Chapter 9
Initiation Process of Starch Biosynthesis

Yasunori Nakamura

Abstract Plants have developed the metabolic system in which a great amount of starch can be synthesized and store them into granules with semicrystalline structure in the plastid. The fine structure of amylopectin, a major component of starch, is a highly organized distinct structure composed of a unit structure called cluster. Thus, it is highly possible that plants have a specific starch biosynthesis initiation different from that of the well-known glycogen biosynthesis initiation found in animals, fungi, and bacteria. Based on a working hypothesis that the starch biosynthesis initiation has two events, i.e., the initiation of amylopectin synthesis and that of starch granule formation, the possible roles of enzymes which are potentially involved in these events and mechanisms underlying the regulation of amylopectin synthesis from simple sugars and starch granule formation are discussed.

Keywords Amylogenin • Amylopectin • Disproportionating enzyme • Glycogen • Glycogen synthase • Glycogenin • Initiation • Phosphorylase • Starch • Starch branching enzyme • Starch granule • Starch synthase

9.1 Introduction

The starch biosynthesis in plant tissues is a tremendous, remarkable, and specific metabolic process despite its apparently simple and monotonous production process, because starch molecules seem to be produced of repetitive reactions between chain elongation catalyzed by starch synthase (SS) and chain branching catalyzed by starch branching enzyme (BE). First, the rate of starch synthesis is tremendously higher compared with other carbon synthetic processes especially in reserve tissues. Second, considering the structure of amylopectin molecule having the numerous cluster units and starch granules that are highly and specifically

Y. Nakamura (✉)
Faculty of Bioresource Sciences, Akita Prefectural University, Shimoshinjo-Nakano, Akita 010-0195, Japan

Akita Natural Science Laboratory, 25-44 Oiwake-Nishi, Tennoh, Katagami, Akita 010-0101, Japan
e-mail: nakayn@silver.plala.or.jp

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organized over several dimension levels (see Chaps. 1 and 3), the syntheses of the polysaccharides and the granular structure must need complex phases operating under various metabolic regulations. Third, in spite of the specific structural features, the homogeneous products with uniform structures are reproduced without accumulation of significant amounts of intermediate glucans and/or dextrins under general physiological and environmental conditions.

To achieve such characteristics, it must be impossible for plants to synthesize amylopectin molecules and starch granules only by a one-step metabolic process. Instead, plants must have at least two processes, the initiation process and the amplification (or reproduction) process. However, the conditions of the initiation process must be different between starch and glycogen biosynthesis. In glycogen biosynthesis in animals and fungi, the initiation process has been well defined, and some primary biochemical steps have been characterized at the molecular level (see review by Roach et al. 2012). In contrast, little is known regarding the initial process of the starch biosynthesis and thus at present the content of the process (most of biochemical events) must be conceptual. What are the requisites for the initiation process in starch biosynthesis? The initiation process in starch synthesis must be much more complex than that in glycogen synthesis because in the former glucans are probably produced from the precursor of amylopectin having the cluster structure. Figure 9.1 illustrates a working hypothesis on the initiation process and the amplification process in starch synthesis in higher plants (Nakamura et al. 2009; Jeon et al. 2010; Nakamura 2014). It is hypothesized that in the initiation process, the precursor of amylopectin is synthesized from simple sugars such as glucose, maltose, and maltotriose, possibly via branched dextrins and glucans having an immature cluster structure, while in the amplification process, mature amylopectin structure is synthesized from the precursor by reproducing the clusters. Under physiological conditions especially when starch is vigorously synthesized, the amounts of intermediates involved in the initiation process are considered to be much lower than those of accumulating starch molecules. Thus, the initiation process is usually hidden behind the amplification process, and it is difficult to detect the biochemical events happening at the starch initiation stage, and in fact almost all of the available information has been derived from the results regarding the amplification process.

Transcriptome analysis indicates that genes highly expressed at the very early developmental stage of reserve tissues/organs in which the initiation process is considered to be dominant are different from genes expressed in these tissues during the amplification process where the starch production is at its highest rate which is achieved by the amplification process (Ohdan et al. 2005), suggesting that enzymes involved in the initiation process are different from enzymes responsible for the amplification process, as described below in detail (Sect. 9.3).

In this chapter, the initiation process of starch biosynthesis in reserve tissues/organs is focused upon and discussed from several possible aspects including features of enzymes and glucans potentially involved in this process. It is assumed that the initiation process includes the synthesis of amylopectin prototype which has the cluster structure and can serve as the precursor for the mature amylopectin

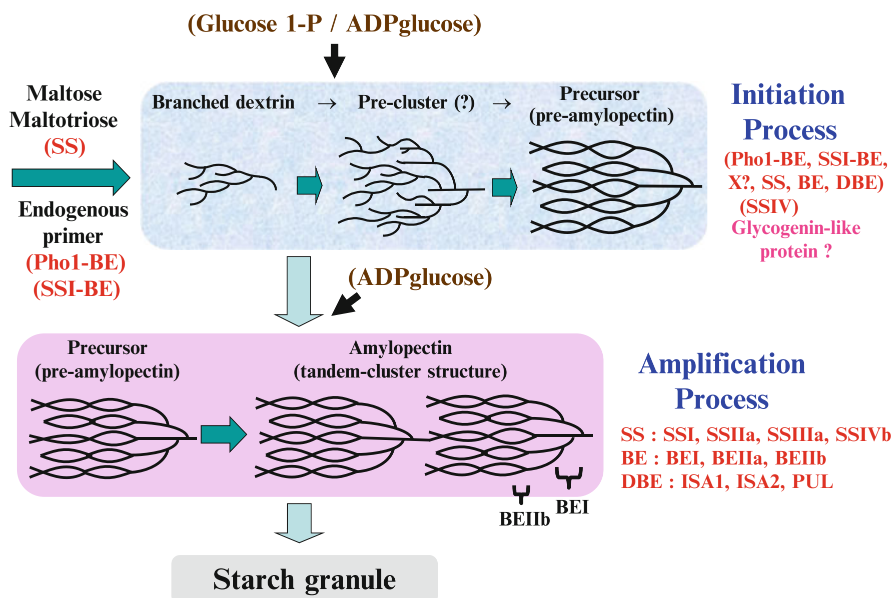

Fig. 9.1 Schematic representation of the initiation process and the amplification process in starch biosynthesis in higher plants

molecules in the subsequent amplification process. The related process in the assimilatory starch synthesis is described in Chap. 6.

9.2 Past and Ongoing Investigations for the Initiation in α-Glucan Biosynthesis

9.2.1 Initiation Process in Glycogen Biosynthesis in Animals, Fungi, and Bacteria

The priming of glycogen synthesis in animals and fungi has been well characterized (see review by Roach et al. 2012). The specialized initiator protein called glycogenin (EC 2.4.1.186) self-glucosylates using a glucose residue of UDPglucose to form a malto-oligosaccharide (MOS) linked by α-1,4 glucosidic bonds (Lomako et al. 1988; Pitcher et al. 1988; Farkas et al. 1991; Cheng et al. 1995) at the highly conserved tyrosine residue (Cao et al. 1993; Mu et al. 1996), which can be subsequently used by glycogen synthase (GS) as primer (Romero et al. 2008). The glycogenin directly interacts with GS to form the majority of glycogen chains. The formation of glycogen molecules is accomplished by concerted and repeated chain-elongation and chain-branching reactions catalyzed by GS and glycogen branching enzyme (BE), respectively.

The glycogen biosynthetic process in bacteria is different from that in animals and fungi because almost all of bacteria have no glycogenin homologues (see review by Wilson et al. 2010). Ugalde et al. (2003) showed that GS from *Agrobacterium tumefaciens* can form MOS by transferring a glucose residue from ADPglucose to an amino acid(s) in the GS protein. Thus, in bacteria the same GS has the capacity for both glycogen initiation and elongation in the absence of added glucan primer.

9.2.2 Investigations for the Initiation Process in Starch Biosynthesis

9.2.2.1 Autoglucosylation: Amylogenin

The expressions of glycogenin-like genes were detected from maize (Rothschild and Tandecarz 1994) and rice (Qi et al. 2005), and the protein was called as amylogenin (Singh et al. 1995). Chatterjee et al. (2005) claimed that knockout of *glycogenin-like protein* gene expression in *Arabidopsis* reduced the amount of starch in the leaf, although they only measured the glucan content by iodine staining method, which is not necessarily the quantitative method. However, a piece of evidences support that this protein is involved in the initiation of cell wall biosynthesis (Delgado et al. 1998; Langeveld et al. 2002; Sandhu et al. 2009). Therefore, at present no definite evidence for an essential role of glycogenin/amylogenin in the initiation process of starch biosynthesis is available.

9.2.2.2 Soluble Starch Synthase IV (SSIV)

There have been several reports indicating the involvement of soluble starch synthase IV (SSIV) in the starch granule initiation in *Arabidopsis* leaves (see Chap. 6 for more details and reviews by D'Hulst and Merida 2010, 2012). Mutation at the *SSIV* locus in *Arabidopsis* reduced the number of starch granules in chloroplasts although SSIV contributed little to the total SS activity in the leaf (Roldán et al. 2007). It is noted, however, that the *ss4* mutants had one or two huge starch granules per chloroplast, suggesting that SSIV is specifically involved in the initial process of starch granule formation, but is not directly involved in the synthesis of starch molecules in the amplification process. Overexpression of *SSIV* gene reportedly enhanced the starch content in *Arabidopsis* leaf and the growth rate (Gámez-Arjona et al. 2011). The *ss4*-related mutations resulted in a wide range of biochemical, physiological, and morphological changes such as starch molecular structure, the contents of sugars, the turnover of starch, the number and morphology of starch granules, the number and size of chloroplasts, and the growth rate and leaf morphology of *Arabidopsis* plant (Roldán et al. 2007; Szydlowsky et al. 2009; Gámez-Arjona et al. 2011; Crumpton-Taylor et al. 2013). Detailed analysis of these phenotypes supports the view that SSIV is directly involved in the starch granule

initiation in *Arabidopsis* leaves (Crumpton-Taylor et al. 2013). Remarkably, the *Arabidopsis* mutants lacking both SSIII and SSIV activities had no detectable starch granules in leaves, while the number of starch granules was apparently not modified in the *ss1/ss2/ss3* triple mutants, but the granule size was strongly reduced (Szydlowsky et al. 2009, 2011). The results indicate that the roles of SSIV and SSIII overlap in the granule initiation process which is indispensable for the starch synthesis, and these roles cannot be substituted by SSI and SSII, or GBSS (see Chap. 6).

However, it is unclear whether the mechanism for the contribution of *SSIV* gene to starch granule formation found in *Arabidopsis* commonly occurs in all the other plant cells. Toyosawa et al. (2015) observed that the single mutation at the *SSIVa* or *SSIVb* locus in rice showed no or little detectable alterations in starch-related phenotypes. However, when both the *SSIVb* and *SSIIIa* genes, the major *SSIV* and *SSIII* genes expressed in developing rice endosperm, were simultaneously defective, the starch granular morphology dramatically changed from the polygonal (wild-type) to the spherical morphology, while the starch content per seed was only slightly reduced (Toyosawa et al. 2015). It is interesting that the spherical starch granules in the *ss3a/ss4b* mutant endosperm were present separately from each other, markedly different from the wild-type polygonal granules packed in a single amyloplast as the compound starch granules (see Chap. 10). The results indicate that SSIIIa and SSIVb have unconventional functions in amyloplast and starch granule developments (see Chap. 13 for details). Thus, at present it is unlikely that SSIVb and SSIIIa play pivotal roles in starch production per se, but they are involved in the determination of starch granule morphology in rice endosperm, although more detailed studies are needed to draw the definitive conclusion.

The SSIII and SSIV are known to share the structural similarity in having a very long N-terminal extension compared with the protein structures of SSI, SSII, and GBSS (Leterrier et al. 2008). Although further studies regarding the contribution of SSIII and SSIV to the starch granule formation are needed, the distinct functional interaction exists between these enzymes (see Chap. 6).

Szydlowsky et al. (2009) found that SSIV could use maltose and maltotriose as glucan primer for glucan synthesis, and the synthetic rate with maltotriose was higher than 90 % of that from amylopectin, suggesting the additional role of SSIV in glucan synthesis, as described below.

9.2.2.3 Plastidial Phosphorylase (Pho1)

The involvement of plastidial phosphorylase (Pho1) in starch biosynthesis has been proposed from in vivo and in vitro studies. A green alga *Chlamydomonas reinhardtii* having plastidial *pho* mutations at the *STA4* locus showed a decreased amount of starch with abnormal shapes of granules and a modified amylopectin structure (Dauvillée et al. 2006). Based on biochemical, molecular, and genetic analyses, they claimed that plastidial Pho is needed for normal starch synthesis in *Chlamydomonas*.

The *pho1* mutation of rice greatly affected the starch phenotype in the endosperm (Satoh et al. 2008). The extent of reduction of starch content in the mutant seeds greatly varied ranging from severely shriveled seed due to no starch accumulation to near normal seed (Fig. 9.2a). The mutant phenotypes were greatly affected by temperatures during growth, and at lower temperature (20 °C) most of seeds became shrunken, while at higher temperature (28–30 °C) the majority of mutant seeds were plump (Fig.9.2b). Despite such drastic reduction of starch amount in seed, the amylopectin fine structure was only slightly altered. Based on these observations, it was proposed that Pho1 is involved in the initiation process of starch biosynthesis, but not in the amplification process in rice endosperm. They also assumed that some unknown factor(s) which can function and/or is(are) expressed only at higher temperatures above 20 °C support(s) the role of Pho1.

Fettke et al. (2010, 2012) have examined a specific incorporation behavior of glucose 1-phosphate (G1P) by potato tuber discs from various transgenic lines and found that the exogenously added G1P was converted to native starch granules in tubers as mediated by Pho1 (Fettke et al. 2010). The rate of incorporation of G1P was much higher than glucose, glucose 6-phosphate (G6P), or sucrose. The amount of carbon incorporated from G1P into starch was abolished only by reduced Pho1 activities, but not inhibited or rather increased by inhibitions of cytosolic and plastidial phosphoglucomutase, cytosolic Pho (Pho2), or cytosolic transglucosidase. The authors also found that incorporations of radioactivity from G1P and sucrose into starch in potato tuber discs differed in amounts in response to temperature during incubation (Fettke et al. 2012). The Pho1-mediated G1P path reached maximal activity at about 20 °C, while the conventional AGPase-mediated sucrose path was markedly activated by higher temperatures above 20 °C. The investigations established that the G1P pathway in which G1P is directly used for starch by Pho1 at least in potato parenchyma cells is regulated by a mechanism different from the primary AGPase pathway for starch biosynthesis.

There have been a lot of pieces of criticisms regarding the involvement of Pho1 in starch biosynthesis. It is often argued that Pho1 is unable to play an important role in starch biosynthesis, considering that actual concentration of Pi is much higher than G1P inside the cellular compartment, despite a good correlation between the activity levels of Pho1 and the rate of starch production in various tissues (Schupp and Ziegler 2004 and References therein). However, in vitro studies showed that rice Pho1 could elongate MOS in the synthetic direction even under physiological conditions of high Pi/G1P concentration ratio (Hwang et al. 2010). Based on the experimental results, Hwang et al. (2010) concluded that Pho1 can play a part in the initiation stage, but not in the amplification process of starch biosynthesis.

Nakamura et al. (2012) found evidence for a close interaction between Pho1 and either BE isozyme from rice in the synthesis of glucans without added primers during the enzymatic reaction (Fig. 9.3). The enhancement of glucan synthesis of Pho1 by BE was not merely due to the increased supply of nonreducing end (acceptor) of glucan substrate, but to a close interaction between both enzymes, which was achieved by activating each of the mutual capacity of the other in terms of increases in catalytic activities and affinities for the glucan (Fig. 9.3b). The glucan

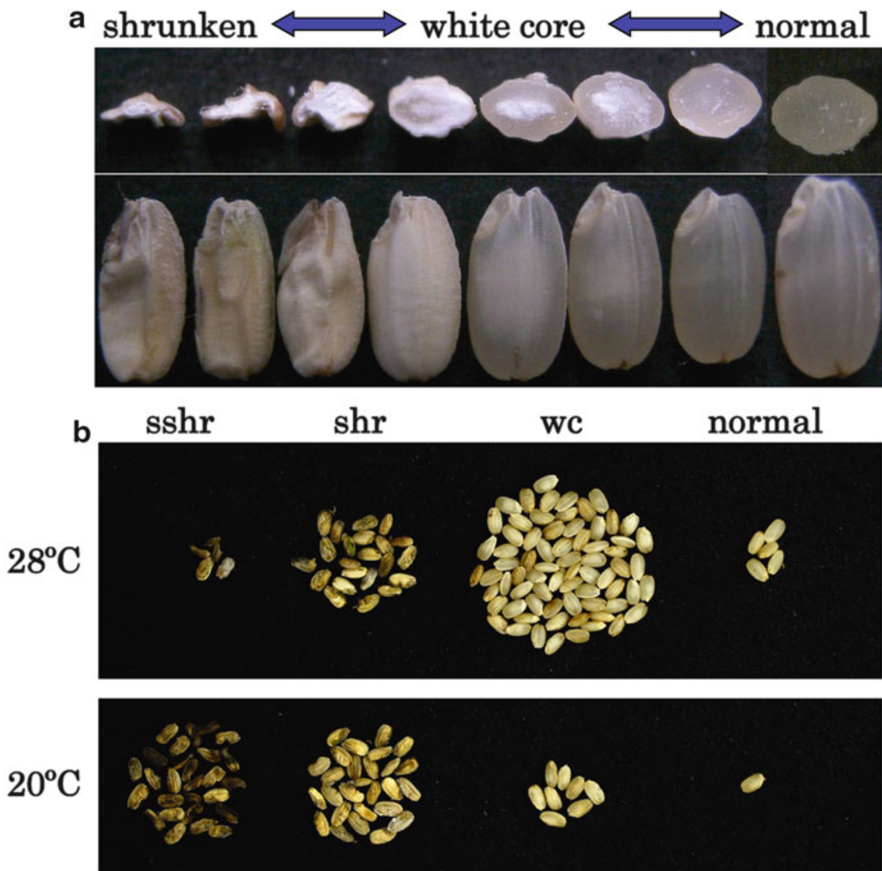

Fig. 9.2 Seed morphology of the rice mutant lines defective in the *plastidial phosphorylase* (*Pho1*) gene. (**a**) Kernels of the homozygous rice *pho1* mutant line ranged from normal to severely shrunken types even when the plant was grown under summer temperature conditions (Satoh et al. 2008). (**b**) Kernels in mature *pho1* mutant seeds of a single panicle were classified into five groups, normal plump, white core (wc), shrunken (shr), severely shrunken (sshr) kernels, and empty seeds (not shown), and these kernels are shown. After flowering, the plants were grown at high (28°) and low (20°) temperatures (Ohdan et al. unpublished)

products were exclusively branched molecules and no linear glucans existed; thus, it is likely that a small amount of endogenous glucan strongly bound to the purified Pho1 preparation from rice endosperm served as primer for the Pho1-BE reaction and all of the products had the branched form.

It is known that Pho1 could easily synthesize very long chains of DP > 100 from MOS (Kitamura et al. 1982), glycogen (Putaux et al. 2006), and amylopectin (Yuguchi et al. 2013) used as primer. The chain preference of Pho1 was similar to GBSS, which elongated glucan chains in a processive manner (Denyer et al. 1999), but was sharply in contrast with that of SS, which could synthesize only short and

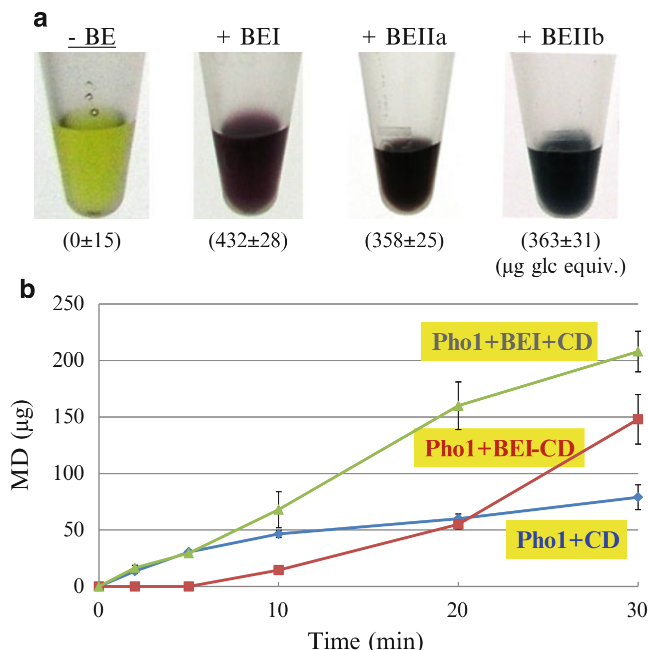

Fig. 9.3 Catalytic interaction between rice plastidial phosphorylase (Pho1) and starch branching enzyme (BE). (**a**) Effects of addition of BE on the in vitro glucan synthesis by Pho1 purified from developing rice endosperm in the absence of added glucan primer, cluster dextrin (CD). (**b**) Effects of concentrations of added glucan primer on the glucan synthesis by Pho1 from rice either in the presence or absence of BE (Data are from Nakamura et al. 2012)

intermediate chains in a distributive manner as long as SS was incubated with the glucan primer added (Denyer et al. 1999; Imparl-Radosevich et al. 2003; Nakamura et al. 2014). For this reason, it is unlikely that Pho1 plays an essential role in the chain elongation of amylopectin molecules because the lengths of amylopectin chains should be strictly restricted to form the cluster structure.

9.2.2.4 Other SS

It is generally believed that the minimum chain length of primer for SS is DP3 (maltotriose) and that SS cannot substantially synthesize glucans in the presence of maltose and glucose (Imparl-Radosevich et al. 2003), although the catalytic activity of SS toward linear MOS and amylose is markedly lower than that toward branched glucan. However, recently, Brust et al. (2013) showed that SSI, SSII, and SSIII from *Arabidopsis* were capable of acting on maltose as glucan acceptor when they were incubated for a prolonged period (22 h), although glucose could not be replaced by maltose (Fig. 9.4). As stated above, *Arabidopsis* SSIV was also able to synthesize

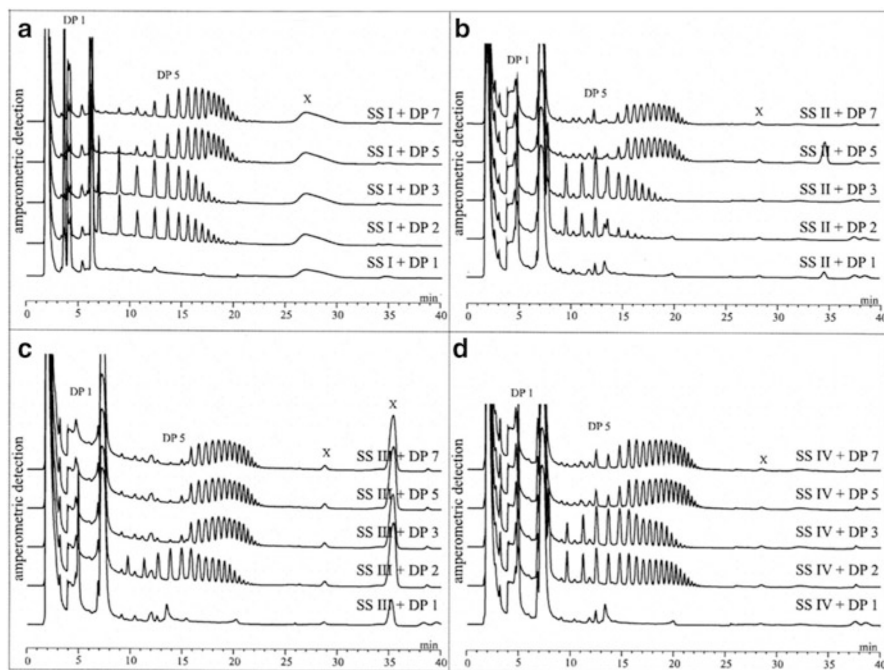

Fig. 9.4 Reactivities of *Arabidopsis* starch synthases (SSI, SSII, SSIII, and SSIV) toward malto-oligosaccharides (MOS) having different chain lengths. The enzymatic action of starch synthases from *Arabidopsis* toward glucose (DP1), maltose (DP2), maltotriose (DP3), maltopentaose (DP5), and maltoheptaose (DP7) at 30 °C for about 22 h in the presence of 3.5 mM ADPglucose and 1.2 mM MOS as primer (Brust et al. 2013)

glucan by using maltotriose and maltose as primer, and this synthesis capacity was much higher than that of SSIII (Szydlowsky et al. 2009).

The results strongly support the view that all the SS isozymes, SSI–SSIV, actually have capacities for the de novo synthesis of MOS having DP≧3 as long as maltose is present in plastids. Since maltose is ubiquitously present in intracellular environments via resulting from the actions of various enzymes such as amylases, Pho, and disproportionating enzyme (DPE) with glucans, this view suggests that plant cells can produce MOS by SS at least to some extent and they do not necessarily need the autoglucosylation process including the specific protein like glycogenin/amylogenin.

Recently, it was found that rice SSI, but not SSIIa and SSIIIa, synthesized glucans in the presence of BE without an addition of exogenous glucan primer (Nakamura et al. 2014) (Fig. 9.5). The role of BE added was not merely to provide SS with the nonreducing end of the glucan, but to enhance the chain-elongation capacity of SS (Fig. 9.5b). In turn, BE was also activated by SSI through its increased affinity for the glucan. Since in addition the branched glucan was also involved in the SSI-BE interacting reaction, the same mechanism might underlie the interactions

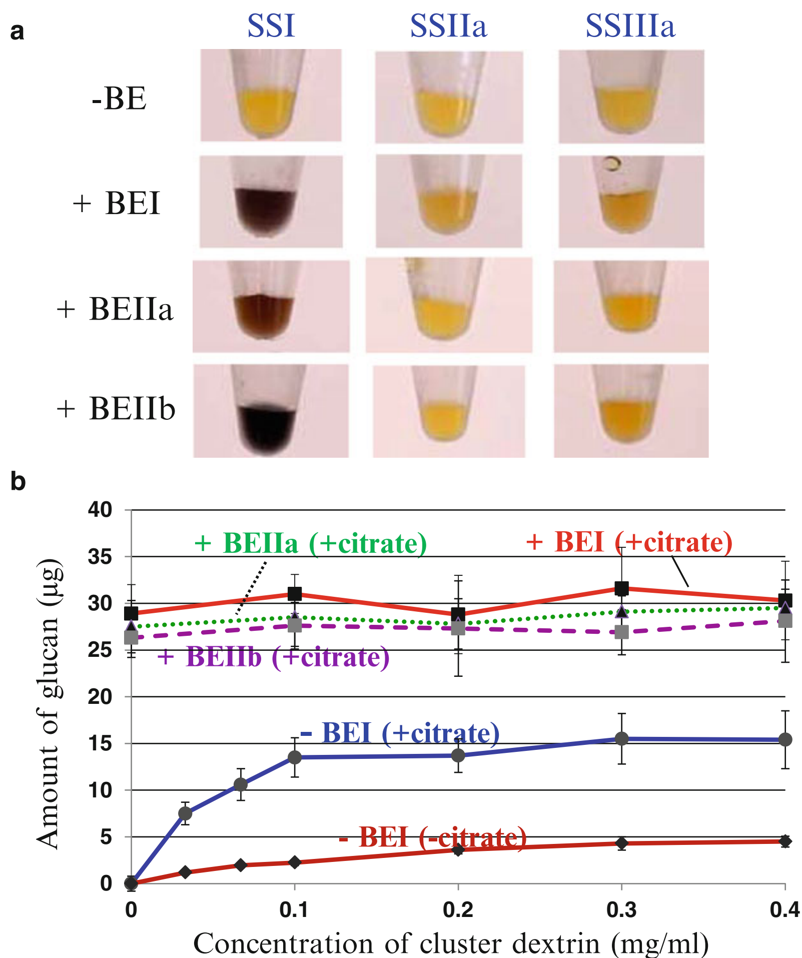

Fig. 9.5 Catalytic interaction between starch synthase (SS) and starch branching enzyme (BE) from rice. (**a**) Effects of addition of BE from rice on the in vitro glucan synthesis by SS from rice in the absence of added glucan primer. The glucans formed were stained with iodine. (**b**) Effects of concentrations of added glucan primer (cluster dextrin) on the glucan synthesis by SS1 from rice either in the presence or absence of BE and/or citrate (Data are from Nakamura et al. 2014)

between both SSI-BE and Pho1-BE. The close interaction between SS-BE in the unprimed synthesis was also reported in *Arabidopsis* enzymes (Brust et al. 2014). At least in rice endosperm, the involvement of branched glucan seemed to be more important for the SSI-BEI interaction than the Pho1-BE interaction because the catalytic activity of SSI was much higher toward branched glucan than to the linear glucan and MOS, whereas Pho1 activity was high toward both branched and linear glucans (see Chap. 5 and review by Fujita and Nakamura 2012). The results suggest

that SSI and BE closely and functionally interact and can efficiently synthesize branched glucans possibly by using an endogenous glucan primer bound to purified SSI preparation.

When SSI from rice was incubated with amylopectin as primer, its elongation was highly specific because the main product during the short incubation period was the DP8 chain formed from acting on very short amylopectin chains of DP6 and 7 (Nakamura et al. 2014). Considering that the maximum chain lengths for the SSI-BEI product were DP10–12, reflecting the chain profile for BEI (see Fig. 5.6d in Chap. 5), these in vitro studies suggest that SSI has dual functions in glucan synthesis, i.e., first, the synthesis of very short chains (DP about 8) of amylopectin in the amplification process and, second, the synthesis of glucan chains longer than the DP8 chains by association with BE in the initiation process of starch biosynthesis.

9.3 Possible Factors Involved in the Initiation of Starch Biosynthesis

Available information suggests that the initiation of starch biosynthesis falls into two categories, i.e., the initiation of formation of starch molecules and that of starch granules, although they must be closely interconnected.

What are features of glucan structures involved in the initiation process of the synthesis of starch differing from the amylopectin synthesis during the amplification process? Practically nothing is known about the glucans and dextrins involved in the initiation process. Preliminary results showed that glucans/dextrins abundant in rice endosperm at the very early developmental stage had some structural features (Nakamura et al. unpublished data). First, the chain-length distribution analysis of the whole glucan chains and the internal segments (chains found in phosphorylase-limit dextrins) indicated that the fine structures of glucans/dextrins possibly involved in the initial stage of glucan synthesis were different from mature amylopectin molecules. In addition, the glucans/dextrins had very short chains of DP2–5, suggesting that that was the results of hydrolysis by amylases, phosphorolysis by Pho1, and/or disproportionation by DPE of outer chains after they were synthesized, whereas mature amylopectin had no or little such short chains, possibly by being protected by such degradation of external chains from actions by these enzymes. Second, the sizes of the glucans/dextrins were much smaller than normal amylopectin. Third, the glucans/dextrins had more hydrophilic properties than starch granules and were not precipitated by a low-speed centrifugation. Fourth, the granular size including the glucans/dextrins was smaller compared with mature starch granules. These results suggest that in the initiation process the intermediate glucans/dextrins with no or immature cluster structure play important roles in amylopectin biosynthesis serving as the precursor of amylopectin-type glucans.

What are candidate enzymes involved in the initiation process? Information from transcriptome analysis of genes encoding starch biosynthetic enzymes of rice plants

is available (Ohdan et al. 2005; Yamakawa et al. 2007, http://ricexpro.dna.affrc.go.jp/Zapping/). Ohdan et al. (2005) classified the four major expression patterns during rice seed development (Fig. 9.6). The first group that includes SSIIIb, BEIIa, and DPE1 was characterized by a high expression level at a very early developmental stage. The second group including SSI, SSIVb, ISA2, and Pho1 showed an intermediate expression level at the initiation seed formation period and rose rapidly to peak at the early developmental stages, and then the level continued to decline in the middle and late developmental stages. The third group composed of SSIIa, SSIIIa, GBSSI, BEI, BEIIb, ISA1, and PUL showed a basal or very low expression level at the initial developmental stage, but rapidly increased to a high level thereafter, and this high level was maintained until seed maturation. The fourth group including SSIIb, SSIIc, SSIVa, GBSSII, and ISA3 was characterized by low expression level at the start and further decreased to a basal level throughout the seed development, whereas some of them were preferentially expressed in leaves. The results suggest that at the early developmental stage of reserve tissue cells, the initiation process of starch synthesis predominantly operates and enzymes involved in the process are vigorously functioning.

The properties of Pho1 match the requirements for the initiation process. Pho1 could synthesize a wide range of chains from short chains to very long chains by using various primers including branched and unbranched glucans (Kitamura et al. 1982; Putaux et al. 2006; Yuguchi et al. 2013), contrasting with SS whose chain preference was distinct depending on each SS type and elongation activity basically decreased with the increase of chain length (Commuri and Keeling 2001; Nakamura et al. 2005; Fujita and Nakamura 2012; Nakamura et al. 2014) (see Sect. 5.3.1.1 in Chap. 5).

SSI might also be potentially involved in the initiation process by closely interacting with BE. It is highly possible that the chain-elongation properties of SSI when the SSI-BE complex synthesized the intermediate glucans/dextrins composed of chains having a wide range of chain lengths (Nakamura et al. unpublished) sharply differed from that when SSI directly reacted to the glucan primer like amylopectin.

What are the advantages of the Pho1-BE and SSI-BE interactions? It should be stressed that both enzyme-enzyme interactions were capable of the glucan synthesis on their own by using the yet unidentified endogenous glucan primers. Thus, their reactions were basically unaffected by surrounding glucans such as mature amylopectin accumulated even at high concentrations, because the Pho1-BE and SSI-BE had much higher affinities for appropriate glucans (Nakamura et al. 2012, 2014) that functioned as primers for the subsequent reactions, compared to other various glucans accumulating in highest amounts in reserve tissues. It is also noted that the minimum chain lengths of branched and linear glucans for BE were DP12 and DP about 48 or larger, respectively (Nakamura et al. 2010; Sawada et al. 2014). This indicates the likely possibility that the contribution of branched dextrins/glucans to the initiation process is much more significant than that of linear MOS/dextrins.

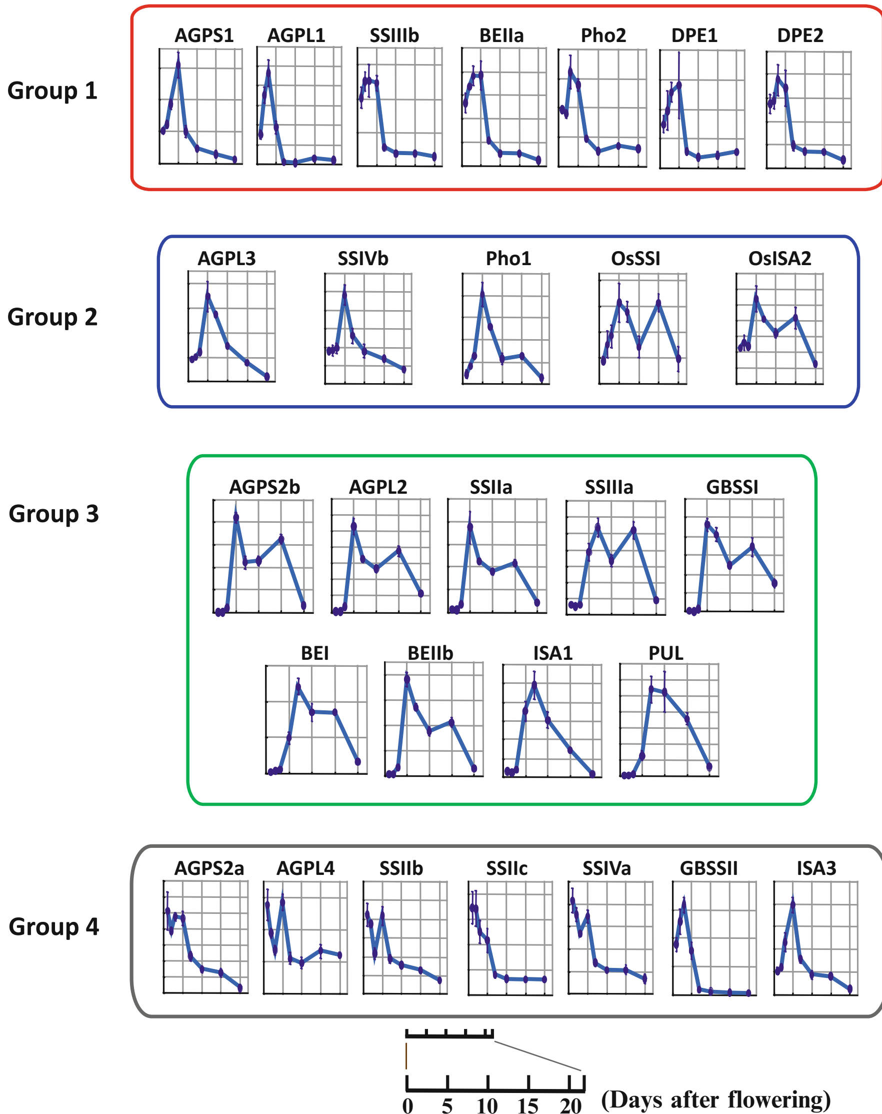

Fig. 9.6 Expression profiles of genes coding for starch synthetic enzymes during development of rice seeds. Genes are classified into 4 groups according to pattern of changes in the transcript levels during development of rice endosperm. The vertical axis shows the amount of the transcript level (see Ohdan et al. 2005 for details)

Plants might have established the specific initiation process in plastids during evolution, which is not found in animals and fungi. Plants must have developed the potential functions of enzymes specifically involved in this process and assigned plastids to perform the highly integrated enzymatic reaction networks by coordi-

nating with other subcellular compartments. SS might have gained the capacity to synthesize MOS from maltose, while Pho1 and SSI in combination with BE have excellent means for the glucan synthesis by using endogenous glucans entrapped with them as primers. Thus, these enzymes have multiple functions in starch biosynthesis: Pho1 would be involved in the initiation process by association with BE, playing a part in the degradation of MOS generated at the amylopectin trimming step by starch debranching enzymes during starch biosynthesis, while SSI could play important roles in the elongation of very short chains of amylopectin (see Sect. 5.3.1.1 in Chap. 5), the synthesis of MOS from maltose and maltotriose (Brust et al. 2013), and the synthesis of branched glucan by forming functional interaction with BE (Nakamura et al. 2014).

Starch granules have distinct size and morphology depending on species, tissues, and developmental stages as well as different genetic backgrounds. Thus, the initiation process for the synthesis of starch granules must be very specific and found in plants. Although several factors are considered to affect directly or indirectly the number, size, and shapes of starch granule structure, the precise mechanism for the initiation of starch granular structure needs to be elucidated.

The specific role of SSIV having some redundancies with SSIII in the starch granule initiation in chloroplasts was revealed by a comprehensive analysis of the *ss4-ss3* mutants of *Arabidopsis* (see Chap. 6 for details). In addition, Yun et al. (2011) proposed that ISA3 was involved not only in starch degradation but also in plastid division in rice endosperm and leaf based on the analysis of rice transformants in which the *ISA3* transcript level was reduced and overexpressed.

Involvement of some enzymes and proteins in plastid division and morphology has also been proposed from the analysis of their localizations in starch granules and inside and outside of plastids by using laser scanning confocal microscopy detecting fluorescence-labeled proteins. Recently, Toyosawa et al. (2015) observed that most of SSIVb proteins were located in the septum-like structure dissecting the inside space of developing rice amyloplast (Yun and Kawagoe 2010), which gave rise to the compound polygonal starch granules in rice endosperm. This sharply contrasts with the observation of Szydlowsky et al. (2009) that SSIV was located in specific regions at the boundaries of starch granules in *Arabidopsis* leaves. Toyosawa et al. (2015) also showed that some SSIIIa proteins were located in the outer envelope and intermembrane spaces of amyloplasts in rice endosperm. These results strongly suggest that SSIVb and SSIIIa have specific secondary functions controlling the division and/or development of amyloplasts in developing rice endosperm, although further concrete evidence is needed.

9.4 Future Perspectives

At present almost nothing is known regarding the molecular mechanism and factors involved in the initiation process of starch biosynthesis and factors involved in this process. No consensus on the consistent definition of the initiation process

has been established. In this chapter, several topics have been described and discussed based on a working hypothesis that the initiation of starch biosynthesis in plant cells is composed of two processes. The first process includes the synthesis from simple carbohydrates such as glucose, maltose, G1P, and ADPglucose to the amylopectin prototype, which is subsequently reproduced at a high rate to form the starch granules in the amplification process. The second is the starch granule initiation process. Since granules including glucans/dextrins synthesized in the initiation process are possibly formed even when they lack the cluster structure, both processes might happen simultaneously at least to some extent and are interrelated. Yet, the possibility that the core region of the granule including cluster-less glucans is used as precursor of the starch granule during the development of the granule packed with mature amylopectin synthesized in the amplification process in its outer starch layer cannot be ruled out. The nature of the initiation process may be different between reserve tissues and leaves and between cereal endosperm and tubers/tuberous roots of potato/sweet potato. In leaves, starch production and consumption are diurnally repeated, and cells may maintain the prototype amylopectin in the very small size of glucans even at the previous night period, which are then used as precursors for amylopectin and starch granules. If this is the case, the true initial process does happen only at the very early leaf developmental stage. On the other hand, developmental and maturing stages of cereal endosperm proceed near synchronously from cell division until the seed desiccation. Thus, the initiation process plays a crucial role in the final production of starch granules in the cereal, and this process might be maintained during the developing stage because amyloplast division and enlargement must be vigorously operating at least until the late developmental stage when amyloplasts are matured. In vegetative tissues such as potato tubers, starch production is accompanied by the process of cell/amyloplast division and expansion, and both the initiation and amplification are not distinctly separated from each other compared with starch synthesis in cereal endosperm. Since these functions are hypothesized based on limited information on characteristics of enzyme actions found in a few plant sources, more detailed analyses of enzymes from various sources must be conducted.

New techniques and approaches required for future studies on starch biosynthesis and described in Chap. 5 are also expected to promote our understanding of the initiation process of starch biosynthesis.

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