternal allele (9). In plants, imprinting occurs mainly in the endosperm, as it occurs mainly in the placenta in mammals (85). Genome-wide surveys in rice and maize have identified hundreds of imprinted genes in endosperms, mostly maternally expressed genes (MEGs) and a few paternally expressed genes (PEGs) (98, 206). In general, MEGs are enriched for hypomethylation at putative promoter and terminator regions, while PEGs are hypomethylated at gene bodies (129).

Although the levels of DNA methylation are regulated by a balanced action of methylation and demethylation, results obtained from studies so far suggest that the hypomethylation of endosperm DNA is driven mainly by the DNA demethylase (116). In *Arabidopsis*, DNA demethylase gene *DEMETER* (*DME*), which is expressed primarily in the central cell of the female gametophyte, removes DNA methylation at distinct regions of the maternal genome, causing it to become hypomethylated, which then leads to a parent-of-origin effect for some endosperm genes (28). As a paralog of *DME*, *ROS1* in *Arabidopsis* is expressed constitutively in all tissues, including endosperms, and functions in preventing the spread of DNA methylation (48). Orthologs of *DME* and *ROS1* have been identified in the genomes of rice (111), maize (168), and wheat (176). RNAi-mediated silencing of the wheat *DME* ortholog decreases the production of the allergenic storage proteins prolamins, offering an avenue to develop new varieties for patients with celiac

diseases (176). In rice, an insertion mutant of *ROS1* is defective in both male and female gametogenesis, and thus produces no grains (111), whereas a weak allele of *ROS1*, *ta2* (as mentioned in Section 3.2), showed an aleurone with multiple cell layers and improved nutrition (93).

3.4.2. Histone modification. Histone modification, especially histone H3 trimethylation on Lys27 (H3K27me3), also plays a crucial role in endosperm development. Forward-genetic screens conducted in *Arabidopsis* for development of mutants of *fertilization-independent endosperm/fertilization-independent seed* (*fie/fis*) led to the discovery of Polycomb Repressive Complex 2 (PRC2), which prevents seed and endosperm development before fertilization (51, 77, 96, 108). The evolutionarily conserved FIS-PRC2 complex, consisting of MEDEA (MEA), FIE, FIS2, and MULTICOPY SUPPRESSOR OF IRA 1 (MSI1), catalyzes H3K27me3 in *Arabidopsis* (90, 130). Mutations in any of the genes encoding PRC2 components result in a maternally inherited gametophytic seed abortion phenotype: That is, 50% of seeds abort in a self-pollinated heterozygous mutant (51, 77, 96, 108). Orthologous genes encoding components of the PRC2 complex have been identified in rice, maize, and barley (70, 158). Two *FIE* orthologs, *FIE1* and *FIE2*, are present in the rice and maize genomes (34, 97). Both *ZmFIE1* and *ZmFIE2* are MEGs (52). *ZmFIE1* is expressed exclusively in endosperms from 6 DAP onwards; *ZmFIE2* is expressed in unfertilized embryo sac and early stage endosperms, and its paternal allele is expressed starting from 5 DAP onwards (34). By contrast, *OsFIE1* is a MEG, whereas *OsFIE2* is not an imprinted gene (97, 204). Hypomethylations at the *ZmFIE1*, *ZmFIE2*, and *OsFIE1* promoter regions occur only in their maternal, not paternal, alleles. As a consequence of these asymmetric DNA methylation patterns, the expression of the maternal allele is induced, while the paternal allele is repressed (52, 204). Overexpression or knockout of *OsFIE1* results in smaller grains (25, 204), suggesting that a balanced level of OsFIE1 is critical for endosperm development. *OsFIE2* is ubiquitously expressed in both vegetative and reproductive tissues (86). Mutations in *OsFIE2*, generated by CRISPR-Cas9-mediated gene editing, led to an impaired endosperm cellularization, and heterozygous *OsFIE2/osfie2* mutants showed a low frequency of autonomous endosperm development (25). Furthermore, *OsEMF2a*, a rice ortholog of the *Arabidopsis* polycomb group gene *EMBRYONIC FLOWER2* (*EMF2*), is a MEG in endosperm and necessary for endosperm cellularization. Mutations of *OsEMF2a* in rice caused autonomous endosperm development and delayed cellularization, as well as disordered genomic imprinting in the endosperm (26, 159). Transcriptome and chromatin immunoprecipitation sequencing (ChIP-seq) analyses suggest that type-I MADS-box TF genes essential for regulating early endosperm development are downstream targets of OsEMF2a-containing PRC2 (26, 159).

3.4.3. Regulation of imprinted gene expression. Analyses of genome-wide allele-specific DNA methylation and H3K27me3 modifications in maize endosperm showed that the paternal genome is hypermethylated and the maternal genome is hypomethylated compared to those in embryos or vegetative tissues (205). Among the thousands of parent-of-origin-dependent differentially methylated regions that have been identified, all are paternally hypermethylated and maternally hypomethylated (205). In general, at least a subset of MEGs is associated with DME-mediated maternal-specific DNA demethylation located in the upstream and 5′ portion of gene body regions, while a majority of PEGs are related to maternal-specific H3K27me3 modifications (104, 205). It is possible that DME-mediated DNA demethylation in the central cell leads to the formation of hypomethylated regions in endosperm cells. Further, PRC2-mediated H3K27me3 modifications are preferentially localized to regions in the endosperm that are targeted by the DME, which links histone modification to DNA demethylation (104). Genomic analyses in *Arabidopsis* and maize have shown that imprinted expression of PEGs is associated with PRC2-mediated H3K27me3 (104, 205). The PRC2 complex functions as an inhibitor of the expression

IMPRINTED GENES IN MAMMALS

Genes are subject to monoallelic and parent-specific gene expression, whereby either the maternal allele (from the chromosome derived from the mother) or the paternal allele (from the chromosome derived from the father) is expressed in a diploid cell. This phenomenon is termed genomic imprinting and is controlled by epigenetic marks in parental germlines without differences in the DNA sequences. In mammals, imprinted gene expression occurs for a small subset of genes, predominately in the placenta, and is believed to have evolved as a mechanism to allocate parental resources to the offspring. Imprinted gene expression is regulated epigenetically by DNA methylation, histone modifications, long noncoding RNAs, and high-order DNA structure. Genes with imprinted expression are inherited with a parent-of-origin effect.

of maternally hypomethylated alleles of PEGs in the endosperm (104). These studies suggest that both DNA methylation and histone modification play important roles in the regulation of imprinted gene expression in the endosperm (see the sidebar titled Imprinted Genes in Mammals).

3.4.4. Functions of imprinted genes. It is interesting to note that genome-wide analyses of imprinted genes show a low degree of conservation among these species (46, 98, 206), although the number conserved between maize and rice is twice as high as that between maize and *Arabidopsis* (206). Mutations in genes coding the PRC2 components such as MEA, FIE, and FIS2 in *Arabidopsis* produce autonomous endosperm without ever undergoing fertilization (51, 96, 108). By contrast, knockout of *OsFIE1* resulted in small seeds that did not display autonomous endosperm development, while autonomous endosperm development was observed occasionally in grains produced in the heterozygous plants of *osfie2* and *osemf2a* mutants (25, 159). Cells in the autonomous endosperms of rice accumulate both starch granules and structures similar to protein storage vacuoles (159). However, this phenomenon has not been observed in autonomous endosperms of *Arabidopsis*. Whether this difference reflects functional differences between persistent and nonpersistent endosperms remains to be studied.

Researchers have proposed that the occurrence of MEGs during evolution serves to ensure equal nutrient allocation across seeds produced on a mother plant (51, 96, 108), as the placenta does in mammals (80), but direct evidence supporting this hypothesis is still limited. Imprinted *MATERNALLY EXPRESSED GENE1* (*MEG1*) encodes a small cysteine-rich peptide and localizes to the cytoplasmic membrane and extracellular matrix of BETL in maize. MEG1 is necessary for the establishment and differentiation of BETL, and, consequently, the loading of sugar to the endosperm indeed supports this hypothesis (31) (**Figure 4**). However, many of those MEGs are not involved in nutrient allocation, which does not seem to support this hypothesis. As an alternative, imprinted gene expression has been proposed as a byproduct of DNA demethylation in the endosperm (45, 60), which may aim to establish the efficient expression of genes involved in storage product accumulation. Further studies are needed to better understand genome imprinting in cereal endosperms.

4. REGULATION OF STORAGE PRODUCT ACCUMULATION IN CEREAL ENDOSPERMS

4.1. Coordinated Actions of Maternal and Filial Tissues in Grain Filling

The accumulation of storage products in cereal endosperms relies on photoassimilates such as sugars and amino acids produced in leaves. Sucrose, as a nonreducing disaccharide, is used in most

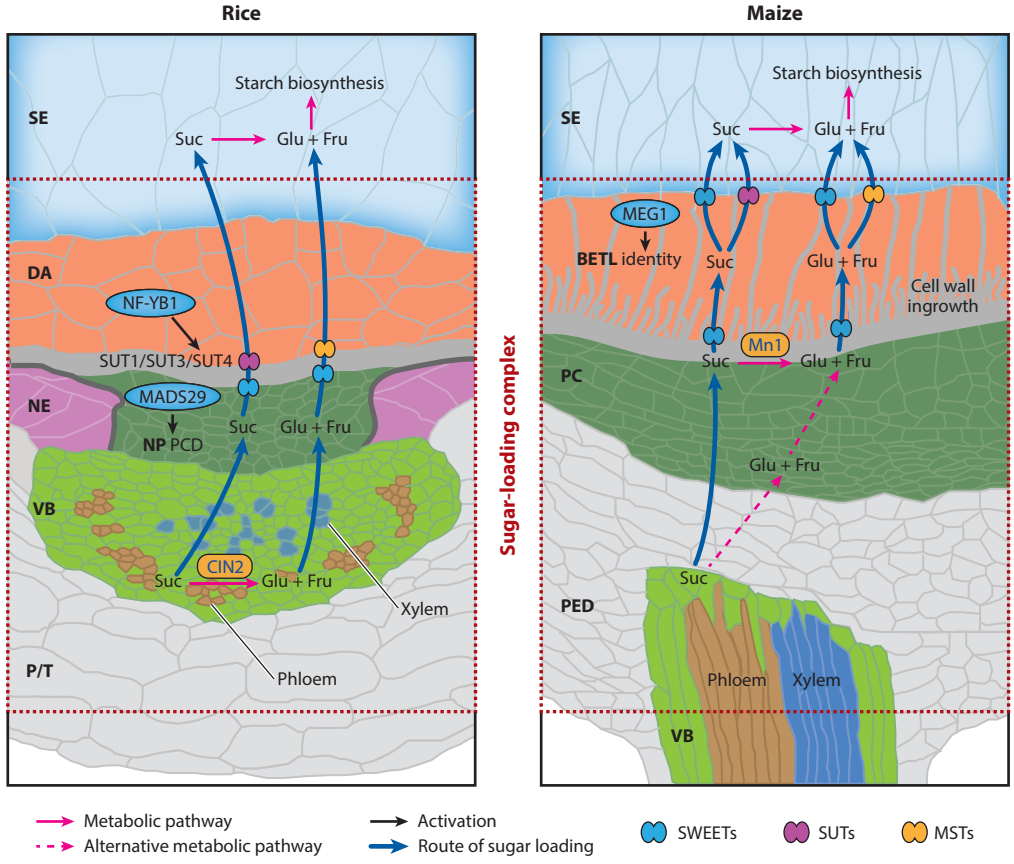

Figure 4

Schematic models for routes of sugar loading into endosperms of rice (*left*) and maize (*right*). In rice, sucrose released from the phloem in the dorsal vascular bundle is either loaded directly to the SE by sugar transporters of SWEETs and SUTs or first cleaved to glucose and fructose by the cell wall invertase CIN2/GIF1 and then loaded to the SE by SWEETs and MSTs. MADS29 promotes the PCD of NP, and NF-YB1 activates the expression of sucrose transporters *OsSUT1*, *OsSUT3*, and *OsSUT4*. In maize, sucrose delivered by the VB is cleaved by the cell wall invertase Mn1 located in the BETL, and cell wall ingrowths in the BETL (regulated by MEG1) facilitate the sugar loading by increasing the cell surfaces. The red dashed boxes indicate the sugar-loading complex. Alternative pathways (*dotted arrows*) may also be present. Abbreviations: BETL, basal endosperm transfer layer; CIN2, CELL WALL INVERTASE 2; DA, dorsal aleurone; Fru, fructose; Glu, glucose; MADS29, MADS-box transcription factor 29; MEG1, MATERNALLY EXPRESSED GENE1; Mn1, MINIATURE1; MST, monosaccharide transporter; NE, nucellar epidermis; NF-YB1, NUCLEAR FACTOR Y B1; NP, nucellar projection; PC, placentachalaza; PCD, programmed cell death; PED, pedicel; P/T, pericarp and testa; SE, starchy endosperm; Suc, sucrose; SUT, sucrose transporter; SWEET, SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTER; VB, vascular bundle.

plant species for long-distance sugar transport through the phloem from source leaves to the sink tissue of grains (102). When they arrive in grains, maternal and filial tissues work together to facilitate the loading of sucrose and other photoassimilates into the endosperm during the grain-filling stage.

4.1.1. Sugar-loading complex. In the caryopsis of cereals (called the kernel in maize), maternal tissues including pericarp, testa, integuments, and nucellus are fused tightly together, encasing the embryo and endosperm (**Figure 2**). Coordinated actions of cell divisions, cell expansion, tissue differentiation, and degeneration occur in these maternal tissues before and after fertilization

to generate a sophisticated structure for effective sugar loading and grain filling (64, 183). The structure, which we named the sugar-loading complex, is composed of the dorsal vascular bundle, nucellar projection, and dorsal aleurone in rice. The nucellar epidermis, with thick cell walls at the side in contact with the endosperm, forms an open gate at the sugar-loading complex to allow sugars to enter endosperms progressively through the complex (**Figure 4**). In maize, a similar structure, consisting of the vascular bundle, placentachalazal tissue, and BETL, is formed at the base of the kernel. The formation of transfer cells with cell wall ingrowth in BETL is regulated maternally by MEG1, as described in Section 3.4 (31) (**Figure 4**).

In rice, *CELL WALL INVERTASE 2* (*CIN2*)/*GRAIN INCOMPLETE FILLING 1* (*GIF1*), which is expressed specifically in the dorsal vascular bundle of the caryopsis, cleaves sucrose to glucose and fructose. Mutation in *CIN2/GIF1* led to compromised grain filling, producing floury endosperm (164). Similarly, in maize, the cell wall invertase Mn1, expressed exclusively in the BETL of the developing kernel, is genetically associated with over 70% of the grain weight (24, 69). Mn1 is also associated with the stability of placentachalazal tissue and the formation of cell wall ingrowth in the BETL (69). These results suggest that, when sucrose arrives in the grain, it may enter directly into the endosperm or be cleaved to hexoses (glucose and fructose) before being loaded to the endosperm (4, 164). At least in rice, these two routes exist in parallel in the sugar-loading complex (4).

4.1.2. The degeneration of maternal tissues. Programmed degeneration occurs in maternal tissues such as the integuments and nucellus at specific stages, which may play a crucial role in grain filling (95, 183). In particular, a B_{sister} family MADS-box TF gene, *OsMADS29*, is expressed specifically in nucellus tissues and is important for the degradation of the nucellar projection. Mutation of *OsMADS29* compromises grain filling in rice (199). OsMADS29 may form a homodimer, or heterodimers with other MADS-box proteins, to activate the expression of a PCD-related papain family Cys protease that may be involved in the degradation of the nucellar projection (106, 199). An OsMADS29 ortholog in *Arabidopsis*, TRANSPARENT TESTA 16 (TT16), also regulates the degradation of the nucellar tissue during endosperm development, suggesting a conserved mechanism that coordinates the degeneration of maternal tissues in monocots and dicots (190).

4.1.3. Sugar transporters. Cytoplasm membrane–localized sugar influx carriers such as sucrose transporters (SUTs) and monosaccharide transporters (MSTs) and sugar efflux carrier SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERs (SWEETs) load sugars against the concentration gradient to developing endosperms (78). In barley, *HvSUT1* is expressed in the endosperm, vascular bundle, nucellar projection, and transfer cell layer (126); in maize, *ZmSUT1* is expressed in the BETL and vascular bundle in pedicel (139); in rice, *OsSUT1* is expressed in the aleurone and in maternal tissues including the nucellus, vascular bundle, and nucellar projection (43). RNAi-mediated silencing of *OsSUT1* led to wrinkled grains with a reduced starch content (137), suggesting that OsSUT1 functions in sucrose transport from maternal tissues to the developing endosperm. *NUCLEAR FACTOR Y B1* (*NF-YB1*), encoding a member of the ancient NF-Y family TFs, is expressed specifically in the aleurone of rice, with a higher level in the dorsal aleurone, and its knockout or downregulation leads to defective grain filling, producing smaller grains with a chalky endosperm. Biochemical studies revealed that NF-YB1 binds directly to the promoter regions of the sucrose transporter genes *OsSUT1*, *OsSUT3*, and *OsSUT4* to activate their expressions and promotes the loading of sucrose directly to the endosperm (4).

SWEETs are membrane-localized sugar uniporters that are important for importing sugars into developing endosperms in both monocots and dicots. In *Arabidopsis*, SWEET11, SWEET12, and SWEET15 regulate sugar efflux from seed coat to endosperm, and a *sweet11 sweet12 sweet15*

triple mutant exhibited retarded embryo development, reduced seed weight, and compromised starch accumulation in the embryo, together with an increased starch accumulation in the seed coat (22). In cereals, *ZmSWEET4c* is expressed in the BETL of maize, and *OsSWEET4* (the ortholog of *ZmSWEET4c*) is expressed in early stage caryopses of rice. Mutations of *ZmSWEET4c* or *OsSWEET4* affect grain filling, suggesting that they have roles in delivering sugars to developing endosperms (144). *ZmSWEET11* is expressed in the aleurone, BETL, placentachalaza, and pedicel (139). Further, the expression of *OsSWEET11* and *OsSWEET15* in rice increases gradually during caryopsis development, with specific expression seen in the dorsal vascular bundle, nucellar projection, nucellar epidermis, and aleurone (99, 193). The *ossweet15* mutant showed normal caryopsis development, *ossweet11* exhibited defective grain filling, and the *ossweet11 ossweet15* double mutant showed an additive phenotype, with severely defective endosperm and elevated starch accumulation in the pericarp, suggesting that OsSWEET11 and OsSWEET15 function redundantly in sugar loading from maternal tissues to the endosperm (193). *OsDOF11* encodes a DOF family TF and is expressed in the vascular bundles of photosynthetic organs and developing caryopses. The rice *dof11* mutant exhibits a semidwarf phenotype, forms fewer tillers, and has short panicles and small grains. Further characterization suggests that OsDOF11 may coordinately activate the expression of downstream genes *OsSUT1*, *OsSWEET11*, and *OsSWEET14* by binding directly to their promoters, since reduced expression levels of these genes were observed in various tissues of the *dof11* mutant (186). Exploration of the molecular machinery underlying sugar loading and grain filling may facilitate the development of crop varieties with improved yields.

4.2. Starch Biosynthesis in the Starchy Endosperm

In cereal endosperms, starch in the form of D-glucose homopolymers is synthesized primarily in the starchy endosperms, not in the aleurone. Starch in plants is in two types: the linear and less branched amylose and the highly branched amylopectin (68). Starch biosynthesis in cereal endosperms is a highly regulated process that requires an active energy supply.

4.2.1. Enzymes involved in starch biosynthesis. Starch biosynthesis involves at least five classes of enzymes: adenosine diphosphate (ADP)-glucose pyrophosphorylases (AGPases), soluble starch synthases (SSs), granule-bound starch synthases (GBSSs), starch branching enzymes (BEs), and starch debranching enzymes (DBEs) (68). AGPase is the rate-limiting enzyme that converts glucose 1-phosphate to ADP-glucose in the cytosol. The elongation of a glucan chain involves the transfer of a glucosyl unit of ADP-glucose to the chain's nonreducing end via an α-1,4-glycosidic linkage. GBSSs and SSs are responsible for the biosynthesis of amylose and amylopectin, respectively (154). One of the GBSSs, GBSSI, also called Waxy based on the name of the *waxy* mutants, is required primarily for the production of amylose. Cultivars harboring defective *GBSSI/Waxy* genes, with high amylopectin contents in endosperms and a sticky texture after cooking, have been identified in many cereal crops, including rice (133), maize (185), wheat (142), barley (145), foxtail millet (169), and sorghum (134). BEs catalyze the formation of α-1,6-glucosidic linkages to produce branched glucans. DBEs have two types, isoamylase (ISA) and pullulanase (PUL), which hydrolyze improper glucan branches (68). These enzymes work together to synthesize starch in an orderly manner.

4.2.2. Transcriptional regulation of starch biosynthesis. TFs regulating starch biosynthesis have been identified in cereals (**Table 1**); most of them regulate multiple genes in the starch biosynthesis pathway. In rice, basic leucine zipper (bZIP)-type TF OsbZIP58, which is also named RISBZ1, activates the expression of at least six starch biosynthetic genes, including

ADP-GLC PYROPHOSPHORYLASE LARGE SUBUNIT 3 (*AGPL3*), *GBSSI*, *SSIIa*, *BE1*, *BEIIb*, and *ISA2*, via direct binding to their promoters (73, 167). In maize, OPAQUE2 (O2), as an OsbZIP58/RISBZ1 ortholog, interacts with a Prolamin-Box Binding Factor (PBF) family TF and activates the expression of *SSIII*, *PYRUVATE ORTHOPHOSPHATE DIKINASE 1* (*PPDK1*), and *PPDK2* (211). A MYB family TF, ZmMYB14, and a DOF family TF, ZmDOF36, activate the expression of several starch biosynthetic genes (180, 189). In barley, WRKY-type TF SUGAR SIGNALING IN BARLEY 2 (SUSIBA2), which is sugar-induced and specifically expressed in endosperm, activates the expression of *ISA1* by binding to its sugar-responsive *cis*-element (148, 149). Overexpression of *HvSUSIBA2* in rice led to increased starch accumulations in stems and grains and decreased carbon allocation to roots (148). bZIP-type TFs TabZIP28 of wheat and TubZIP28 of the wild einkorn wheat (*Triticum urartu*) promote the transcriptions of *AGPase* genes in developing endosperms (143). While the previous TFs constitute examples of transcriptional activators, a few transcriptional repressors have been identified that control the starch biosynthesis. RICE STARCH REGULATOR1 (RSR1) is an APETALA2/ethylene-responsive element binding protein (AP2/EREBP) family TF that negatively regulates the expression of multiple starch biosynthetic genes in endosperms. A transfer DNA (T-DNA) insertional mutant of *RSR1* displayed elevated expression of starch biosynthetic genes, along with increased amylose content, grain size, and grain weight (42), while RNAi-mediated silencing of the *RSR1* orthologs in wheat led to elevated expressions of several starch biosynthetic genes and increased starch content and grain weight (92). Further, a dehydration response element-binding (DREB)-type TF of SALT-RESPONSIVE ERF1 (SERF1) in rice binds directly to the promoter regions of *GBSSI* and *RPBF*, inhibiting their expressions in endosperms, and the expression level of *SERF1* correlates negatively with starch content and grain size (135). Together, these results suggest that a TF-based regulation network is present in endosperms that precisely regulates the spatial and temporal expressions of genes involved in starch biosynthesis.

Several lncRNAs are also involved in repressing starch biosynthesis. Overexpression of *LncRNA_2308*, *LncRNA_1267*, or *LncRNA_1631* in rice impaired the expression of *GBSSI*, resulting in reduced starch content and grain weight (212). The transcriptional repressor *RSR1*, located near *LncRNA_1267* in the genome, is upregulated in *LncRNA_1267*-overexpressing rice plants, suggesting that *LncRNA_1267* may impair the expression of *GBSSI* through regulatory control of *RSR1* (212). These results suggest the presence of multiple layers of transcriptional regulations of starch biosynthesis in cereal endosperms.

Co-expression analyses uncovered the presence of complex networks regulating storage product biosynthesis in the endosperm. For example, the expression of *GBSSI* in rice is regulated by a heterodimeric complex consisting of MYC family TF BINDING PROTEIN-5 (OsBP-5) and EREBP family TF OsEBP-89, as well as possibly another heterotrimeric complex consisting of NF-YB1, NF-YC12, and basic helix-loop-helix (bHLH) family TF bHLH144, as RNAi-mediated silencing of *OsBP-5* or mutations in *NF-YB1*, *NF-YC12*, or *bHLH144* led to greatly reduced starch contents in rice endosperm (14, 216). It seems that a TF can regulate the expression of multiple starch-synthesis-related enzyme genes, and, conversely, a given starch synthesis enzyme is likely to be regulated by multiple TFs. To some extent, this may explain why the mutation or knockout of a single TF often does not cause a detectable endosperm phenotype. A regulatory network with crosstalk may allow the endosperm to establish resilient responses to internal and external changes. Genetic analyses with readouts of grain filling and starch profiles are undoubtedly powerful in identifying regulatory genes in starch biosynthesis, and recent developments in multiplexed gene editing may provide further insight into the regulatory network involved in starch biosynthesis, especially the distinct expressions and functions of different isoforms of starch biosynthetic enzymes in endosperms and the source tissues of leaves.

4.2.3. Energy supply. Starch biosynthesis in cereal endosperms requires an active energy supply. As the power generators in all eukaryotic cells, mitochondria convert chemical energy into ATP, which can be utilized directly for starch biosynthesis (39). The mitochondrion is a semiautonomous organelle that, like the chloroplast, imports most of its functional proteins, encoded by the nuclear genome, from the cytoplasm. Mutations in these genes may therefore interfere with mitochondrial function (**Table 1**). Among these mitochondria-targeted proteins, pentatricopeptide repeat (PPR) proteins are commonly known to be critical for endosperm development and storage product accumulation. Most PPR proteins exhibit sequence-specific RNA-binding activity and are targeted to mitochondria and/or chloroplasts, where they play essential roles in editing, splicing, and stabilizing RNAs (5). Mutations in mitochondria-targeted PPRs often lead to defective endosperm development in maize. In particular, *dek2* (120), *dek10* (119), *dek35* (23), and *dek41* (214) maize exhibit 32%, 27%, 20%, and 13% reductions in kernel weights, respectively, mainly due to reduced starch and/or storage protein biosynthesis. Similar results have been observed for several *ppr* mutants in rice. The *opaque and growth retardation 1* (*ogr1*), *small kernel 1* (*ossmk1*), *floury endosperm10* (*flo10*), and *radicleless 1* (*rl1*) mutants exhibit variable defects in rice grain filling (75, 88, 181, 182). Mutations in other genes encoding mitochondria-targeted proteins, such as the transcription termination factor SMALL KERNEL 3 (ZmSMK3) in maize (114) and nicotinamide adenine dinucleotide (NADH) dehydrogenase 1 alpha subcomplex subunit 9 (OsNDUFA9) in rice (61), also caused defective accumulation of storage products. Taken together, these results suggest that mitochondria play a critical role that may be entirely related to energy supply in grain filling and starch biosynthesis of cereals.

4.3. Storage Protein Accumulation in Cereal Endosperms

Storage proteins produced in cereal endosperms are classified into four types—prolamins, glutelins, globulins, and albumins—according to their solubilities in various solvents (105). The alcohol-soluble prolamin is the major type in maize and wheat endosperms, while the alkaline- and acid-soluble glutelin is the major type in the rice endosperm (71). Water-soluble globulin and albumin in cereal endosperms are easy for humans to digest but account for only a minor fraction of total storage proteins.

4.3.1. Expressional regulations of storage protein genes. Many storage proteins are encoded by multiple genes. For example, the rice genome has 34 genes coding for prolamins, 15 for glutelins, 3 for globulins, and 7 for albumins (72). Most of these genes are expressed specifically in developing endosperms (72, 125). Efforts have been made to identify critical *cis*-elements in their promoter regions controlling expression specificity, which led to the identification of several conserved motifs, such as GCN4, prolamin-box (P-box), and AACA (187). The GCN4 motif is recognized by bZIP family TFs, including wheat STORAGE PROTEIN ACTIVATOR (SPA) (3), maize O2 (177), BARLEY LEUCINE ZIPPER 1 (BLZ1) and BLZ2 (110, 162), and rice OsbZIP58/RISBZ1 (112), which activate the expression of storage protein genes. The P-box motif is recognized by a Dof family TF PBF (101, 161, 192), and the AACA motif is recognized by MYB family TFs such as OsMYB5 in rice (152) and HvGAMYB in barley (38). A minimal 197-bp *Glutelin-B1* promoter fragment containing the GCN4, P-box, and AACA motifs confers specific and strong expression of the reporter gene in rice endosperm (177, 178). In the promoter regions of storage protein genes, GCN4 and P-box motifs are often present together or separated by a few nucleotides (100, 187, 192).

Other novel TFs regulating the expression of storage protein genes have also been identified recently. In maize, two O2 HETERODIMERIZING PROTEINs (OHPs), OHP1 and OHP2,

are bZIP family TFs, which activate the expression of *27-kD γ-zein* through binding to its promoter (210). ZmbZIP22 binds directly to the ACAGCTCA box in the *27-kD γ-zein* promoter, activating its expression, and interacts with PBF1, OHP1, and OHP2, suggesting that ZmbZIP22 cooperates with these TFs to regulate the expression of the *27-kD γ-zein* gene (83). ZmMADS47 interacts with O2 to transactivate the genes encoding α-zein and the 50-kD γ-zeins by binding the CATGT motif of their promoters (123). In wheat, SPA HETERODIMERIZING PROTEIN (SHP), the ortholog of BLZ1, acts as a transcriptional repressor to negatively regulate *Gluteinin-B1-1* expression through binding to its promoter (16).

At the storage product accumulation stage, abundant storage proteins and starch are synthesized synchronously in cereal endosperms, suggesting a coordinated regulation of these two storage products. Some TFs play an essential role in coordinating the expressions of starch biosynthesis and storage protein genes. Maize O2 and PBF1, in addition to their roles in storage protein accumulation, also regulate starch biosynthesis by binding directly to promoters of starch biosynthesis genes (211). Upstream regulatory regions of these storage protein and starch biosynthesis genes have shared motifs, which may allow coregulation of these two types of storage products by common TFs, such as the NAM/ATAF/CUC (NAC) family TFs. ZmNAC128 and ZmNAC130 bind to the ACGCAA motif of *16-kD γ-zein* and *AGPS2* promoters to activate their transcription (209), while OsNAC20 and OsNAC26 also bind to the same motif in *16-kD prolamin* and *BEI* for their transcriptional activations (166). In wheat, TaNAC019 binds directly to a specific motif in promoter regions of *Glu-1* (encoding high molecular weight gluten), *SuSy1* (encoding sucrose synthase), and *SSIIa* to activate their expressions (44). In maize, O11, an endosperm-specific bHLH TF, regulates directly the expressions of *O2* and *PBF1*, coordinating the starch biosynthesis and storage protein accumulation (40). Further, maize B3 domain TF ABSCISIC ACID INSENSITIVE 19 (ZmABI19) may function as an upstream regulator to activate the expressions of *O2*, *PBF*, *ZmbZIP22*, *ZmNAC130*, and *O11* (194). It would be important and challenging to establish the transcriptional regulation network underlying the coordination of starch biosynthesis and storage protein accumulation.

4.3.2. Messenger RNA localizations. In developing rice endosperm, messenger RNAs (mRNAs) for prolamin, glutelin, and α-globulin are localized on two distinct subdomains of cortical endoplasmic reticulum (ER): prolamin and α-globulin on protein body ER (PB-ER) and glutelin on cisternal ER (*cis*-ER) (27, 87, 174). In maize, prolamin and 11S globulin RNAs are concentrated on the PB-ER and *cis*-ER, respectively, suggesting that the distinct distributions of storage protein mRNAs on the ER may be conserved in cereal endosperms (175). These distinct distributions of prolamin and glutelin RNAs in rice depend on multiple *cis*-RNA sequences (known as zipcodes) at their coding and 3′-untranslated regions. Deletion of these zipcodes led to mislocalization of these mRNAs (53, 173). Some RNA-binding proteins (RBPs) bind to these zipcodes to form messenger ribonucleoprotein (mRNP) complexes that are exported from the nucleus into the cytoplasm (195). Mutations of RBP-L and RBP-P caused mislocalization of glutelin and prolamin RNAs and conferred other general growth defects, including dwarfism, late flowering, and low fertility (155, 156). Moreover, a cytoplasm-localized cytoskeletal-associated RBP, OsTudor-SN, is a component of the RNA transport particle and regulates storage protein biosynthesis by mediating the transport, localization, and anchoring of their RNAs to the cortical ER (29, 163).

4.3.3. Trafficking and packaging of storage proteins. All storage proteins are secretory proteins, which are synthesized on rough ER and later transported to different protein bodies via either Golgi-dependent or Golgi-independent pathways (141). In both rice and maize, prolamins aggregate inside the ER lumen and form intracisternal protein granules that later bud off as

spherical protein body I (PB-I) through a Golgi-independent pathway (140). In wheat, prolamins are either packaged into protein bodies through a Golgi-independent pathway or sequestered into storage protein bodies through vesicles via a Golgi-dependent pathway (21). Rice glutelin precursors and α-globulin are transported to the Golgi apparatus to form dense vesicles that are then targeted to protein storage vacuoles. Within these vesicles, glutelin precursors are cleaved into α-acid and β-basic subunits, which together with α-globulin form irregularly shaped protein body II (PB-II) (32). Mutants defective in the Golgi-dependent secretion pathway have been identified by screening for the *glutelin precursor overaccumulation* (*gpa*) phenotype in rice. Molecular genetic analyses of these mutants revealed that *GPA1* encodes the GTPase Rab5a (172), *GPA2* [also called *VACUOLAR PROTEIN SORTING 9A* (*VPS9A*)] encodes a guanine nucleotide exchange factor of Rab5a (91), *GPA3* encodes a plant-specific Kelch-repeat-containing protein (127), *GPA4* encodes the evolutionarily conserved membrane protein GOLGI TRANSPORT1B (GOT1B) (170), and *GPA5* encodes a plant-unique phox-homology (PX)-domain-containing protein as the Rab5a effector (128). GPA1/Rab5a, GPA2/VPS9a, and GPA3 form a heterocomplex that assists in the transport of glutelin precursors from the Golgi apparatus to protein storage vacuoles (127). Interestingly, the mutation of *GPA3* also led to the docking of the mispackaged glutelin precursor to extracellular spaces (127). GPA4/GOT1B functions in ER exit of storage proteins through mediating the proper assembly of the coat protein complex II prebudding complexes (170). GPA5 interacts with GPA1/Rab5a and GPA2/VPS9a and may mediate the tethering and subsequent fusion of dense vesicles with protein storage vacuoles (128). Further, *GLUTELIN PRECURSOR 3* (*GLUP3*) encodes VACUOLAR PROCESSING ENZYME 1 (VPE1), and its loss-of-function mutant showed a greatly reduced cleavage of the glutelin precursor into α-acid and β-basic subunits (79). *ENDOSPERM STORAGE PROTEIN MUTANT 2* (*ESP2*) encodes a protein disulfide-isomerase-like 1-1 (PDIL1-1) that helps to release glutelin precursors out of the ER (153). In maize, *FLOURY1* (*FL1*) encodes a novel zein protein body membrane protein with three transmembrane domains and a C-terminal plant-specific domain of unknown function (DUF593). FL1 interacts with the 22-kD α-zein through its DUF593 domain to facilitate the localization of this zein in the protein body (59). A maize myosin XI motor protein, Opaque1 (O1), regulates the protein body formation by mediating ER motility. The endosperm cells in the *o1* mutant exhibited dilated ER structures and misshapen protein bodies (165). O10 is a cereal-specific protein body protein that is essential for the ring-shaped distribution of 22-kD and 16-kD zeins and controls protein body morphology in maize endosperm (196). Obviously, the storage protein biosynthesis, trafficking, and packaging in cereal endosperms provide a unique system in which to study intracellular protein movement and deposits.

4.3.4. A free-trade storage product biosynthesis factory. PCD has been reported to be in the early stage of starchy endosperm development in several cereal crops (184, 200, 201), and this is still a controversial issue, as a real PCD in the starchy endosperm cells may compromise storage product accumulation. Further, DNA fragmentation as a common mark of PCD has been observed in maize and wheat but not in rice (201). As both starch biosynthesis and storage protein accumulation require active gene expressions and functional organelles, we propose that the PCD observed in the starchy endosperm occurs in a very specialized form in which only the cytoplasmic membrane loses integrity, while membranes in most intracellular organelles, such as the nuclei, mitochondria, plastids, ER, and Golgi apparatus, remain functional and intact (**Figure 5**). This may lead to the formation of a large free-trade compartment in shared cytoplasm that allows sugars and amino acids arriving in the starchy endosperm to pass freely within it and, consequently, effective starch biosynthesis and storage protein accumulations (184) (**Figure 5**).

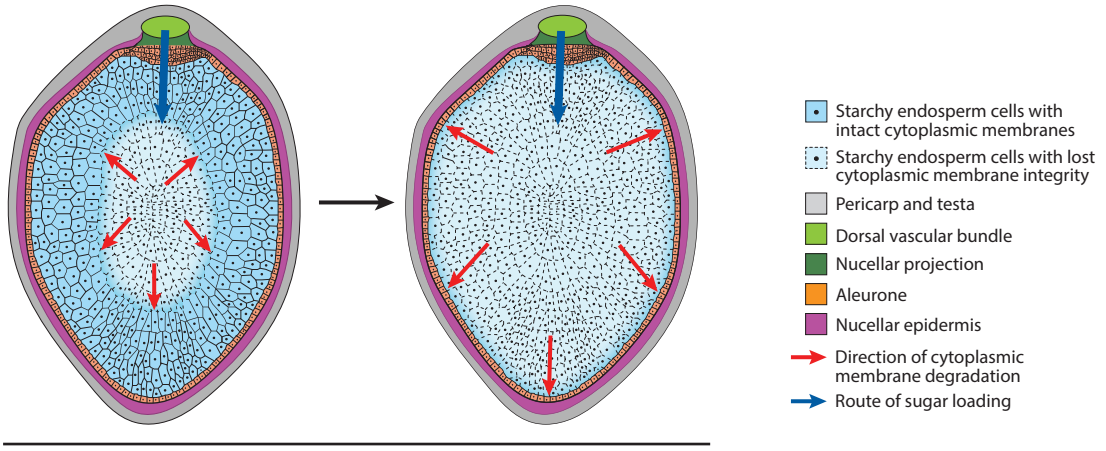

Figure 5

A proposed model of a free-trade storage product biosynthesis factory in cereal endosperms (using rice as an example). At 8 DAP, the central region of rice starchy endosperm starts to lose its cytoplasmic membrane integrity (*red arrows*). The process moves progressively toward the periphery of the starchy endosperm, leading to the formation of a free-trade starchy endosperm in which sugars are able to move freely among cells with a shared cytoplasm. The formation of the free-trade storage product biosynthesis factory enables effective starch biosynthesis in a large endosperm compartment (*light blue*), using sugars delivered from the dorsal vascular bundle into the compartment. The so-called PCD at this stage may cause only a partial degeneration of cytoplasmic membranes, while membranes of the nucleus and other organelles remain intact and functional. Abbreviations: DAP, days after pollination; PCD, programmed cell death.

5. FUTURE PERSPECTIVES

Cereal endosperms are not only important food sources but also fascinating models in which to study cell cycle regulation, cell fate determination, epigenetic regulation, transcriptional regulation, protein trafficking, and PCD. The development of high-throughput genomic and gene editing tools provides new avenues to elucidate the molecular machineries acting in these biological events. Further, cereal endosperms are bioreactors for biofortification and drug production. Golden Rice was developed by producing β-carotene in the endosperm to combat vitamin A deficiency (113). Purple Endosperm Rice was developed through the transgenic expression of 13 anthocyanin biosynthesis genes, which are either silenced or expressed at low levels in wild-type rice endosperm, under the control of endosperm-specific promoters (215). Similar approaches have been taken to produce vitamin B1 (147), the antiviral lectin griffithsin (160), the human serum albumin (55), and multiple antihypertensive peptides (122) in rice endosperms. Studies of cereal endosperms will undoubtedly facilitate the improvement of cereal crops to create varieties with better yields and improved cooking and nutrition qualities.

SUMMARY POINTS

1. The development of cereal endosperms proceeds through coenocytic nuclear division, cellularization, tissue differentiation, and storage product accumulation, during which two major tissues are formed: the outer aleurone (as the default state) and the inner starchy endosperm (the advanced state).

2. The subaleurone cells located below the aleurone usually form starchy endosperm, but they may switch their cell fates to form aleurone when processes related to starchy endosperm differentiation, such as DNA demethylation or adenosine triphosphate (ATP) biogenesis, are compromised.
3. Aleurone is rich in proteins, lipids, vitamins, and micronutrients, and the nutritional value of cereal grains could potentially be improved by increasing the number of aleurone cell layers.
4. Concerted actions of maternal and filial tissues in cereal grains facilitate the formation of a sugar-loading complex, in which sugar transporters located in aleurone cells load sugars into a developing endosperm against the concentration gradient.
5. The loss of cytoplasmic membrane integrity in the starchy endosperm cells of cereals may allow sugars to move freely among these cells, leading to efficient starch biosynthesis.
6. Imprinted gene expression is regulated in cereal endosperms through allele-specific DNA methylation and histone modification.

FUTURE ISSUES

1. Like most angiosperms, all cereal crops have a coenocytic endosperm at the beginning of their development, but the evolution of this phenomenon, its developmental advantage, and the regulatory machinery underlying this process remain to be elucidated.
2. Although the mechanism underlying aleurone and starchy endosperm differentiation has been elucidated in recent years, key regulators remain to be identified.
3. We proposed a hypothetical model for a metabolic advantage deriving from the loss of membrane integrity in the cereal starchy endosperm; however, we do not know how this process is regulated or how this can occur while the membrane integrity of other organelles, such as the nucleus, mitochondria, and plastids, is maintained.
4. Previous studies suggest that active signal transduction occurs between the embryo and endosperm, but what kinds of signal molecules are involved and how they are perceived remain largely unknown.
5. Very few imprinting genes identified in endosperms are conserved across species; thus, the roles and evolutionary trajectories of these imprinting genes need to be elucidated.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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