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Insight into starch biosynthesis in cereal endosperm

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Abstract
Major storage reserve present in cereal endosperm is starch that forms the basis of yield in the most important crops of the world. Storage starch is composed of two D-glucose homopolymer, a near-linear polymer amylose and a highly branched glucan amylopectin. In endosperm, amylose is synthesized by ADP-glucose pyrophosphorylase and granule-bound starch synthase, whereas amylopectin synthesis requires coordinated series of enzymatic reactions catalyzed by ADP-glucose pyrophosphorylase, soluble starch synthase, branching and debranching enzyme. A recent advance in understanding the function of enzyme isoforms has provided new insight in addition to core reactions. This review describes our current view of understanding of storage starch biosynthesis inside the amyloplast of developing cereal endosperm.

Keywords: Starch, amylose, starch synthase, starch branching enzyme, debranching enzymes, amylopectin

Introduction
Cereals represent top three crops viz. wheat, rice and maize in terms of area and production worldwide currently (Tetlow *et al*., 2017) [21]. In general, economic part of cereal crops are heterotrophic starch storing organ. Globally, annual starch production of cereal seeds (rice, maize etc) and roots and tubers (cassava, taro, potato) is approximately 2 billion tons and 700 million tons respectively (http://faostat.fao.org), much of which is utilized for non food purposes. Starch based diet accounts for the major proportion of daily calorie intake of humans and significant fraction of livestock feed. A higher fraction of starch products that are consumed by human and animal affects their health, particularly quality characteristics of starch (Venn *et al*., 2004) [25]. Moreover, a rapid increase in world's population and growing loss of agricultural lands to urbanization and climate change prompts to screen and breed plants with higher starch content. In particular, increased prosperity of world emerging economies aggravated higher crop yield demand for livestock production as meat consumption has increased (Parry *et al*., 2010) [19]. It is therefore important to understand the basis of starch biosynthesis in cereal endosperm in order to produce higher yields and ensure greater food security.

Starch biosynthetic pathway
In higher plants, starch is synthesized inside the plastid for both the short term storage as transitory starch (over a day-night cycle in chloroplasts of photosynthesizing tissue e.g. leaves) or long term storage as storage starch (storage starch in amyloplasts of seeds and tubers). Sucrose synthesized in leaves is imported by storage tissues that act as a carbon source of production and energy and starch synthesis in amyloplast. Starch is basically a D-glucose homopolymer that is composed of amylose and amylopectin. Amylose is essentially a near-linear molecule composed of 1, 4 linked α-D- glucan chains. Amylopectin, on the other hand, is a regularly branched larger polymer with α-1, 6- branch points (Jeon *et al*., 2010) [10]. The position and frequency of α-1, 6- branch points accounts for the water insoluble nature of starch. The exact proportion of both the components of starch, shape and size of starch granules vary between species and between the organs of the same plant. This diversity in both composition and physical parameters give rise to their varying properties and application.
Starch biosynthesis in amyloplast of cereal endosperm involves biosynthesis of soluble precursor, followed by coordinated reactions of α-(1→4) - linked glucan chain elongation, α-(1→6) branching and debranching of specific branch linkages.

Synthesis of precursor adenosine 5' diphosphate-glucose (ADP-Glc) from adenosine 5' triphosphate (ATP) and glucose-1-phosphate is catalyzed by ADP-Glc pyrophosphorylase (AGPase). α-(1→4)- linked glucan chain elongation is catalyzed by ADP-Glc-dependent transferases, known as starch synthases (SS), α-(1→6)-linked branch points formed by starch branching enzymes (SBE) and debranching is catalyzed by debranching enzymes (DBE). Each of these enzyme classes is divided into different subunits and isoforms. In cereals, some enzymes are characterized by further sub-division into different tissue specific isoforms and in some cases with different subcellular compartmentalization (Tetlow *et al*., 2011) [23]. In the present review, distinct roles of each of these enzymes in cereal endosperm is summarized

ADP glucose pyrophosphorylase: The first committed step in starch biosynthesis

AGPases catalyzes the the first committed step of starch biosynthetic pathway present in all higher plants that form ADP-Glc and pyrophosphate (PPi) from Glc-1-P and ATP. Hence, it plays an important role in regulating carbon flux from imported sucrose to amyloplast synthesized starch in developing endosperm. Plant AGPases is a heterotetramer ($\alpha_2\beta_2$) made up of two large (LSU) and two small (SSU) subunits with slightly different molecular masses (Tetlow *et al*., 2004) [22]. SSUs are responsible for catalytic activity, whereas LSU modulate the regulatory property of enzyme that increases the allosteric response of SSU to 3-phosphoglyceric acid (3-PGA) and inorganic phosphate (Ballicora *et al*., 2005) [1]. In cereals, a significant proportion of AGPases is located in cytosol and a minor portion is found in amyloplast. Rice AGPase gene family consists of four LSU, OsAGPL1, OsAGPL2, OsAGPL3 and OsAGPL1 and two SSU genes OsAGPS1 and OsAGPS2. Experimental evidences related to subcellular localization suggests that only OsAGPS2b and OsAGPL2 are localized in cytosol, whereas other proteins are localized in plastid. The AGPases catalyzed reaction is reversible and is in equilibrium. For the formation of starch, ADP-Glc formation is favoured by the removal of PPi. Formation of ADP-Glc by cytosolic AGPases is driven by PPi consuming reactions, while formation of ADP-Glc in plastid is driven by cleavage of PPi by pyrophosphatase (Gross *et al*., 1986) [8]. Consequently, precise mode of action of cytosolic AGPases is a debatable question. Plastidial AGPase is subjected to allosteric regulation by 3-phosphoglycerate (activator) and inorganic phosphate (Pi, inhibitor) as well as redox activation via reduction of intermolecular disulphide bridge between the conserved cysteine residues of small subunits (Tiessen *et al*., 2002) [24].

Starch synthase mediated elongation of α-chains

Starch synthase (SS) belongs to glucosyltransferase family of enzymes that catalyze the transfer of glucosyl unit of ADP-Glc to the non-reducing end of a glucan chain. ADP formed during this reaction in amyloplast is shipped in counter exchange for cytosolic ADP via BT1 transporter. Various isoforms of starch synthases identified in cereal endosperm include granule bound starch synthase (GBSS), SSI, SSII, SSIII and SSIV (James *et al*., 2003) [11]. In rice 10 starch synthase isoforms has been identified (Hirose *et al*., 2004) [10].

Granule- bond starch synthase

GBSS exists in two isoforms viz. GBSSI which is restricted to storage tissues (seed endosperm) and GBSSII which is restricted to non-storage plant tissues where transitory starch accumulates. GBSSI is encoded by the waxy (wx) locus in cereal endosperm. GBSSI is so named due to its exclusive localization within granule matrix and is involved in the synthesis of amylose component of starch. Amylose elongation via GBSSI is mediated by the addition of Glc from ADP-Glc leading to formation of linear polymer. In addition, GBSSI is also involved in extension of long glucans of amylopectin.

Soluble starch synthase

Based on the results of expression studies of soluble starch synthase in rice endosperm, these enzymes are classified into three groups: early, late and steady expressers. Early expressors in rice seed include *SSIIb* and *SSIIIb* at pre storage phase of endosperm development and late expressors include *SSIIa* and *SSIIIa* that are abundant during starch biosynthesis at later stage (Ohdan *et al*., 2005) [8].

Starch synthase I

Biochemical studies conducted so far with cereal endosperm revealed that SSI and SSII are the major enzymes in cereal endosperm involved in starch biosynthesis. SSI accounts for 70% of total SS activity (Fujita *et al*., 2006) [7]. SSI utilizes α-glucan chains of degree of polymerization (DP) 6-7 as substrates to produce products with DP 8-12 (Commuri *et al*., 2001) [4]. Experimental evidences from barley endosperm revealed that it shows no affinity towards maltotriose and maltotetraose as substrate (Wilkens *et al*., 2014) [26]. RNAi mediated suppression of SSI in wheat resulted in accumulation of very short chains (DP 6-7), significant reduction in amylose content and altered granule morphology (Mcmaugh *et al*., 2014) [15]. The products of SSI act as a substrate for SSII.

Starch synthase II

SSIIa is the product of *sugary2* gene and is involved in the formation of intermediate length glucan-chains. Its mutation results in accumulation of short chains of DP 6-10 and reduced accumulation of chains of DP 12-30 (Zhang *et al*., 2004) [29]. Despite being accounting for minor portion of total SS activity, the loss of SSIIa results in major effects on starch structure, reduced crystallinity, altered granule morphology and rise in amylose content (Cao *et al*., 1999) [3]. Cooking quality of rice grain can be assessed by soaking polished rice grains in solutions of 4 M urea or 1.3M KOH to evaluate the disintegration of endosperm localized starch. This disintegration of rice grains correlates with gelatinization temperature which influences its cooking quality. Major gene controlling this trait is *alk* (alkali disintegration)/ *gel* (gelatinization). Starch granule of indica cultivars is more resistant compared to japonica which degrades with ease. Structurally amylopectin is classified as L- or S- type in rice and are predominant in *indica* and *japonica* cultivars respectively. S-type of amylopectin has a higher proportion of short chains of DP 10 relative to that of L-type. Thus amylopectin of *japonica* cultivars has a higher proportion of short chains resulting in lower gelatinization temperature. Further genetic mapping studies conducted so far showed a tight linkage between *SSIIa* gene and *alk* locus (Nakamura *et al*., 2002) [24]. At protein levels, SSIIa is present in higher amount in *indica* compared to *japonica* cultivars that converts amylopectin from S-type to L-type by forming chains of DP 13-25 utilizing short chains of DP < 11 (Nakamura *et al*., 2005) [16].

Starch synthase III

SSIII is second only to that of SSI in terms of total activity of SS in cereal endosperm such as rice, maize and barley (Li *et al*., 2011) [14]. It has two tissue specific isoforms viz. SSIIIa and SSIIIb found in endosperm and leaf respectively (Dian *et al*., 2005) [5]. Primary role of this enzyme is the synthesis of amylopectin chains of DP ≥30 utilizing intermediate chains as substrate. Maize endosperm with mutation in SSIII produces endosperm with dull and glassy appearance and thus named as dull 1 mutant. Mutants lacking SSIII showed reduction in long glucan chains, altered granule morphology and crystallinity.

Starch synthase IV

Most recently discovered form of SSs is SSIV and is phylogenetically related to SSIII (Dian *et al*., 2005) [5]. It exists in two isoforms viz. SSIVa and SSIVb localized in endosperm and leaf respectively (Leterrier *et al*., 2008) [13]. Mutant studies conducted so far establishes its role in priming of starch granule formation and determination of starch granule number.

Branching enzyme

Position and frequency of branch points in amylopectin diversify higher plant starches contributing towards its water insoluble nature and varied structural characteristics. Branching enzyme catalyzes the formation of branch points by hydrolytic cleavage of α-1, 4 linkage in polyglucan and transfer of released reducing ends to C6 hydroxyls to form α-1,6 glucan linkage (Drummond *et al*.,. 1972) [6]. Based on biochemical and physicochemical properties, two classes of branching enzymes (BE) exist in cereals namely BEI and BEII. Cereals possess two distinct isoforms of BEII: BEIIa and BEIIb.

Branching enzyme I

BEI preferentially transfer longer glucan chains (upto DP 30, majority being DP 10-13) and has a high affinity for amylose as a substrate. BEI exists as a single isoform and predominantly expressed in the developing endosperm. Phylogenetic analysis revealed that it evolved prior to monocot-dicot diveregence and its retention in plants indicates its requirement for starch synthesis and plant fitness (Guan *et al*., 1997) [9].

Branching enzyme II

BEIIb specifically expresses in cereal endosperm whereas BEIIa is ubiquitously present (Yamanouchi *et al*., 1992) [27]. BEIIa mutations in rice and maize showed no significant changes in amylopectin chain profiles suggesting it plays a supporting role to other BE isoforms. Mutation in maize BEIIb, *amylose extender* (*ae*) produces a high amylose starch (Yun *et al*., 1993) [18]. In wheat suppression of both the isoforms produces high amylose starch. Double mutant of rice (deficit in BEIIb/GBSSI) lacks amylose, produces very few short chain DP ≤17 and greatly produces elongated chains. Similar data has been reported in rice having BEIIb mutation. Thus, BEIIb plays a role in formation of amylopectin A chains. Also, BEIIb influences physicochemical properties of starch granules and it becomes more resistant to gelatinization.

Debranching enzymes

DBE belong to α-amylase "super family" of enzymes and has a starch binding domain. DBE has two classes: isoamylase (ISA) and pullulanase (PUL). ISA mainly debranches amylopectin and phytoglycogen whereas PUL acts upon pullulan and amylopectin. ISA and PUL has three and one isoforms present in plants.

Isoamylase

Isoamylase plays a major role in editing excessively branched or in eliminating improper branches of amylopectin produced by branching enzymes.

Pullulanase

In contrast to isoamylase, physiological function of PUL is well defined. Significant PUL activity has been detected in developing cereal endosperm in addition to its function in starch degradation during seed germination (Beatty *et al*., 1999) [2]. Though PUL is expressed during the entire seed development period, peak activity is detected in middle and later stages in rice endosperm suggesting its involvement in starch synthesis.

Starch phosphorylase

Two types of starch phosphorylase are present in plants: Pho1 and Pho2 located in plastid and cytosol respectively. It catalyzes the transfer of glucosyl units from Glc-1-P to the non-reducing end of α-1,4-linked glucan chains producing Pi (biosynthetic reaction) or utilization of Pi to produce Glc-1-P from the removal of Glc from an α-1,4-linked glucan chain (degradative reaction). Cytosolic SP metabolises α-glucans that are resulting from degradation of starch and hence not involved in starch biosynthesis. SP is present in all starch synthesizing tissue and its expression correlates with starch accumulation in developing endosperm. Mutants lacking Pho1 shows altered characteristics of starch granule and shrunken endosperm when plants were subjected to lower temperature indicating its role in accumulation of starch under different environmental conditions (Satoh *et al*., 2008) [20].

Conclusion

Starch biosynthetic pathway is a key metabolic pathway in cereal endosperm that forms the basis of yield and quality characteristics in the most important crops of the world. Rapid advancement in starch biosynthetic pathway beyond basic reaction in the identification of regulatory elements and function of specific isoforms of each enzyme involved will open up potential targets for breeding purposes.

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