

Starch biosynthesis in cereal endosperms: An updated review over the last decade

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ABSTRACT

Starch is a vital energy source for living organisms and is a key raw material and additive in the food and non-food industries. Starch has received continuous attention in multiple research fields. The endosperm of cereals (e.g., rice, corn, wheat, and barley) is the most important site for the synthesis of storage starch. Around 2010, several excellent reviews summarized key progress in various fields of starch research, serving as important references for subsequent research. In the past 10 years, many achievements have been made in the study of starch synthesis and regulation in cereals. The present review provides an update on research progress in starch synthesis of cereal endosperms over the past decade, focusing on new enzymes and non-enzymatic proteins involved in starch synthesis, regulatory networks of starch synthesis, and the use of elite alleles of starch synthesis-related genes in cereal breeding programs. We also provide perspectives on future research directions that will further our understanding of cereal starch biosynthesis and regulation to support the rational design of ideal quality grain.

Key words: cereal, starch biosynthesis, regulation network, endosperm development, quality improvement

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INTRODUCTION

Starch is a natural polymer composed of glucose that is unique to plants and algae. The metabolism of starch is the hub of energy metabolism and is widely involved in almost all aspects of plant growth and development. Storage starch, synthesized in the seeds, tubers, corms, and roots of plants, is the main substance used by plants to store carbohydrates and is the most important energy source for all living organisms, as well as an important industrial raw material and additive (Zeeman et al., 2010). The degradation of storage starch can also affect seed germination and pre-harvest sprouting (Tai et al., 2021). Storage starch is a semi-crystalline, insoluble particle composed of two α -glucose polymers, linear amylose and highly branched amylopectin linked by α -1,4- and α -1,6-glycosidic linkages (Zhu et al., 2020b). The composition of amylose and amylopectin, and the hierarchical fine structures they form, determine the diversity of starch physicochemical properties, cereal grain quality, and industrial uses. Extensive research has been carried out on storage starch in fields such as plant science, food science, polymer science, and various industrial fields. For details, please refer to several excellent reviews and book chapters published previously (Jeon et al., 2010; Kötting et al., 2010; Zeeman et al., 2010; Shen et al., 2011; Tetlow et al., 2015; Wang et al., 2015;

Pfister and Zeeman, 2016; Zaman and Sarbini, 2016; Crofts et al., 2017; Thalmann and Santelia, 2017; Kaur et al., 2018; Seung and Smith, 2018; Xia et al., 2018; Abt and Zeeman, 2020; Jukanti et al., 2020; Seung, 2020; Sharma et al., 2020; Zhou et al., 2020a; Tai et al., 2021).

The cereal endosperm is a major source of storage starch in plants. In the first decade of the 21st century, great progress was made in the functional analysis of key starch synthesis-related genes (SSRGs) in cereal endosperms and their correlations with the physicochemical properties of starches with different components and structures. This work has been well summarized previously (Jeon et al., 2010; Kötting et al., 2010; Zeeman et al., 2010). In the past 10 years, a series of advances have been made in the area of starch biosynthesis within the cereal endosperm. This review aims to update recent findings in cereals, especially in the endosperm of major cereals, including rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*).

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The main text of the review is divided into five parts. In the first part, we briefly introduce the physicochemical properties of starch and its applications in food and non-food industries. In the second part, we detail the starch biosynthesis pathway in the endosperm of cereals based on progress made in the past decade and on previous reviews. In the third part, we summarize the regulatory network and mechanism of starch synthesis in cereal endosperms. In the fourth part, we discuss genetic improvement of cereal quality using accumulated knowledge on the genes involved in starch synthesis and its regulation, especially the SSRGs. Finally, we highlight the challenges that remain to be overcome in cereal endosperm starch research, and we raise prospects for future research.

PHYSICOCHEMICAL PROPERTIES AND APPLICATIONS OF CEREAL STARCH

Physicochemical properties of cereal starch

Starch physicochemical properties, including hardness, stickiness, gelatinization, retrogradation, digestibility, crystallization, and elasticity, are key factors for the manufacture and functional features of starch-based foods and industrial raw materials. To understand the relationship between the composition/structure of starch and its properties, starch characteristics are widely parameterized. Amylose content (AC) is the most classic parameter that reflects the composition of starch, whereas diverse parameters have been established for starch structure (Li et al., 2020a). Among them, chain-length distribution (CLD) is the fundamental component of starch structure and physicochemical properties. The CLD of amylose has been divided into three fractions based on the degree of polymerization (DP): short (DP 100–500), medium (DP 500–5000), and long (DP 5000–20 000) amylose chains (Li et al., 2016). Alternatively, it has been divided into two fractions: fraction 1 (DP 100–1000) and fraction 2 (DP 1000–20 000) (Li et al., 2017a). The CLD of amylopectin has been divided into diverse chain categories in different reports (Zhu, 2018). Generally, it has been divided into four fractions based on the amylopectin cluster model. These are the A (DP 6–12), B₁ (DP 13–24), B₂ (DP 25–36), and B₃ (DP >36) chains, of which the A and B₁ chains account for more than 90% (Yu et al., 2019; Jukanti et al., 2020). In addition, a series of indicators and instruments have been summarized and developed to quantify the physicochemical properties of starch. These include the gelatinization properties of starch, which are mainly indicated by gelatinization temperature (GT), and the crystallization properties, which are determined by crystallinity degree (Tan et al., 1999; Dankar et al., 2018).

After decades of exploration, the correlations among quantitative indicators, starch physicochemical properties, and cereal grain quality have been largely revealed and established. For starch composition, AC shows a negative correlation with starch stickiness, digestion rate, crystallinity degree, and transparency of polished rice (AC < 13%) and a positive correlation with starch hardness, elasticity, and retrogradation rate (Gong et al., 2019; Huang et al., 2020a; Li et al., 2020a; Zhang et al., 2020b). Therefore, AC is the most critical indicator that determines the physicochemical properties of starch and its end use. For the CLD of amylopectin, short (A and B₁) amylopectin chains form amylopectin crystalline

regions, and longer A and B₁ chains can form longer double helices that require a higher temperature to dissociate than do shorter double helices in the crystalline region (Noda et al., 1998; Li et al., 2020a). Thus, the numbers of amylopectin A chains and B₁ chains are negatively and positively correlated with GT, respectively, and longer amylopectin A and B₁ chains reduce the starch digestion rate (Martinez et al., 2018). Starch that contained a higher proportion of amylopectin with DP 70–100 showed a less viscous but more elastic texture (Li et al., 2016). For the CLD of amylose, shorter amylose medium chains are relatively easily retrograded, whereas shorter amylose medium-to-long chains and the amount of short-to-medium chain amylose help to reduce the starch digestion rate (Li et al., 2020a).

Applications of cereal starch in food and non-food industries

Starch from cereal endosperms is used mainly as food, providing about 50% of the daily energy needed by the human body (Figure 1A and 1C). Consumer preferences and dietary habits in different regions of the world result in diverse needs for starchy food and require cereal starches with diverse physicochemical properties (Huang et al., 2020a). For instance, cereals with a lower AC, such as waxy corn and soft rice, tend to be preferred in Southeast Asia, despite being less healthy because of their high digestibility and high glycemic index (GI) (Dong et al., 2019a; Parween et al., 2020). Cereal grains are also crucial ingredients for animal feeds (Figure 1D).

As the second most abundant carbohydrate in nature after cellulose, starch's plentiful sources, easy access, low cost, and natural, renewable, and biodegradable characteristics have led to its widespread use in industrial applications. Using physical, chemical, or enzymatic methods such as molecular cutting, rearrangement, oxidation, or introduction of substituents into starch molecules can produce modified starches with altered, enhanced, or new physicochemical properties. Modified starch overcomes the shortcomings of natural starch, such as poor freeze-thaw stability, low solubility, easy retrogradation, and low transparency, further expanding its range of applications in related industries (Masina et al., 2017; Wang et al., 2020c). In food processing, modified starch is widely added to food as a partial fat substitute to provide the shape, taste, thickening, gelling, adhesiveness, and stability required by the food system while avoiding the perception that high-fat meat products will cause cancer (Heinen et al., 2009; Kapelko-Žeberska et al., 2015; Kaur et al., 2018) (Figure 1B). Starch is also used in the alcoholic beverage industry and as a feedstock in bioethanol production for clean energy (Zeeman et al., 2010) (Figure 1E). In the textile industry, modified starch is an important material for warp sizing, printing paste, and silk printing paste (Hebeish et al., 2008) (Figure 1F). Low- and non-amylose starch is very sticky and is an ideal industrial adhesive (Onusseit, 1992) (Figure 1G). Starch with a molecular structure similar to that of cellulose in papermaking fiber is also widely used in the papermaking industry to improve the strength, stiffness, smoothness, gloss, and whiteness of paper (Shen et al., 2011) (Figure 1H). Starch-based biodegradable plastic is considered to be an ideal replacement for traditional plastics in packaging (Gross and Kalra, 2002) (Figure 1H). Modified starch is one of the most important

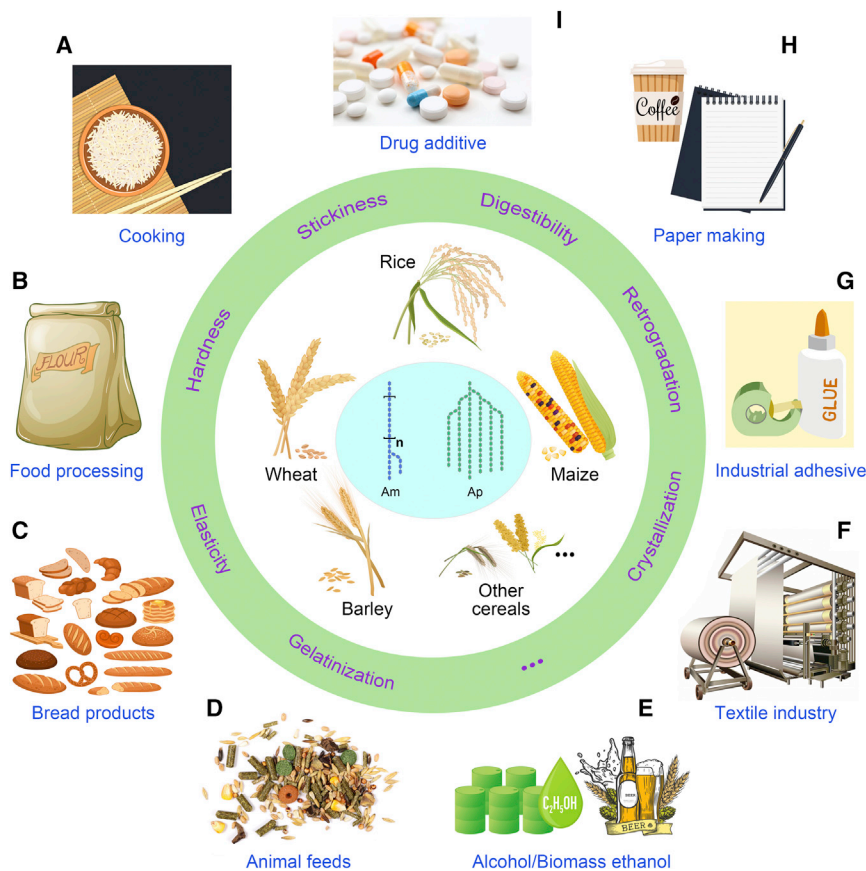


Figure 1. Physicochemical properties and industrial applications of cereal starch.

The major cereal grains are shown within the circle, and the middle circle highlights the physicochemical properties of cereal starch, including hardness, stickiness, gelatinization, retrogradation, digestibility, crystallization, and elasticity. The pictures outside the circle (A–I) present the applications of cereal starch in food and non-food industries. Am, amylose; Ap, amylopectin.

the whole genome, the types and functions of SSREs are highly conserved in rice, maize, wheat, and barley, despite the differences in copy number and isoenzyme number owing to variations in chromosome number among cereal species (Ohdan et al., 2005; Radchuk et al., 2009; Yan et al., 2009; Kang et al., 2013a) (Table 1). A brief depiction of the starch biosynthesis pathway in the cereal endosperm is shown in Figure 2. For specific information, please refer to previous reviews (Jeon et al., 2010; Zeeman et al., 2010; Abt and Zeeman, 2020).

Generation of substrates and primers for starch synthesis

AGPase is responsible for the synthesis of ADP glucose (ADPG), the major substrate for starch synthesis (Pfister and Zeeman, 2016). In higher plants, AGPase is a heterotetramer composed of two large subunits (AGPL) and two small subunits (AGPS). The isozymes of AGPL and AGPS can be classified into cytosolic and plastidial types according to their cellular localization (Saripalli and Gupta, 2015). The cytosolic type accounts for most of the total AGPase activity in the cereal endosperm, and mutation of its encoding genes often leads to severe grain-filling defects (Crompton-Taylor et al., 2011; Huang et al., 2014; Wei et al., 2017). ADPG, synthesized in the cytosol of the developing endosperm, is transported to the amyloplast through the plastid ADPG transporter BT1 (Brittle 1), which is located in the amyloplast envelope (Kirchberger et al., 2007; Cakir et al., 2016; Li et al., 2017b) (Figure 2A).

Malto-oligosaccharides (MOSs) are required as primers for starch synthesis (Seung and Smith, 2018) (Figure 2B). The *de novo* synthesis of the original MOSs remains unclear. However, MOSs have been found to be continuously generated through trimming of nascent amylopectin by isoamylase 1 (ISA1) and ISA2 during starch synthesis or released by α/β -amylases during starch degradation (Myers et al., 2000; Fulton et al., 2008; Abt and Zeeman, 2020). Recombinant SSI-SSIV and GBSSI from barley and *Arabidopsis* elongated MOSs as short as maltose with low affinities *in vitro* (Brust et al., 2013; Cuesta-Seijo et al., 2016). PHO1 (also known as PHS1 and SP) was reported to interact with SSIV in *Arabidopsis* and may function in the extension of MOSs (Malinova et al., 2018). In rice, PHO1 assembles with DPE1 to use a broader range of sugars to

excipients for various new preparations in the pharmaceutical industry (Elvira et al., 2002) (Figure 1I). Starch also has remarkable potential for the preparation of nanofibers using the electrospinning process. Electrospun starch nanofibers have applications in drug delivery, tissue engineering, and the manufacture of wound dressings, and their continued development and applications are expected to further expand the use and commercial value of starch (Liu et al., 2017).

STARCH BIOSYNTHESIS PATHWAY IN CEREAL ENDOSPERMS

The pathway of starch biosynthesis in the endosperm is highly conserved among cereals. A number of key enzymes, including ADP-glucose pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), soluble starch synthase (SS), starch branching enzyme (SBE), debranching enzyme (DBE), disproportionating enzyme (DPE), and starch/ α -glucan phosphorylase (PHO), have been found to participate in the synthesis of starch in the cereal endosperm (Zeeman et al., 2010; Seung and Smith, 2018). Each of the starch synthesis-related enzymes (SSREs) in cereals is present in a variety of isozymes, and these isozymes can form heterologous multi-enzyme complexes with other isozymes or homomultimers with themselves *in vivo* to perform their functions (Utsumi et al., 2011; Crofts et al., 2015). In addition, some novel non-enzymatic proteins, such as PROTEIN TARGETING TO STARCH (PTST), have also been found to participate in starch synthesis in the cereal endosperm (Peng et al., 2014; Seung et al., 2015). From the perspective of

Enzymes/non-enzymatic proteins	Rice (<i>Oryza sativa</i> L.)		Maize (<i>Zea mays</i> L.)		Wheat (<i>Triticum aestivum</i> L.)		Barley (<i>Hordeum vulgare</i> L.)	
	Gene name	Acc. No./ID	Gene name	Acc. No./ID	Gene name	Acc. No./ID	Gene name	Acc. No./ID
ADPG pyrophosphorylase (AGPase, EC 2.7.7.27) ^a	<i>OsAGPS1</i>	AY028315	<i>ZmAGPS1</i>	AY032604	<i>TaAGPS1</i>	AY727927	<i>HvAGP-S1</i>	AAO16183
	<i>OsAGPS2a</i>	EF122437	<i>ZmAGPS2a-1/Bt2</i>	AF330035	<i>TaAGPS2-a</i>	X66 080	<i>HvAGP-S2a</i>	CAA88449
			<i>ZmAGPS2a-2</i>	DQ118038				
	<i>OsAGPS2b</i>	AP004459	<i>ZmAGPS2b</i>	AF334960	<i>TaAGPS2-b</i>	EU582678	<i>HvAGP-S2b</i>	CAA88450
	<i>OsAGPL1</i>	AY028314	<i>ZmAGPL1/Sh2</i>	BT016868	<i>TaAGPL1</i>	Z21969	<i>HvAGP-L1</i>	CAA47626
	<i>OsAGPL2/GIF2</i>	D50317	<i>ZmAGPL2</i>	Z38111	<i>TaAGPL2</i>	DQ406820	<i>HvAGP-L2</i>	AAC49729
	<i>OsAGPL3</i>	NM-001065811	<i>ZmAGPL3</i>	EF694838				
	<i>OsAGPL4</i>	NM-001057719	<i>ZmAGPL4</i>	EF694839				
ADPG transporter	<i>OsBt1</i>	Os02g0202400	<i>ZmBt1</i>	M79333	<i>TaBt1</i>	BT008958.1	<i>HvBt1</i>	AY560327.2
Granule-bound starch synthase (GBSS, EC 2.4.1.21)	<i>OsGBSSI</i>	AB425323	<i>ZmGBSSI</i>	AY109531	<i>TaGBSSI</i>	AF286320	<i>HvGBSSIa</i>	AAM74051
							<i>HvGBSSIb</i>	AAM74054
	<i>OsGBSSII</i>	AY069940	<i>ZmGBSSIIa</i>	EF471312	<i>TaGBSSII</i>	AF109395		
			<i>ZmGBSSIIb</i>	EF472248				
Soluble starch synthase (SS, EC 2.4.1.21)	<i>OsSSI</i>	AY299404	<i>ZmSSI</i>	AF036891	<i>TaSSI</i>	AJ292521	<i>HvSSI</i>	AAF37876
	<i>OsSSIIa/SSII-3/ALK</i>	AF419099	<i>ZmSSIIa/Su2</i>	AF019296	<i>TaSSIIa/Sgp1</i>	AJ269503	<i>HvSSIIa/Sex6</i>	AAN28307
	<i>OsSSIIb/SSII-2</i>	AF395537	<i>ZmSSIIb-2</i>	EF472249	<i>TaSSIIb</i>	EU333947	<i>HvSSIIb</i>	AAN28307
			<i>ZmSSIIb-1</i>	AF019297				
	<i>OsSSIIc/SSII-3</i>	AF383878	<i>ZmSSIIc</i>	EU284113	<i>TaSSIIc</i>	EU307274		
	<i>OsSSIIIa/SSIII-2/Flo5</i>	AY100469	<i>ZmSSIIIa/du1</i>	AF023159	<i>TaSSIIIa</i>	AF258608	<i>HvSSIIIa</i>	AAF87999
	<i>OsSSIIIb/SSIII-1</i>	AF432915	<i>ZmSSIIIb-1</i>	EF472250	<i>TaSSIIIb</i>	EU333946	<i>HvSSIIIb</i>	AAL40942
			<i>ZmSSIIIb-2</i>	EF472251				
	<i>OsSSIVa/SSIV-1</i>	AY373257	<i>ZmSSIV</i>	EU599036	<i>TaSSIV</i>	AY044844	<i>HvSSIV</i>	AAK97773
	<i>OsSSIVb/SSIV-2</i>	AY373258						
	<i>OsSSV</i>	EU621837.1	<i>ZmSSV</i>	NM_001 130 131.1				

Table 1. Enzymes and non-enzymatic proteins involved in starch synthesis during cereal endosperm development.

(Continued on next page)

Enzymes/non-enzymatic proteins	Rice (<i>Oryza sativa</i> L.)		Maize (<i>Zea mays</i> L.)		Wheat (<i>Triticum aestivum</i> L.)		Barley (<i>Hordeum vulgare</i> L.)	
	Gene name	Acc. No./ID	Gene name	Acc. No./ID	Gene name	Acc. No./ID	Gene name	Acc. No./ID
Starch branching enzyme (SBE, EC 2.4.1.18)	<i>OsSBEI/SBE1</i>	EF122471	<i>ZmBEI</i>	AY105679	<i>TaBEI</i>	Y12320	<i>HvSBEI</i>	AAP72268
	<i>OsSBEIIa/SBE4</i>	AB023498	<i>ZmBEIIa</i>	EF433557	<i>TaBEIIa</i>	AF286319	<i>HvSBEIIa</i>	AAC69753
	<i>OsSBEIIb/SBE3</i>	D16201	<i>ZmBEIIb/Ae</i>	EU333945	<i>TaBEIIb</i>	AY740401	<i>HvSBEIIb</i>	AAC69754
	<i>OsSBEIII</i>	AK066930	<i>ZmBEIII</i>	ZMU18908	<i>TaBEIII</i>	JQ346193		
Debranching enzyme (DBE, EC 3.2.1.68 and EC 3.2.1.41)	<i>OsISA1</i>	AB015615	<i>ZmISA1/Su1</i>	ZMU18908	<i>TaISA1</i>	AF548380	<i>HvISA1</i>	AAM46866
	<i>OsISA2</i>	NM-001061991	<i>ZmISA2</i>	EU976060	<i>TaISA2</i>	JX473824	<i>HvISA2</i>	BAD08581
	<i>OsISA3</i>	NM-001069968	<i>ZmISA3</i>	AY172634	<i>TaISA3</i>	JN412069	<i>HvISA3</i>	BAD89532
	<i>OsPUL</i>	D50602	<i>ZmPUL/Zpu1</i>	AF080567	<i>TaPUL</i>	EF137375	<i>HvPUL</i>	AAD34348
Starch/ α -glucan phosphorylase (PHO, EC 2.4.1.1)	<i>OsPHOL/PH O 1</i>	AF327055	<i>ZmPHOL/Sh4/SP</i>	EU857640	<i>TaPHOL</i>	EU595762	<i>HvPHOL/PHS1</i>	KF195562
	<i>OsPHOH</i>	NM-001051358	<i>ZmPHOH</i>	EU971442	<i>TaPHOH</i>	AF275551	<i>HvPHOH/PHS2</i>	KF147849
Disproportionating enzyme (DPE, EC 2.4.1.25)	<i>OsDPE1</i>	AB626975	<i>ZmDPE1</i>	BT061520	<i>TaDPE1</i>	DQ068045		
	<i>OsDPE2</i>	AK067082	<i>ZmDPE2</i>	BT055804	<i>TaDPE2</i>	BQ294920		
Protein targeting to starch (PTST)	<i>OsGBP</i>	LOC_Os02g04330	GPM177	NP_001132796.1			<i>HvPTST1</i>	HORVU6Hr1G018500
	<i>OsFL O 6</i>	LOC_Os03g48170			<i>TaBGC1</i>		<i>HvFL O 6/Fra</i>	HORVU4Hr1G004470

Table 1. Continued

Most of the information was collected from previous reports (Ohdan et al., 2005; Radchuk et al., 2009; Yan et al., 2009; Jeon et al., 2010; Kang et al., 2013a; Ma et al., 2013; Soliman et al., 2014; Liu et al., 2015b; Cuesta-Seijo et al., 2017; Wang et al., 2019; Abt and Zeeman, 2020); some genes are predicted by sequence alignment, and their authenticity and function require further verification; Acc. No., accession number.

^aThe naming of AGPS1 and AGPS2 is contrary to that of Yan et al. (2009), Radchuk et al. (2009) and Kang et al. (2013a), but consistent with their original citations (Johnson et al., 2003; Ohdan et al., 2005) and the NCBI website.

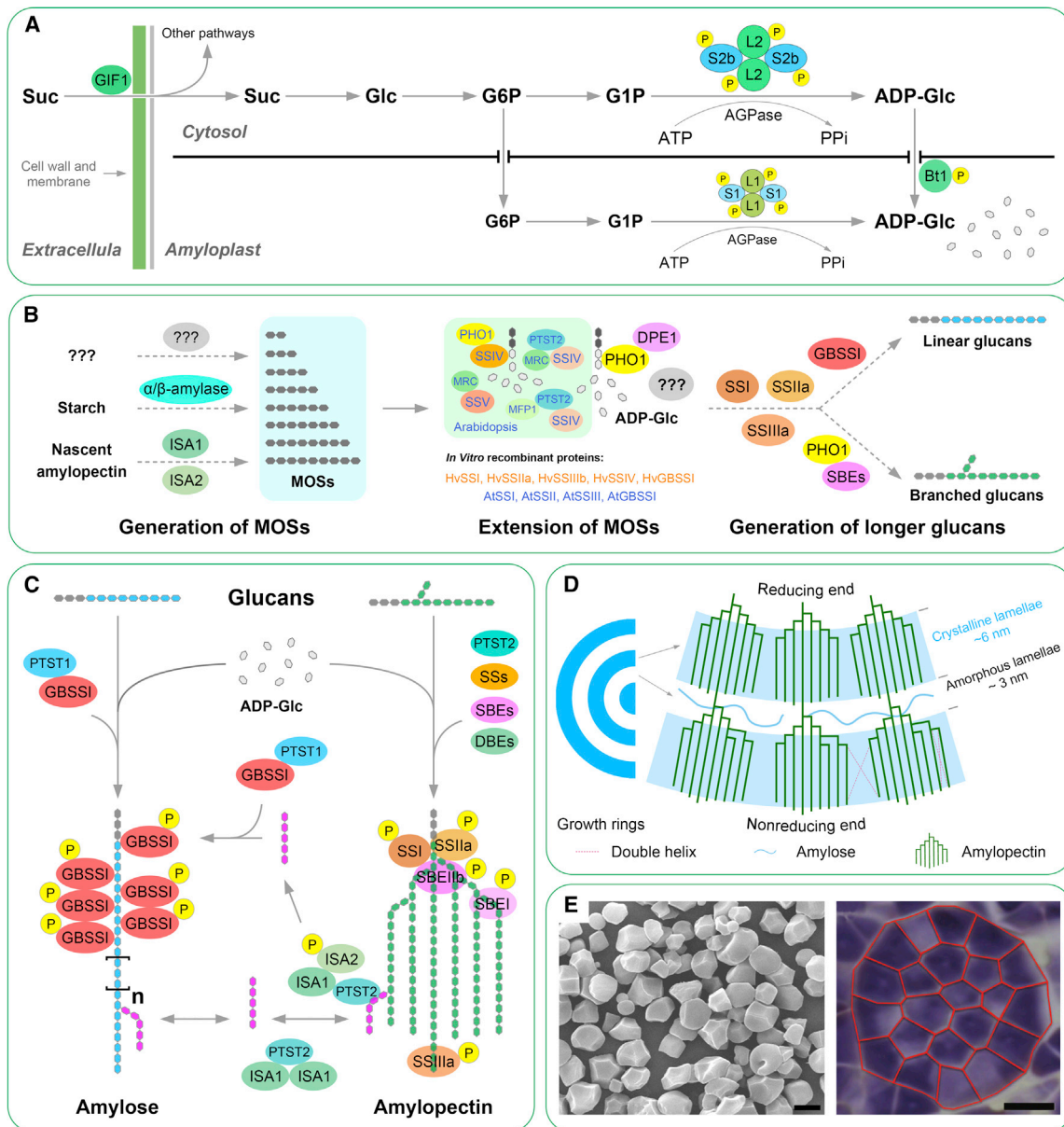


Figure 2. Starch biosynthesis in the cereal endosperm: rice as an example.

(A) Generation and transport of ADP glucose (ADPG), the major substrate for starch synthesis. Sucrose synthesized by photosynthesis is unloaded and distributed by GRAIN INCOMPLETE FILLING 1 (GIF1) (Wang et al., 2008), then processed into ADPG in the cytosol and amyloplast by AGPase after a series of reactions and transport. In higher plants, AGPase is a heterotetramer composed of two large subunits (AGPL) and two small subunits (AGPS). ADPG synthesized in the cytosol is transported to the amyloplast through Brittle 1 (Bt1).

(B) Generation of primers for starch synthesis. The initiation of starch synthesis requires the availability of malto-oligosaccharides (MOSs). MOSs have been found to be continuously generated through trimming of nascent amylopectin by ISA1 and ISA2 in the process of starch synthesis or released by α/β -amylases during starch degradation (Myers et al., 2000; Fulton et al., 2008; Abt and Zeeman, 2020). However, the *de novo* synthesis of the original MOSs in cereals is still unclear. In rice, the PHO1-DPE1 complex enhanced synthesis of long MOSs *in vitro*, and an unknown factor(s) can compensate for the function of PHO1 at room temperature (Sato et al., 2008; Hwang et al., 2016). SSV and its interaction partners, including PHO1, PTST2, MFP1, and MRC, were found to participate in the extension of MOSs in *Arabidopsis* (Malinova et al., 2018). In addition, recombinant SSSs and GBSSI from barley and *Arabidopsis* were demonstrated to elongate MOSs as short as maltose *in vitro* (Brust et al., 2013; Cuesta-Seijo et al., 2016). MOSs are further processed into linear glucans and branched glucans, which act as primers to initiate the synthesis of amylose and amylopectin, respectively. PHO1 and SBEs combine to promote the extension of MOSs and the synthesis of branched glucans by activating each other's mutual capacities in rice (Nakamura et al., 2012).

(C) Synthesis of amylose and amylopectin. Amylose in cereal endosperms is synthesized by GBSSI, whereas amylopectin is synthesized by coordinated cooperation of SSSs, SBEs, and DBEs. PTST1 and PTST2, two non-enzymatic proteins, are also involved in the starch synthesis pathway. The isozymes of starch synthesis-related enzymes in cereals form heterologous multi-enzyme complexes with other isozymes or homo-multimers with themselves *in vivo*

(legend continued on next page)

synthesize MOSs and enhance the synthesis of long MOSs *in vitro* (Hwang et al., 2016). PHO1 was also demonstrated to combine with SBEs to promote the extension of MOSs and the synthesis of branched glucans in rice, and similar interactions were also observed in protein extracts from the endosperms of wheat, maize, and barley (Tetlow et al., 2004; Nakamura et al., 2012; Subasinghe et al., 2014; Cuesta-Seijo et al., 2017). However, the functions of PHO1 and DPE1 remain controversial, as only the isolated cases mentioned above have been reported.

Synthesis of amylopectin

Amylopectin is synthesized by at least SS, SBE, and DBE (Kötting et al., 2010; Crofts et al., 2015) (Figure 2C). SS is responsible for the extension of the α -1–4 glycosidic bond in amylopectin; it has the largest number of isoforms and the most complex functions among the SSREs (Ohdan et al., 2005). SSI, SSII, and SSIII are generally believed to be responsible for the elongation of α -glucan chains during amylopectin synthesis, whereas SSIV and its interaction partners, including PHO1, PTST2, MAR BINDING FILAMENT-LIKE PROTEIN 1 (MFP1), and MYOSIN-RESEMBLING CHLOROPLAST PROTEIN/PROTEIN INVOLVED IN STARCH INITIATION 1 (MRC/PII1), are involved in starch granule initiation (Jeon et al., 2010; Crofts et al., 2015; Seung et al., 2018; Abt and Zeeman, 2020). The novel starch synthase SSV was recently identified, and it shows conserved sequences and gene structures in all green plants (Liu et al., 2015b). SSV shows no enzymatic activity but interacts with MRC to manipulate starch granule initiation in *Arabidopsis* chloroplasts, similar to its close relative SSIV (Abt and Zeeman, 2020; Abt et al., 2020).

SBE is the only enzyme that acts on glucan to produce branches connected by α -1,6-glycoside bonds, and it generally includes three types in cereals: SBEI (SBE1), SBEII, and SBEIII (Han et al., 2007; Tian et al., 2009). SBEII, including SBEIIb (SBE3) and SBEIIa (SBE4), plays a major role during amylopectin synthesis in the cereal endosperm (Tian et al., 2009; Sawada et al., 2018). Significantly, SBEIIa mainly compensates for the function of SBEI, instead of SBEIIb, by forming amylopectin medium chains in the rice endosperm (Sawada et al., 2018). SBEIII, which shares low similarity with SBEI and SBEII, has been found in many higher plants, including rice, maize, and wheat, and only *TaSBEIII* was identified to be associated with the synthesis of both A and B granules in wheat grains (Han et al., 2007; Kang et al., 2013b).

DBE, comprising ISA1, ISA2, ISA3, and pullulanase (PUL), hydrolyzes α -1,6-glycosidic bonds and corrects the wrong branches in starch synthesis to ensure the orderly synthesis of amylopectin. ISA1, also known as Sugary 1 (Su1) and Pre-harvest sprouting 8 (PHS8), is critical for the orderly synthesis of amylopectin in cereal endosperms (James et al., 1995; Burton et al., 2002; Du et al., 2018). Mutation of ISA1 causes a massive accumulation of highly branched glucan and phytoglycogen in grains and also causes pre-harvest sprouting (Du et al., 2018). ISA1 can form homo-oligomers with itself or heterologous complexes with ISA2, in which ISA1 plays a catalytic role and ISA2 plays a regulatory role (Utsumi et al., 2011). Moreover, ISA1, ISA2, and SSIII can act synergistically to inhibit the accumulation of phytoglycogen (Lin et al., 2011).

In cereal endosperms, the enzymes involved in amylopectin synthesis usually act in the form of multi-enzyme complexes (Kötting et al., 2010; Crofts et al., 2017). For instance, trimers composed of SSI, SSIIa, and SBEIIa/SBEIIb have been observed in the developing endosperms of wheat, maize, and rice; within these trimers, SSIIa is located between SSI and SBEII and is responsible for binding to starch granules (Hennen-Bierwagen et al., 2008; Tetlow et al., 2008; Liu et al., 2012; Hayashi et al., 2018).

Synthesis of amylose

Amylose in cereal endosperms is synthesized by granule-bound starch synthase I (GBSSI), which is encoded by the *Waxy* (*Wx*) gene (Shure et al., 1983; Wang et al., 1990) (Figure 2C). GBSSI controls amylose synthesis in the form of oligomers after being phosphorylated on the surface of starch granules (Liu et al., 2013). All three PTST proteins are involved in the synthesis of starch, but only PTST1 is involved in the synthesis of amylose (Seung et al., 2017). PTST1 interacts with GBSSI to help the latter's localization to the surface of starch granules (Seung et al., 2015). The C-terminal carbohydrate binding module (CBM) of PTST1 is required for correct GBSSI localization, and mutation of CBM can cause GBSSI to remain in the plastid stroma (Wang et al., 2020b). The *ptst1* mutants in *Arabidopsis* and barley showed significantly reduced GBSS protein accumulation without amylose synthesis, consistent with the *gbss* mutants (Seung et al., 2015; Zhong et al., 2018). The *OsGBP* gene in rice is homologous to *PTST1* in *Arabidopsis* and barley, but the reduced AC in the endosperm of the *osgbp* mutant is still much higher than that of glutinous rice,

to perform their functions. These complexes include, but are not limited to, trimers composed of SSI, SSIIa, and SBEIIa/SBEIIb (Utsumi et al., 2011; Crofts et al., 2015). ISA1 can form homo-oligomers with itself or heterologous complexes with ISA2, and PTST2 interacts with ISA1, enabling its binding to starch granules (Utsumi et al., 2011; Peng et al., 2014). PTST1 interacts with GBSSI to help the latter's localization to the surface of starch granules, and GBSSI then controls amylose synthesis in the form of oligomers after being phosphorylated (Liu et al., 2013; Wang et al., 2020b).

(D) Schematic diagram of the internal growth ring structure of starch granules formed by amylose and amylopectin. Adjacent α -1,4-glucan chains (DP \geq 10) in or between amylopectin can form double helices and self-assemble to form crystalline lamellae in the starch granule matrix (Pfister and Zeeman, 2016). Amylopectin branch points and amylose form amorphous lamellae. The crystalline lamellae alternate with the amorphous lamellae containing branch points of amylopectin and amylose to form concentric growth rings with a period of 9 nm (Abt and Zeeman, 2020).

(E) Scanning electron micrograph of extracted starch granules from mature rice grains (left) and a schematic diagram of compound starch granules formed by multiple starch granules (traced as red polygons) in the amyloplast (right) (Wei et al., 2010; Matsushima et al., 2015). Scale bar, 5 μ m.

Suc, sucrose; Glc, glucose; G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; ATP, adenosine triphosphate; PPI, pyrophosphate; ADP-Glc, adenosine diphosphate-glucose; MOSs, malto-oligosaccharides; GBSS, granule-bound starch synthase; SS, soluble starch synthase; SBE, starch branching enzyme; DBE, debranching enzyme; ISA, isoamylase; DPE, disproportionating enzyme; PHO, starch/ α -glucan phosphorylase; PTST, protein targeting to starch; MFP1, MAR BINDING FILAMENT-LIKE PROTEIN 1; MRC, MYOSIN-RESEMBLING CHLOROPLAST PROTEIN; P, phosphorylation.

suggesting that other mechanisms are involved in localizing GBSSI to starch granules (Wang et al., 2020b).

Initiation and morphogenesis of starch granules

Starch in the cereal endosperm exists in the form of semi-crystalline, insoluble starch granules (Abt and Zeeman, 2020) (Figure 2D). The morphology of starch granules, including number, size, shape, and distribution in amyloplasts, is diverse among cereal endosperm cells. The amyloplast in the endosperm of maize and most members of the Triticeae (including wheat and barley) contains only one simple starch granule, and the starch granules of Triticeae are bimodal in size (Myers et al., 2011; Seung and Smith, 2018). In the rice endosperm, multiple starch granules initiate in amyloplasts and fuse into a larger compound structure during grain maturation (Matsushima et al., 2015) (Figure 2E). The compound starch granules are considered to be the ancestral form in the Gramineae, and the number of starch granules per amyloplast is determined in the early developmental stage (Matsushima et al., 2013, 2015).

The matrix of starch granules is composed mainly of amylopectin. Thus, enzymes and proteins involved in amylopectin synthesis, such as ISA1, SBEs, SSIII, SSIV, SSV, and PTST2, also function in the initiation and morphogenesis of starch granules. As mentioned above, ISA1 is vital for ensuring the correct formation of starch granules instead of highly branched glucan or phyto-glycogen (Burton et al., 2002). FLOURY ENDOSPERM6 (FLO6), which is homologous to PTST2 in *Arabidopsis*, B-GRANULE CONTENT 1 (BGC1) in wheat, and Franubet (Fra) in barley, modulates the formation of compound starch granules in the rice endosperm through interaction with ISA1, enabling its binding to the starch granules (Peng et al., 2014; Saito et al., 2018; Chia et al., 2019). The double mutation of ISA1 and SBEL1a eliminated the sugary endosperm, resulting in normal starch granule formation (Lee et al., 2017). Gradually decreasing the expression of SBEs can lead to the formation of various heterogeneous starch granules, including polygonal, aggregate, elongated, and hollow granules (Wang et al., 2018). Studies in wheat indicated that SSIV and BGC1 are required for proper granule initiation, as *TaSSIVb-D* mutation led to a decrease in starch granules per chloroplast (Guo et al., 2017), and mutation of either SSIV or BGC1 resulted in multiple initiations of amyloplasts and the formation of compound granules (Hawkins et al., 2021). Mutation of SSIVb (SSIV-2) or SSIIIa did not show even a slight effect on starch granule morphology in the rice endosperm, but their combination resulted in small and spherical starch granules (Toyosawa et al., 2016). SSIII also functions in starch granule initiation in the absence of SSIV, and the absence of SSV negatively affects the number of starch granules in *Arabidopsis* chloroplasts, similar to that of SSIV (Szydlowski et al., 2009; Abt et al., 2020).

In cereals, starch granule morphology after mutation of the *Wx* gene is mostly normal, indicating that amylose is not essential for the formation of starch granules (Seung and Smith, 2018). However, mutations of PTST1 or OsGBP, which interacts with GBSSI, cause dramatic changes in starch granule morphology (Seung et al., 2015; Zhong et al., 2018; Wang et al., 2020b).

REGULATORY NETWORK OF STARCH SYNTHESIS IN CEREAL ENDOSPERMS

Starch metabolism is regulated by various upstream pathways. In the past decade, considerable progress has been made in revealing the upstream regulatory network of starch synthesis in the endosperms of a number of cereals, especially rice, as summarized in Figure 3.

Transcriptional regulation

At the transcription level, the expression of SSRGs is regulated by a series of transcription factors (TFs) (Table 2). The basic leucine zipper (bZIP) family of TFs plays important roles in cereal endosperm development. For example, Opaque2 (O2) not only transactivates the expression of key SSRGs and sucrose synthase (SUS)-encoding genes but also binds to the GCN4 motif in the promoters of almost all zein genes in maize, indicating its role as a hub for corn kernel development (Li et al., 2015; Zhang et al., 2016; Deng et al., 2020). The prolamins binding factor (PBF) from the DNA binding with one finger (Dof) family interacts with O2 to additively regulate the expression of certain SSRGs and zein genes or directly activates the expression of these genes (Zhang et al., 2016). OsbZIP58 (also known as RISBZ1) and rice PBF (RPBF) are the homologs of maize O2 and PBF, respectively. OsbZIP58 and RPBF show both complementarity and functional redundancy with one another to regulate the transcription of genes involved in starch and protein synthesis during endosperm development (Yamamoto et al., 2006; Kawakatsu et al., 2009; Wang et al., 2013). OsbZIP58 and RPBF also directly regulate the expression of the gene encoding lysine ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), a key regulator of free lysine content in plants (Kawakatsu and Takaiwa, 2010). Similar conclusions were also obtained with the O2 and PBF homologs in wheat and barley (Guo et al., 2020b; Orman-Ligeza et al., 2020).

In addition to O2 and its homologs, several other bZIP TFs have also been shown to participate in the transcriptional regulation of SSRGs in maize, rice, wheat, and barley. ZmbZIP22 positively regulates prolamins synthesis in corn kernels by binding to the promoter of the gene encoding 27-kDa γ -zein and negatively affects starch synthesis by regulating key genes involved in sugar and starch metabolism (Li et al., 2018a; Dong et al., 2019b). ZmbZIP22 interacts with PBF but not with O2 (Li et al., 2018a). ZmbZIP91 positively regulates the expression of SSRGs in the maize leaf and endosperm by binding to ACTCAT elements in their promoters (Chen et al., 2015). Knockout or knockdown of OsbZIP76, which regulates endosperm cellularization in rice, caused precocious cellularization in the rice caryopsis and enhanced the expression of SSRGs, leading to earlier starch accumulation (Niu et al., 2020). OsbZIP33/REB/RISBZ2 interacts with GCN4 motifs in the promoters of *Wx*, *SBE1*, and α -globulin genes in rice (Yang et al., 2001; Cai et al., 2002). In the wheat endosperm, TubZIP28/TabZIP28 binds to the promoters of genes encoding cytosolic AGPase, enhancing their transcription (Song et al., 2020). In barley, more than 10 bZIP TFs were identified with expression patterns similar to those of SSRGs, two of which could bind to the promoter of *HvAGPS* (Zhong et al., 2021).

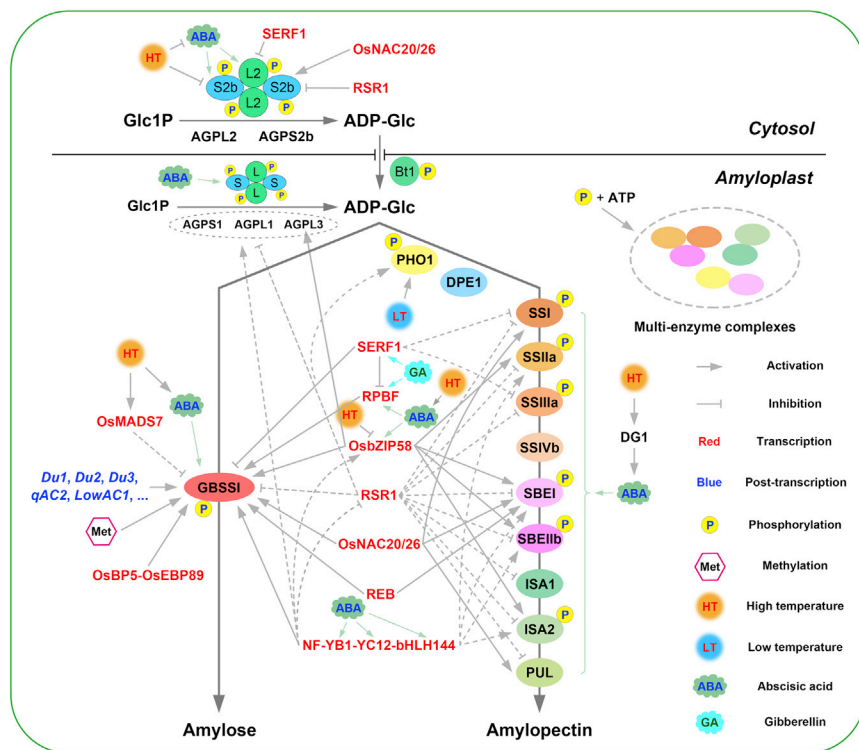


Figure 3. Regulatory network of starch synthesis in the cereal endosperm: rice as an example.

At the transcriptional level, several key transcription factors (red font) and methylation are involved in the stimulation or inhibition of starch synthesis-related genes (SSRGs). At the post-transcriptional level, some *Du* (*Dull*) genes (*Du1*, *Du2*, *Du3*, etc.) and quantitative trait loci (QTLs) (*qAC2*, *LowAC1*, etc.) have been reported to regulate amylose synthesis by manipulating the splicing efficiency of *Wx* mRNA. At the post-translational level, phosphorylation affects starch synthesis by promoting the formation of multi-enzyme complexes. Gibberellin (GA) modulates the expression of SERF1 and RPBF in starch synthesis to promote starch accumulation in the developing rice endosperm. Abscisic acid (ABA) synthesized in rice and maize leaves directly activates the expression of most SSRGs and multiple hub transcription factors in the rice caryopsis after long-distance leaf-to-caryopsis ABA transport. Defective grain-filling 1 (DG1)-mediated long-distance ABA transport is sensitive to high temperature (HT) during seed development. HT also downregulates the expression of SSRGs and the stability of their encoded enzymes, especially genes encoding AGPase and GBSSI, resulting in chalky grains and defective starch accumulation. Low temperature (LT) promotes the function of PHO1. The arrowed

lines indicate activation, and the bar-ended lines indicate inhibition. The dotted lines indicate that the transcription factor can regulate the transcription of the target gene, but there is no direct interaction evidence. The abbreviations for the enzymes, transcription factors, and genes are the same as those listed in Tables 1 and 2.

Recently, some NAM/ATAF/CUC (NAC) TFs involved in the regulation of starch and storage protein synthesis in developing cereal grains were cloned. Three NAC members related to starch synthesis were identified in maize: ZmNAC126, ZmNAC128/ZmNAC34, and ZmNAC130. Among these, ZmNAC126 modulates starch accumulation in corn kernels by increasing starch synthesis and inhibiting starch degradation (Xiao et al., 2020). The functions of ZmNAC128/ZmNAC34 and ZmNAC130 are redundant. They compete for a 6-bp consensus sequence (ACGCAA) in the promoters of starch and zein synthesis-related genes, such as *Bt2* and 16-kDa γ -zein, to regulate the accumulation of starch and storage protein in corn kernels (Peng et al., 2019; Zhang et al., 2019d). ZmaNAC36, which is highly expressed in the maize endosperm and is co-expressed with most of the SSRGs, may also play a role in starch synthesis in the maize endosperm (Zhang et al., 2014b). In rice, OsNAC20 and OsNAC26 bind directly to key genes of starch and protein synthesis in the endosperm. However, the high similarity of their amino acid sequences has resulted in redundancy, which was verified by the fact that only the *osnac20/26* double mutant showed significantly decreased starch and storage protein content in the endosperm (Wang et al., 2020a). In the linked region of rice chromosome 11, a gene cluster encoding four NAC members (ONAC025, ONAC127, ONAC128, and ONAC129) was identified (Fang et al., 2008). ONAC127 and ONAC129 are not directly involved in starch synthesis in the rice endosperm, but they do form heterodimers that participate in apoplastic transport and the heat stress response to regulate rice grain filling, including starch accumulation (Ren et al., 2021). In wheat, the endosperm-specific TaNAC019 was also

found to regulate glutenin and starch accumulation by directly activating the expression of relevant genes (Liu et al., 2020; Gao et al., 2021).

A group of nuclear factor Ys (NF-Ys), which are predominantly expressed in the rice endosperm, were also reported to be regulators of rice endosperm development (Yang et al., 2017; Zhiguo et al., 2018). Recent findings showed that OsNF-YB1 is specifically expressed in the aleurone layer of the rice developing endosperm and regulates grain filling and endosperm development by interacting with other TFs, including OsbZIP76, OsERF115, OsNF-YC11, OsNF-YC12, and bHLH144 (Bai et al., 2016; Bello et al., 2019; Xiong et al., 2019; Niu et al., 2020). For instance, NF-YB1 binds with NF-YC12 and bHLH144 to form an NF-Y heterotrimer complex (NF-YB1-YC12-bHLH144), and mutation of each gene in the complex can alter starch synthesis in the rice endosperm (Bello et al., 2019). A rice LEC1-like TF known as OsNF-YB9 functions in starch synthesis by affecting SSRG expression and starch granule morphology (Niu et al., 2021).

Furthermore, the maize MYB TF ZmMYB14 binds to the promoters of multiple SSRGs, including *ZmBT1*, to activate their expression (Xiao et al., 2017). ZmMYB138 and ZmMYB115 are candidate TFs involved in the regulation of starch synthesis in the maize endosperm, as suggested by expression correlation analysis (Hu et al., 2021). ZmABI4, ZmERE156, and HvSUSIBA2 are TFs related to the modulation of SSRGs such as *SSIIa*, *SSI*, and *ISA1* by abscisic acid (ABA) and/or sugar signaling (Sun et al., 2003; Hu et al., 2012; Huang et al., 2016). OsMADS7, OsBP-5, and OsEBP89 were identified in rice as

TF	Locus	TF family	Key references
<i>Oryza sativa</i> L.			
OsZIP58/RISBZ1	LOC_Os07g08420	bZIP	Kawakatsu et al., 2009; Wang et al., 2013; Xu et al., 2020
OsZIP33/REB/RISBZ2	LOC_Os03g58250	bZIP	Yang et al., 2001; Cai et al., 2002
OsZIP76	LOC_Os09g34880	bZIP	Niu et al., 2020
OsNAC20	LOC_Os01g01470	NAC	Wang et al., 2020a
OsNAC26	LOC_Os01g29840	NAC	
ONAC127	LOC_Os11g31340	NAC	Ren et al., 2021
ONAC129	LOC_Os11g31380	NAC	
NF-YB9	LOC_Os06g17480	NF-Y	Niu et al., 2021
NF-YB1	LOC_Os02g49410	NF-Y	Zhiguo et al., 2018; Bello et al., 2019
NF-YC12	LOC_Os10g11580	NF-Y	
bHLH144	LOC_Os04g35010	bHLH	
OsMADS7	LOC_Os08g41950	MADS-box	Zhang et al., 2018
OsMADS29	LOC_Os02g07430	MADS-box	Yin and Xue, 2012
OsMADS6	LOC_Os02g45770	MADS-box	Zhang et al., 2010
RSR1	LOC_Os05g03040	AP2/EREBP	Fu and Xue, 2010
OsEBP-89	LOC_Os03g08460	AP2/EREBP	Zhu et al., 2003
RPBF	LOC_Os02g15350	DOF	Yamamoto et al., 2006; Kawakatsu et al., 2009
SERF1	LOC_Os05g34730	DREB	Schmidt et al., 2014
OsBP-5	LOC_Os03g43810	MYC-like	Zhu et al., 2003
<i>Zea mays</i> L.			
ZmABI19	Zm00001d011712	B3 domain	Yang et al., 2020
Opaque2/O2	GRMZM2G015534	bZIP	Zhang et al., 2016; Deng et al., 2020
ZmbZIP22	GRMZM2G043600	bZIP	Li et al., 2018a; Dong et al., 2019b
ZmbZIP91	GRMZM2G043600	bZIP	Chen et al., 2015
PBF	GRMZM2G146283	DOF	Zhang et al., 2016
ZmDOF36	GRMZM2G137502	DOF	Wu et al., 2019
ZmNAC36	GRMZM2G154182	NAC	Zhang et al., 2014b
ZmNAC126	Zm00001d005028	NAC	Xiao et al., 2020
ZmNAC128/ZmNAC34	GRMZM2G062650	NAC	Peng et al., 2019; Zhang et al., 2019d
ZmNAC130	GRMZM2G154182	NAC	
ZmMYB14	GRMZM2G172327	MYB	Xiao et al., 2017
ZmEREB156	GRMZM2G421033	AP2/EREBP	Huang et al., 2016
Opaque11/O11	GRMZM2G147685	bHLH	Feng et al., 2018
ZmMADS1a	GRMZM2G160687	MADS-box	Dong et al., 2019c
ZmABI4	GRMZM2G093595	AP2/ERF	Hu et al., 2012
<i>Triticum aestivum</i> L.			
TaSPA	TraesCS1B02G343500, TraesCS1D02G332200	bZIP	Guo et al., 2020b
TubZIP28/TabZIP28	TRIUR3_00 571, AML47732	bZIP	Song et al., 2020
TaNAC019-A, B, D	TraesCS3A02G077900, TraesCS3B02G092800, TraesCS3D02G078500	NAC	Liu et al., 2020; Gao et al., 2021

Table 2. Transcription factors involved in starch synthesis during cereal endosperm development.

(Continued on next page)

TF	Locus	TF family	Key references
TaRSR1	JX473823	AP2/EREBP	Kang et al., 2013a
TaPBF/LSY3	TraesCS5A02G155900, TraesCS5B02G154100, TraesCS5D02G161000	DOF	Orman-Ligeza et al., 2020
<i>Hordeum vulgare</i> L.			
HvSUSIBA2	AY323206 (GenBank)	WRKY	Sun et al., 2003
PBF/LSY3	HORVU5Hr1G048700	DOF	Orman-Ligeza et al., 2020

Table 2. Continued

AP2/EREBP, APETALA2/ethylene-responsive element binding protein; AP2/ERF, APETALA2/ethylene-responsive factor; bZIP, basic leucine zipper transcription factor; DOF, DNA binding with one finger; MYB, myeloblastosis; NAC, NAM/ATAF/CUC domain transcription factor; NF-Y, nuclear factor Y; PBF, prolamin box binding factor.

being associated with *Wx* gene regulation and its response to high temperature ([Cai et al., 2002](#); [Zhu et al., 2003](#); [Zhang et al., 2018](#)). Rice Starch Regulator1 (RSR1), an APETALA2/ethylene-responsive element binding protein (AP2/EREBP), is a negative regulator of SSRGs that is expressed predominantly in seeds of rice and wheat ([Fu and Xue, 2010](#); [Kang et al., 2013a](#)). Suppression of *OsMADS29* expression inhibits starch biosynthesis and grain filling in the rice endosperm by causing defective programmed cell death ([Yin and Xue, 2012](#)). *OsMADS6* is highly expressed in the florets and endosperm and regulates starch accumulation by regulating genes that encode AGPase ([Zhang et al., 2010](#)).

Upstream of these TFs, the DEHYDRATION-RESPONSE ELEMENT BINDING (DREB)-type TF SALT-RESPONSIVE ERF1 (SERF1) was reported to manipulate the synthesis and degradation of starch by timing the expression of *RPBF* in rice ([Schmidt et al., 2014](#)). O11 is a central hub of maize endosperm development and nutrient metabolism that directly regulates related key TFs, including O2 and PBF ([Feng et al., 2018](#)). The B3 domain-containing TF ZmABI19 can coordinate the expression of O11, O2, and other key TFs and has been defined as a hub of hubs ([Yang et al., 2020](#); [Zhan, 2020](#)).

Methylation regulation

Previous studies have shown that genes preferentially expressed in the rice endosperm, including SSRGs, usually show reduced expression because of methylation, indicating that methylation is also involved in the regulation of starch synthesis ([Zemach et al., 2010](#)). Recently, similar overall methylation patterns were found in the SSRGs expressed in the maize endosperm ([Hu et al., 2021](#)). In the developing maize endosperm, the coding regions of low-expression SSRGs are highly methylated, and expression levels of *SBE1* and *Su1* correlate significantly and negatively with predicted DNA methylation marks ([Hu et al., 2021](#)). Thus, DNA methylation may act as a switch in the global regulation of starch biosynthesis. Moreover, high CpG methylation in *Wx* is closely related to low AC in rice grains, and two neighboring CpG islands were detected in the promoter region of *Wx*, indicating that DNA methylation is involved in the regulation of amylose synthesis ([Anacleto et al., 2019](#)).

Post-transcriptional regulation

At the post-transcriptional level, some *Du* (*Dull*) genes and quantitative trait loci (QTLs) were reported to regulate amylose synthe-

sis by manipulating the splicing efficiency of *Wx* mRNA in the rice endosperm. *Du1* and *Du2* showed similar genetic interactions with *Wx* alleles: the level of GBSSI protein in both the *du1* and *du2* mutants was decreased by inefficient splicing of *Wx^b* pre-mRNA, whereas neither mutant affected the expression of the *Wx^a* allele. The *Du1* gene encodes a member of the pre-mRNA processing (Prp1) family that is a component of the U4/U6 small nuclear ribonucleoprotein required for spliceosome assembly ([Zeng et al., 2007](#)). *Du3* modulates *Wx* pre-mRNA splicing by encoding the rice homolog of a cap-binding protein 20-kDa subunit (CBP20), a component of the heterodimeric nuclear cap-binding complex. The novel QTL *qAC2* and the novel dull gene *LowAC1*, which encodes an RNA recognition motif protein, were recently shown to have a function related to *Wx^b* pre-mRNA splicing, similar to that of *du1* and *du2* ([Takemoto-Kuno et al., 2015](#); [Igarashi et al., 2021](#)). The post-transcriptional regulation of *Wx* may also be involved in grain filling at high temperatures, which will be discussed below.

Post-translational regulation via phosphorylation

Post-translational phosphorylation is an important aspect of starch synthesis regulation: the formation of multi-enzyme complexes depends on protein phosphorylation, and broad-spectrum phosphatase treatment can prevent the formation of high-molecular-weight protein complexes ([Grimaud et al., 2008](#); [Tetlow et al., 2008](#); [Kötting et al., 2010](#); [Ahmed et al., 2015](#)). ³²P-labeled autoradiography and phosphorylated proteomics further confirmed the phosphorylation of enzymes involved in the formation of multi-enzyme complexes in the starch synthesis pathway ([Tetlow et al., 2004](#); [Walley et al., 2013](#)). In the developing maize endosperm, the phosphorylation sites of SSREs, including AGPase large and small subunits, PHO1, SBEs, ISA2, and Bt1, were identified using high-throughput proteome, transcriptome, and phosphorylated proteome analysis ([Walley et al., 2013](#)). For example, a systematic study of the phosphorylation sites of maize SBEIIb demonstrated that three serine residue sites, Ser²⁸⁶, Ser²⁹⁷, and Ser⁶⁴⁹, can be phosphorylated ([Makhmoudova et al., 2014](#)). These phosphorylation sites form salt bridges to stabilize the SBEIIb protein structure, and their mutation caused a decreased phosphorylation level of recombinant SBEIIb. The three phosphorylation sites show different degrees of conservation in different crop species and SBE isoforms, indicating functional differentiation among them. The difference in the conservation of Ser²⁸⁶ among the SBEs may explain why phosphorylation

can change the activities of SBEIIa and SBEIIb but has no effect on the activity of SBEI in wheat (Tetlow et al., 2008). Several phosphorylation sites were also found in the rice GBSSI protein, such as the S415P substitution that was shown to change the phosphorylation level of GBSSI, thereby modulating its activity to a moderate level in *Wx^{lv}* (Zhang et al., 2019a). In wheat, the amount of phosphorylated AGPase increased during the progress of seed development and was positively correlated with relative increases in AGPase activity and starch accumulation (Ferrero et al., 2020).

Regulation by hormones

Hormones play vital roles in all stages of plant growth and development, including endosperm starch synthesis. In the process of starch metabolism, gibberellin (GA) functions mainly in starch degradation by regulating *Ramy1A* through the TF *GAMYb*, but it also modulates the expression of the key TFs SERF1 and RPF in starch synthesis to promote starch accumulation in the developing rice endosperm (Schmidt et al., 2014). Previous studies showed that ABA could greatly enhance AGPL1 (Apl3) induction by sugar in cultured rice cells (Akihiro et al., 2005). In maize, the novel TF ZmEREB156 was found to mediate the regulation of ABA and sucrose in starch synthesis (Huang et al., 2016). Sugar accumulation resulting from mutation of ISA1 decreased the expression of OsABI3 and OsABI5 and reduced sensitivity to ABA (Du et al., 2018). ABA induces the accumulation of *ZmSSI* mRNA in maize endosperm. The *ZmSSI* promoter contains a CACCG motif that interacts with the ABA pathway of the TF ABI4 *in vitro* (Hu et al., 2012). ABI4 enhances the activity of promoters containing the CACCG motif, including *ZmSSI* (Hu et al., 2012). A recent study indicated that starch synthesis in developing seeds is regulated mainly by leaf-derived ABA. ABA synthesized in rice leaves directly activates the expression of most SSRGs and multiple hub TFs in the rice caryopsis following long-distance leaf-to-caryopsis ABA transport. Defective grain-filling 1 (DG1), a functionally conserved multi-drug and toxic compound extrusion transporter in cereals, regulates grain filling by manipulating long-distance leaf-to-caryopsis ABA transport (Qin et al., 2021).

Response to temperature

The environment is also a key determinant of starch synthesis in cereal endosperms, mainly through temperature fluctuations during the reproductive phase (Fan et al., 2019). The *pho1* mutant showed shrunken rice grains when grown at 20°C, whereas most grains were plump when grown at 30°C, suggesting that low temperature promotes the function of PHO1 and that an unknown factor(s) can compensate for PHO1 function at room temperature (Sato et al., 2008). The negative effect of high temperature (HT) on starch synthesis is more severe and more common during the reproductive period of cereals. At the genetic level, HT significantly downregulates the expression of SSRGs and induces the expression of genes encoding α -amylase, resulting in chalky grains and defective starch accumulation (Zhang et al., 2018). AGPase and GBSSI are among the SSREs that are severely affected by HT during grain ripening (Denyer et al., 1994; Boehlein et al., 2007; Yamakawa et al., 2007). HT significantly destabilizes AGPase heterotetramers, dramatically reducing ADPG synthesis (Saripalli and Gupta, 2015). Unlike the AGPase from potato

tubers, the AGPases of rice and maize endosperms lack a conserved QTCL (Gln-Thr-Cys-Leu) motif and are therefore heat labile (Boehlein et al., 2007). An insertion of cysteine in the N terminus of AGPS2b in maize and rice enhanced the heat stability of their heterotetramers (Linebarger et al., 2005; Hwang et al., 2019). The L379F mutation of rice AGPS2b was also found to improve the heat stability of AGPase by increasing the number of hydrogen bonds between two AGPS2b subunits and the intermolecular interactions between AGPL2 and AGPS2b (Hwang et al., 2019).

HT inhibits *Wx*-encoded GBSSI and AC accumulation, resulting in rice grains with poor eating quality (Zhang et al., 2018). One of the mechanisms is that HT induced the alternative splicing of *OsbZIP58*, thereby reducing the transcription of the *Wx* gene (Xu et al., 2020). HT promotes the formation of the truncated *OsbZIP58 β* protein relative to the full-length *OsbZIP58 α* protein, and *OsbZIP58 β* showed lower transcriptional activity than *OsbZIP58 α* under HT conditions (Xu et al., 2020). Specific suppression of the floral organ identity gene *OsMADS7* in the rice endosperm stabilized AC under HT stress without reducing spikelet fertility (Zhang et al., 2018). The post-transcriptional regulation of *Wx^p* is a critical mechanism for maintaining a stable AC at HT. Four QTLs that can stabilize rice AC at HT by increasing the splicing efficiency of *Wx* pre-mRNA (*qSAC3*, *qHAC4*, *qHAC8a*, and *qHAC8b*) were detected in a genome-wide survey of chromosome segment substitution lines derived from a cross between the *indica* variety 9311 and the *japonica* variety Nipponbare (Zhang et al., 2014a, 2019b).

Furthermore, inhibition of α -amylase gene expression resulted in fewer chalky grains under HT (Hakata et al., 2012). The presence of heat-stable PGD1 or PGD2, two heat-stable isoforms of 6-phosphogluconate dehydrogenase, in the endosperm amyloplast mitigated maize yield losses caused by HT stress (Ribeiro et al., 2020). Recently, HT was also found to affect starch accumulation in the rice and maize endosperms by regulating the efficiency of DG1-mediated leaf-to-caryopsis ABA transport. HT increased *DG1* expression, causing increased transport of ABA to the caryopsis and thus altering the expression of SSRGs (Qin et al., 2021).

GENETIC IMPROVEMENT OF CEREAL STARCH QUALITY

Research on SSRGs in cereals has resulted in numerous breakthroughs in cereal breeding for grain quality improvement, especially the application of elite natural and mutagenic alleles of key SSRGs (Tian et al., 2009; Chen and Bao, 2016; Zeng et al., 2017). The genetic engineering approach has also been widely used in the genetic modification of SSRGs.

The *Wx* gene that controls the synthesis of amylose is the most widely used gene in cereal breeding (Huang et al., 2020a). Since the cloning of the *Wx* gene in the 1980s, transgenic lines with various ACs have been established using antisense RNA, homologous recombination, and overexpression, and the effects of different ACs on starch physicochemical properties have been clarified (Shimada et al., 1993; Terada et al., 2000, 2002; Itoh et al., 2003; Liu et al., 2015a). In recent years,

diverse *Wx* alleles have been cloned in rice, maize, wheat, and barley (Asare et al., 2012; Guzmán and Alvarez, 2016; Luo et al., 2020). Cloning of numerous null *wx* alleles in maize has enabled the convenient breeding of waxy maize with a unique chewing texture. The combination of one, two, or three null *wx* (*wx-A1*, *wx-B1*, and *wx-D1*) alleles allows the breeding of partial waxy wheat with a reduced AC and amylose-free waxy wheat (Guzmán et al., 2012). Flours with different ACs and physicochemical properties can also be obtained by mixing waxy flour and non-waxy flour in different proportions. The *Wx* gene was selected preferentially during rice domestication (Anacleto et al., 2019). In rice, at least nine functional *Wx* alleles responsible for various ACs between 0% and 30% have been cloned, including *Wx^{lv}*, *Wx^a*, *Wxⁱⁿ*, *Wx^b*, *Wx^{mwl/a}*, *Wx^{op/hp}*, *Wx^{mq}*, *Wx^{mp}*, and *wx* (Zhang et al., 2019a). Significant achievements have been made through the introgression of *Wx^b* alleles (low to medium AC) and *Wx^{op/hp}*, *Wx^{mq}*, and *Wx^{mp}* alleles (low AC) to reduce the AC of targeted rice varieties (Huang et al., 2020a). Recently, the rare *Wx* allele *Wx^{mwl}/Wx^{ja}* (AC ~13.5%), derived from the homologous recombination of *Wxⁱⁿ* and *Wx^b*, was identified through genome-wide association analysis and map-based cloning (Zhang et al., 2020b; Zhou et al., 2020b). Rice carrying *Wx^{mwl}/Wx^{ja}* showed an excellent eating and cooking quality, similar to soft rice, but its grains remained transparent, unlike the opaque endosperm of soft rice cultivars (Zhang et al., 2020a; Zhou et al., 2020b). Moreover, a series of novel *Wx* alleles was created by editing the promoter and coding region of rice *Wx*, and similar strategies could be adopted to create more novel *Wx* alleles (Huang et al., 2020b; Zeng et al., 2020; Xu et al., 2021). Direct editing of the *Wx* gene in superior germplasm could reduce the impact of linkage drag on yield (Gao et al., 2020).

The *ALK* gene, also known as *SSIIa* and *SSII-3*, controls GT and is another crucial gene in rice quality improvement breeding (Gao et al., 2003). Three typical *ALK* alleles were identified based on three key single-nucleotide polymorphisms (SNPs) among rice cultivars (Nakamura et al., 2005; Waters et al., 2006; Gao et al., 2011). *ALK^a* and *ALK^b* encode inactive *japonica*-type *SSIIa*ⁱ that produces a low GT. *ALK^c* encodes the active *indica*-type *SSIIa*ⁱ that produces a high GT. *ALK^b* induces a slightly lower GT but has a wider distribution among rice subpopulations than *ALK^a*, and both their GTs and distributions are lower than those of *ALK^c* (Chen et al., 2020). In addition, *ALK^d*, caused by a G/T SNP in exon 1 of the *ALK^c* allele, was identified from the high-GT *indica* rice cultivar Zhonghui 9308, which had a higher GT and improved retrogradation properties (Zhang et al., 2020a). The *SSIIa*-deficient mutant *ssIIa*, with no *SSIIa* activity or *SSIIa* protein and a lower GT, was also identified following *N*-methyl-*N*-nitrosourea mutagenesis of the *japonica* rice cultivar Kinmaze (Miura et al., 2018). In rice breeding programs, *ALK* alleles that produce low and medium GTs are generally preferred.

Sweet corn is an important direction in maize breeding. The improvement of sweet corn involves multiple genes and their allelic variants, including *Su1*, *Su2*, *Se1*, *Bt1*, *Bt2*, and *Sh2*. Mutant alleles of isoamylase 1, designated *su1*, were first used for sweet corn breeding, and *su1* hybrids were mostly planted before 1985 (Schultz and Juvik, 2004). However, the rapid conversion of sugar to starch and the moisture loss in *su1* sweet corn hybrids after harvest caused a rapid decline in ear

quality, restricting their storage, transport, and marketability (Schultz and Juvik, 2004). The *su1* allele and the *se1* mutant can be combined to breed enhanced sweet corn. The *se1* gene is a recessive modification gene of *su1* that is inherited independently from *su1* (Zhang et al., 2019c). After the use of *su1*, *sh2* was used extensively in commercial varieties to breed super-sweet corn; the *sh2-i* allele, which encodes a partially inactivated AGPase, was obtained using ethyl methanesulfonate (EMS) mutagenesis (Lal et al., 1999). The combination of *sh2-i* with *su1* and *sh2* can produce multi-gene compound sweet corn varieties that overcome the genetic defects of the single *sh2* gene (Dodson-Swenson and Tracy, 2015). Knockout of *Wx* using genome editing also facilitated sucrose accumulation in corn kernels, and the simultaneous abolition of *Wx* and *Sh2* created super-sweet and waxy compound corn (Dong et al., 2019a).

Slow-digestible starch and resistant starch (RS) with a low GI are beneficial for the prevention and treatment of type 2 diabetes, obesity, and other blood glucose diseases. Increasing their content is a topic of considerable interest in current cereal research and breeding (Jukanti et al., 2020). Increasing AC and extra-long chains of amylopectin in cereal grains are the keys to GI reduction because of their negative correlation with starch digestion rate and positive correlation with RS content (Xia et al., 2018; Huang et al., 2020a). As early as the 1940s and 1950s, *ssii* and *amylose extender* (*ae*) mutants were used for high-amylose maize breeding (Li et al., 2019). A series of *ae* mutants characterized by loss of *SBEIIb* activity were identified in maize, among which *ae 1.2* lost *SBEIIb* catalytic activity and was unable to bind to amylopectin because it lacked a 28-amino-acid peptide (Val²⁷²-Pro²⁹⁹). The granule morphology and physicochemical characteristics of *ae 1.2* are distinct from those of the regular frameshift *ae* mutant as well as the wild type (Liu et al., 2011). The effects of each *SBE* isoform on cereal grain AC and RS content were determined using RNA interference and antisense RNA technology (Regina et al., 2006, 2010; Carciofi et al., 2012; Zhu et al., 2012). *SBEIIb* mutation in rice and maize significantly increased the AC and RS content (Li et al., 2019). Enhanced AC and RS content were also observed in the *SBEIIb* and *SBEIIa* double mutant but not in the *SBEIIa* single mutant (Zhu et al., 2012). However, this is incongruent with reports in which the opposite effects were observed in wheat and barley (Regina et al., 2006, 2010). Consequently, *sbe-rs*, a natural allele of *SBEIIb/SBE3* that can be used for rice breeding, was cloned through the mapping of Jiangtangdao 1, a rice cultivar with a high RS content and low GI (Yang et al., 2012). Two mutant alleles of *SBEIIb* that produced moderate changes in starch gelatinization and AC and a fine-tuned texture were obtained by screening a mutant population of the *japonica* rice variety Nipponbare, and they were named *altered gelatinization 1* (*age1*) and *age2* (Nakata et al., 2018). In addition, low GI breeding lines with different mutation combinations of *SBEIIa* and *SBEIIb* have been obtained through map-based cloning and EMS mutagenesis in wheat (Hazard et al., 2015; Regina et al., 2015; Schönhofen et al., 2016). More recently, genome editing technology has successfully enabled the development of novel rice and wheat germplasms with a low GI through mutation of *SBEs* (Sun et al., 2017; Guo et al., 2020a; Li et al., 2020b). In addition to *SBEs*, mutations in *SSIIa* and *SSIIla* also reportedly increased

the grain AC of cereals (Shimbata et al., 2012; Zhu et al., 2013; Zhou et al., 2016; Miura et al., 2018; Li et al., 2019).

PERSPECTIVES

Comprehensive analysis of the functions of SSRGs

Fully and systematically revealing SSRG functions, interactions, and effects on grain quality is the foundation of starch-related research and its applications; it is essential, albeit not highly innovative. Nevertheless, research on SSRGs has mainly focused on isoforms that are predominantly expressed in the cereal endosperm, whereas the roles of SSRGs that are expressed at low levels in the endosperm or predominantly expressed in other tissues remain unclear (Jeon et al., 2010; Pfister and Zeeman, 2016). Recent studies have found that some SSRGs that are weakly expressed in cereal endosperms nonetheless play significant roles in endosperm starch synthesis and quality formation (Tian et al., 2009; Lee et al., 2017; Zeng et al., 2017). For example, knockdown or knockout of *SSIIb* (*SSII-2*), which is expressed at a low level in the endosperm, suppressed the expression of *SSIIa*, *SSIIb*, and *Wx*, resulting in decreased AC, altered amylopectin CLD, and novel low-amylose rice with soft, transparent grains (Li et al., 2018b; Huang et al., 2021). Numerous mutation combinations with various starch properties can be isolated from different SSRG mutations, but only a few such combinations have been obtained, significantly limiting relevant studies (Jeon et al., 2010). The emergence of genome-editing technology provides the possibility for the systematic creation of related mutants, fully revealing SSRG functions, interactions, and effects on grain quality (Chen et al., 2019; Zhu et al., 2020a).

In-depth study of the regulatory network of starch synthesis

The cloning of *ZmABI19* and *O11*, which directly regulate key TFs of corn kernel development such as O2 and PBF, has shed light on the further upstream regulatory network of starch synthesis in cereal endosperms (Feng et al., 2018; Yang et al., 2020). Recent research has demonstrated that some microRNAs (miRNAs) modulate starch synthesis by affecting TFs that regulate SSRG expression and that DNA methylation may function as a switch to regulate starch synthesis by manipulating SSRG and miRNA expression levels in the early stage of grain development (Cheng et al., 2021; Hu et al., 2021). Furthermore, phosphorylation is widely involved in the regulation of starch synthesis (Makhmoudova et al., 2014). However, little is known about the protein kinases and phosphorylases that regulate SSRE phosphorylation, and this topic warrants further work to parse the underlying mechanisms.

Underlying mechanism of the metabolic connection between starch and other storage substances in cereal endosperms

The reduction of starch synthesis in cereal endosperms can lead to a general increase in the accumulation of multiple primary and secondary metabolites, such as sugars, fatty acids, amino acids, and phytosterols. It can also trigger extensive metabolic changes throughout the plant via transcriptional reprogramming (Baysal et al., 2020). TFs that regulate SSRGs also directly regulate genes involved in storage protein synthesis and lysine synthesis

(Kawakatsu and Takaiwa, 2010; Zhang et al., 2016). These observations outline a complex regulatory network in which TFs coordinately regulate the accumulation of storage substances in cereal endosperms through the dynamic balance and distribution of carbon and nitrogen; however, only a small portion of this network has been analyzed.

Toward the rational design of cereal starches

The physicochemical properties of starch are closely related to the grain quality of cereals. Further optimization of starch synthesis in cereal endosperms is an important direction in future cereal breeding for grain quality improvement. Nonetheless, there are still many hurdles to overcome. Substantial quality improvements that result from alterations in starch synthesis are often accompanied by negative effects on grain yield and other aspects of grain quality, such as grain chalkiness (Li et al., 2018b). An increase in grain AC may drastically reduce starch digestion rate and reduce eating and cooking quality; the inhibition of *SBEIIb* or *SSIIIa* led to an increase in AC and a reduction in grain yield (Zhu et al., 2012; Zhou et al., 2016). Compared with SSRGs predominantly expressed in the endosperm and pleiotropic TFs, SSRGs with a relatively low level of expression in the endosperm appear to be more suitable for crop breeding because their mutations tend to cause moderate changes in starch synthesis (Huang et al., 2021). Furthermore, fine-tuning of the expression or enzyme activity of SSRGs with predominant expression in the endosperm is also likely to be useful in developing elite cereal varieties with improved grain quality without compromising yield traits (Huang et al., 2020b; Xu et al., 2021).

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AUTHOR CONTRIBUTIONS

Qiaoquan Li, L.H., Qianfeng Li, and C.Z. conceived the project. L.H. and H.T. collected the references and prepared the figures and tables. L.H. and Qiaoquan Li wrote the manuscript. All authors read and approved the final version of the manuscript.

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