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Molecular Assembly of Starch Granules: Interplay of Metabolizing Enzymes in Rice

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Abstract

Starch is of fundamental importance both for plant development and for energy needs upon consumption. Molecular assembly of starch granules is an important event controlling not only starch metabolism but also affect protein and lipid metabolism. Restructuring of starch granule in planta therefore affect plant metabolism. Great progress have been made by studying both crop and model systems and we approach the point of knowing the enzymatic machinery responsible for creating the massive, insoluble starch granules. Micro-structure of starch granule has been modulated by harmonising metabolic enzymes and their intermediates. Here, we summarize our current understanding of starch biosynthetic enzymes and their role in starch packing. The functional aspects of differently assembled intermediates have also been discussed. We flag-up recent observations suggesting a significant degree of flexibility during the synthesis of starch and its packing, which is contributed by enzymatic (starch synthases, branching and de-branching) as well as non-enzymatic cell matrix components (lipids, proteins, phenolics, phosphate monoesters etc). We conclude that novel experimental, both at molecular and biochemical levels along with theoretical approaches will be important to understand metabolic adjustments and redirection, tuning to various types of starch with different functional applications.

Key words: Resistant starch, starch packing, starch branching enzymes, debranching enzymes, retrograde starch.

Introduction

Starch is composed of two polymers of glucose, amylose and amylopectin and is stored in the plastid (chloroplast in leaves, amyloplasts in non-photosynthetic tissues) as insoluble, semi-crystalline granules. Starch, which is the major dietary source of carbohydrates, is the most abundant storage polysaccharide in plants, and occurs as granules in the chloroplast of green leaves and the amyloplast of seeds, pulses, and tubers (Ellis *et al.,* 1998). Starch either can accumulate during the day-time photosynthesis as "transitory starch" or can accumulate in non-photosynthetic tissues and heterotrophic organs (e.g. seeds, roots and tubers) as "storage starch". Metabolic pathways regulating starch synthesis and assembly have been extensively discussed (Zeeman *et al.,* 2010). Transcriptional expression of genes involved in starch metabolism have been investigated and found to be modulated by metabolic intermediates (Tetlow *et al.,* 2004).

Starch has multiple diverse applications from industrial to health-related benefits. The structure of starch is important for effective mobilization during the grain germination process. Starch metabolism has been comprehensively characterized in rice. There are many varieties of rice grain in the world, which vary considerably in their starch accumulation, and metabolising activity. Starch polymer, amylose is a linear and relatively short polymer of glucose units linked by a (1 → 4) bonds. Amylopectin is a branched and longer polymer where glucose units are arranged linearly through a (1→4), with branches emerging via a (1→6) bonds occurring every twenty-four to thirty glucose units (Shaik *et al.,* 2014). It is well known that starch with a higher amount of amylose is more resistant to digestion (Tetlow, 2011). In addition to the amylose content, cooking and cooling processes can influence starch digestibility. The process and degree of gelatinisation affect retrogradation of rice starch. Gelatinisation is the irreversible collapse of molecular order (breaking of H bonds) within the starch granule, leading to starch solubilisation during hydrothermal treatment. This affects granular properties like swelling and crystal melting (Atwell *et al.,* 1988). This leads to the higher starch availability to human digestive enzymes (Tester and Sommerville, 2003).

A type of starch got recent recognition due to its slow / incomplete digestion which doesn't contribute to blood sugar spike and act as a matrix for fermentation by helping microbiome to grow. This non-digestible starch fraction is known as resistant starch (RS) (Englyst *et al.,* 1992).

The concept regarding digestion of RS has evoked new interest in the bioavailability of starch and in its use as a source of dietary fiber with immense health benefits but its mechanism of synthesis as well as structure has not been explored yet. Resistant starch is mainly of five types – RS1, RS2, RS3, RS4 and RS5. RS1 represents starch that is resistant because it is in a physically inaccessible form such as partly milled grains and seeds and in some very dense types of processed starchy foods. RS2 represents starch that is in a certain granular form and resistant to enzyme digestion mainly due to tight packaging at granular level and relatively dehydrated. RS3 represents the most abundant fraction and is mainly retrograded amylose formed during cooling of gelatinized starch. RS4 is a type of RS where novel chemical bonds other than (1→4) or (1→6) are formed and can be obtained by various types of chemical treatments. RS5 is a complex with lipids, which resist amylolysis (Figure 1).

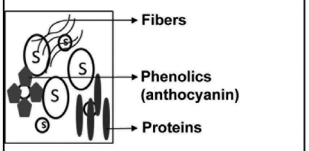
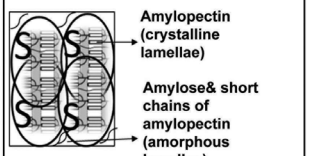
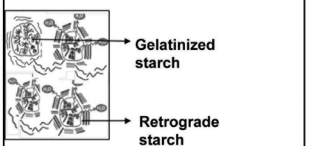
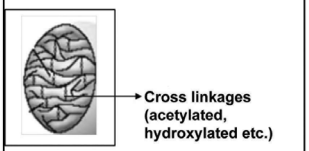
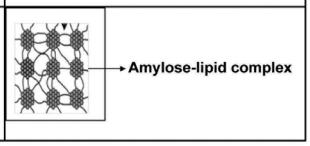
RS type	Description	Example	Putative Structure
RS1	Physically inaccessible starch	Coarsely ground or whole-kernel grains	 Fibers; Phenolics (anthocyanin); Proteins
RS2	Packed crystalline structure	High-amylose starch	 Amylopectin (crystalline lamellae); Amylose& short chains of amylopectin (amorphous lamellae)
RS3	Retrograded starch	Cooked and cooled starchy foods like rice	 Gelatinized starch; Retrograde starch
RS4	Chemically modified starch	Cross-linked starch (acetylated, hydroxylated, octenyl succinate starch etc.)	 Cross linkages (acetylated, hydroxylated etc.)
RS5	Amylose-lipid complex	Steric acid-complexed high-amylose starch	 Amylose-lipid complex

Figure 1. Resistant starch types and their putative structures

In this pursuit, authors have summarized the current understanding of starch biosynthetic enzymes and their role in starch packing behind the functional aspects of starch. The existing reports suggesting the role of enzymes as well as other cellular matrix components towards imparting flexibility and resistance during digestion, has been highlighted in this review.

Variation in RS content – possible factors

A great source of variation has been observed in RS content among the germplasm due to genetic, environmental and mutation effects. Deciphering the molecular switches, which turn normal starch to digestion resistant phenotype, is potential for future genetic engineering. Starch structure varies with their genetic origin. Additional genetic variation occurs within the genotype due to allelic variation in starch biosynthesis, branching and de-branching enzyme genes. Even though commercial rice varieties, like basmati there is little variation in RS levels (<2%), but the wild and pigmented germplasm contains substantial variation in RS content (Birt *et al.,* 2013). The field environmental conditions have an impact on starch biogenesis as well as packing, which determines the starch digestibility. It might be possibly due to the altering of the activity of various starch enzymes by temperature and moisture. Environmental variation in RS content is difficult to predict and control; therefore, it has not been used as a tool for increasing RS levels. Mutations at the loci's of starch synthases as well as branching enzymes had resulted in starch with higher apparent amylose content or long branched chain of amylopectin which resulted with more percentage of digestion RS compared to normal germplasm. These variations delivered some valuable insights on the role of various enzymes (synthase, branching, debranching and other) during granule biogenesis in coordination to environmental stimuli, which result in tight crystalline packaged structure contributed by a hierarchical intricate organization at different levels.

Starch structure and packing

Rice has polyhedral starch granules with varying size of 3-8μm in diameter. Despite these differences, microscopic analysis of granule structure has shown some features, which appear to be constant. All granules have been shown to exhibit concentric sphere morphology, with alternating semi-crystalline and amorphous growth shells. The levels of starch organization include glucose strings, which form the basic components – amylose and amylopectin. Later it attains a double helical structure and further organize to form lamellae, lamellae combines to form super helices, which further form as blockets and growth rings, which made up to form a starch granule (Figure 2).

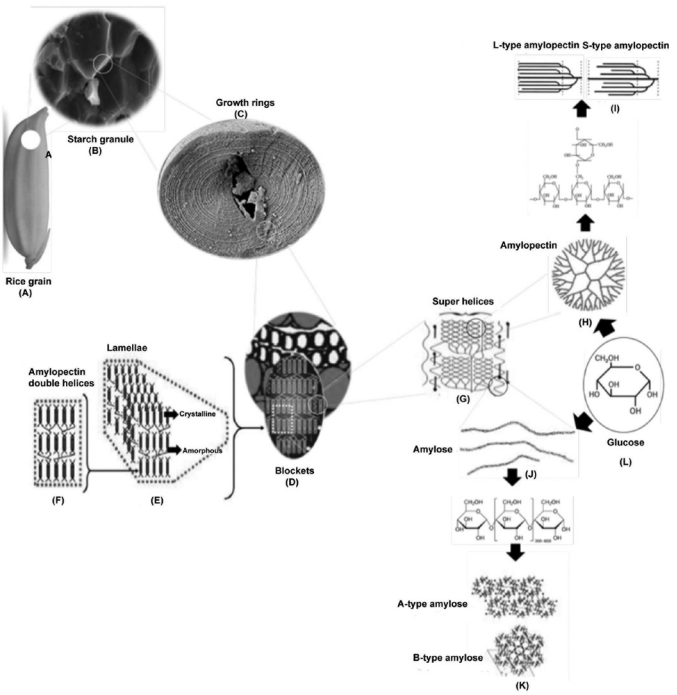


Figure 2. Schematic representation of starch hierarchical organization

(A) Starch accumulate in the endosperm of rice grain (B) Scanning electron micrograph of starch granule (C) Starch granule with growth rings radially organized and extending from hilum (D) Blockets, the small units of granules. Blockets consist of super helices comprising (E) Crystalline and (F) amorphous lamellae formed by double helices and branched segments of amylopectin (G) Double helices and branched fragment along with amylose form super helices (H) Glucosyl units showing α-(1,4)- and α-(1,6)-linkages known as amylopectin (I) Two types of amylopectins differing in branching pattern – S & L type (J) Glucosyl units showing α-(1,4) form linear single strand helices known as amylose (K) Amylose exist in two allomorphs – A&B type (L) Glucose, monomeric unit of starch synthesis.

Structural peculiarities are translated to physiological variations in digestibility and hence there are majorly three types of starch– rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS). RDS is a rapid source of energy, while SDS provides slow and sustained energy release. RS is of great interest as it belongs to special class of dietary fiber, which enriches the gut microbiome.

Despite similar organizational hierarchy, it has been observed that RS2 granules have some structural peculiarities. They contain tiny pores or channels that extend from the granule surface into the interior parts. The number and diameter of these external openings vary among the genotypes and these act as the target place for amylolytic enzymes that enter and enlarge the pores into tunnels during digestion. The variation in RS due to environmental and mutations of various enzymes also throw light into the starch packing difference in normal vs. RS granules. Amylose-amylopectin chains are crystallized in a hexagonal lattice (B allomorph), where they pack as an array of left-handed parallel-stranded double helices fashion, which in turn form the lamellae. Short chains of amylopectin have reduced tendency to form double helical structure, less crystallite thus easily digestible, while long chains exert more molecular order and optimum packing. Such tightly packed helices contribute to the crystalline lamella and loosely packed contributed by short chains contribute to amorphous lamellar regions. Although both these amorphous and crystalline regions are arranged concentrically, crystalline regions contributed by tight packing are resistant to digestion. The presence of higher amylose also contributes to RS3 during processing, as its recrystallization on cooling occurs rapidly than amylopectin. Re-association into tight crystalline, termed as 'retrogradation' reduces starch digestibility. Higher percentage of RS1 especially in exotic, wild or pigmented germ plasm might be possibly due to the presence of various minor components, which make starch inaccessible.

Phenolics like anthocyanins, tannins, catechins have found to play role in physically trapping starch granules reducing its digestibility. Starch phosphate monoesters in native starch are essentially found along with amylopectin, which also possibly a matrix component contributing RS1 other than major components like fibers and proteins. Lipids are also found in low amounts (up to 1.5%) in much starch, especially cereal starch, in the form of free fatty acids and lysophospholipids. The lipids in cereals are associated with the amylose fraction. In these plants, the proportion of lipid-complexed amylose varies from 13 to 43%, known as RS5, an variant having resistance to amylolysis due to structural differences. Other than the components in the matrices, which make them inaccessible for enzymatic digestion, the most critical factor is the tight packing by the interplay of various key enzymes which play in coordination during starch granule biogenesis resulting in varied proportion of RS.

Allelic diversity in starch biosynthesis

Starches with higher amylose content or with longer-branched amylopectin have been reported to have higher tendency towards crystalline packing which rapidly retrograde and thus slow down the enzymatic degradation in the digestive track (RS2 & RS3). The ratio of amylose to amylopectin being a critical parameter contributing towards RS, various enzymes have been contributing in this process (Figure 3).

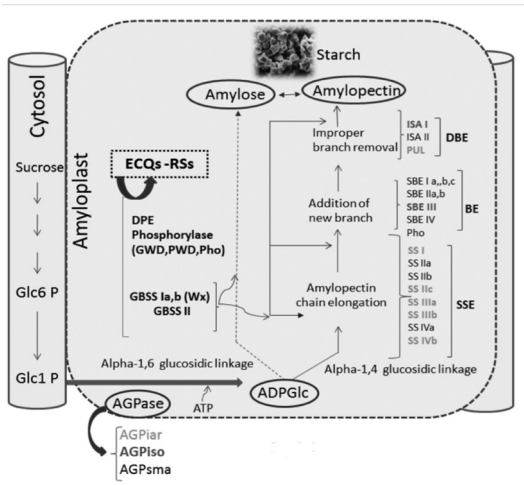


Figure 3. Integration of starch biosynthesis pathway genes (alleles & isoforms) to starch packing (ECQ-RS) involved in or plays distinct roles in different steps of starch synthesis.

Dotted lines shows amylose synthesis pathway genes, solid lines shows amylopectin synthesis genes, genes in different colour codes and bold letters are involved in rice starch grain ECQs and RS types (AC-green, GC-purple, and GT- orange and GP-black). ECQ, eating and cooking quality; RS, resistant starch; AC, amylose content, GC, gel consistency; GT, gelatinization temperature; GB-granular packing; AGP, ADP-glucose pyrophosphorylase; AGPlar, AGP large subunit; AGPiso, AGP large subunit isoform; AGPsma, AGP small subunit; GBSS, granule-bound starch synthase; Wx, waxy gene; SSE, soluble starch synthase enzymes; SBE, starch branching enzyme; Pho, plastidial phosphorylase; belong to branching enzymes (BE) as well to GP; ISA, isoamylase; PUL, pullulanase; ISA and PUL belong to starch debranching enzyme (DBE); GWD, glucan water dikinase; PWD, phosphoglucan water dikinase; DPE, Dis-proportionating enzyme belongs to ECQs and RSs.

Amylose is synthesized by ADP glucose pyrophosphorylase (AGPase) and granule-bound starch synthase I (GBSSI), whereas amylopectin is synthesized by concerted reactions catalyzed by AGPase, soluble starch synthase (SS), starch-branching enzyme (BE) and starch-debranching enzyme (DBE). AGPase catalyzes the first reaction in starch synthesis, producing the activated glucosyl donor ADP-glucose(Glc). GBSSI and SSs act specifically to elongate amylose and amylopectin, respectively. Higher GBSS activity contributes for RS3, as higher amylose content retrograde faster. The enzyme GBSS has also been reported to play role in synthesizing 'super-long' chains in amylopectin (Hanashiro *et al.,* 2008).

Branching enzymes (BEs) and DBEs, which extend and trims the glucose strings critically determines the chain length as well as the packing of granules generating RS2. BEs are involved in generating alpha-1,6 glycoside bonds by cleaving internal alpha-1,4 glycoside bond and transferring the released reducing ends to C6 hydroxyls, thereby forming a new branched chain. Degree of polymerisation (DP); DP>33 – Long chains; 13<DP<33 – Intermediate chains; <13 – Short chains produced by BEs along with DBEs like isoamylase (ISO) and pullulanase (PUL), play role in efficient tight packed structure. Simultaneously, starch degradation is initiated by the addition of phosphate groups at the C6-position and C3-position of individual glucosyl residues that act to disrupt the packing of the glucans at the granule surface. Two enzymes, glucan water dikinase (GWD) and phosphoglucan water dikinase (PWD) respectively, catalyze these phosphate additions. The hydrolysis of the resulting glucan and phosphoglucan chains is carried out by a set of enzymes including the phosphoglucan phosphatases, β-amylases, DBE; ISA3, α-amylase (AMY3), α-glucanphosphorylase and the disproportionating enzyme 1 (D-enzyme 1; an α-1,4-glucanotransferase). In the cytosol glucose is converted to substrates for either sucrose synthesis, glycolysis or the oxidative pentose phosphate pathway by a number of enzymes including the disproportionating enzyme 2 (D-enzyme 2; an α-1,4-glucanotransferase), α-glucanphosphorylase, hexokinase and phosphoglucomutase. DBEs hydrolyze 1,6-glycoside bonds and play an essential role in the formation of amylopectin. Additionally, a-glucanphosphorylase (Pho) is involved in storage starch synthesis. In rice, the loss of plastidialphosphorylase (Pho1) causes smaller sized starch granules to accumulate and modifies the amylopectin structure, resulting in abnormal endosperm phenotypes, such as white core, shrunken and pseudonormal endosperms (Satoh *et al.,* 2008). Pho1 may play an important role in the glucan initiation process by synthesizing glucan primers from short-chain malto oligosaccharides (MOSs; Hwang *et al.,* 2010).

Dis-proportionating enzyme (DPE) is an α-1,4-glucanotransferase that catalyzes the cleavage of α-1,4-glucosidic bonds of glucans, transferring the glucosyl groups to the non reducing end of another glucan or free glucose(Glc) and releasing Glc or aglucan chain, depending on the cleavage site. Different isoforms of starch-synthesizing enzymes control amylose and amylopectin content in rice, which in turn greatly influence rice cooking, eating and textural quality. Activity of one or more isoforms of starch synthesizing enzymes results in various forms of starch structure based on the amylopectin chain length and average external, internal and core chain length distribution and hence results in varying physicochemical and cooking quality (Pandey *et al.,* 2012). Genome of rice consists of at least 27 genes encoding starch-biosynthesizing enzymes dispersed in 7 groups based on their isoforms, six for AGPase, two for GBSSI (Waxy gene), eight for SS, three for BE, four for DBE, two for Pho and two for D-enzyme (Ohdan *et al.,* 2005). Among them, the functions of the former six groups have been well studied; however, not much is known about the exact role of DPE1 in starch metabolism in developing rice endosperm, although endosperm-specific over-expression or suppression of DPE1 affected the amylose content, starch structure and morphological and physicochemical properties of starch granules. The activities of other major starch synthesizing enzymes were not found changed in DPE1-overexpressed or suppressed seeds. DPE1 and a-1, 4-D-glucanotransferase, has been thought to be involved in storage starch synthesis in cereal crops. However, the precise function of DPE1 remains to be established. DPE1 over expression decreased amylose content and resulted in small and tightly packed starch granules, whereas DPE1 suppression increased amylose content and formed heterogeneous-sized, spherical, and loosely packed starch granules (Dong *et al.,* 2015).

The reduction of SBEIIb activity is another route to increase amylose content. However, much higher amylose contents have been obtained in rice by targeting SBEIIa, SBEIIb and SBEI, (Fasahat *et al*. 2014) as well amylopectin-synthesizing enzymes, such as SSIIIa, SSIIa, SSIVb, BEI, BEIIb, and PUL have also been found to target amylopectin synthesis simultaneously (Ordonioa and Matsuokab, 2016). Branching enzyme proteins consist of three common characteristic domains, carbohydrate-binding, catalytic amylase and a-amylase C-terminal domain (Pfister and Zeeman, 2016). These genes/enzymes both ways, either directly or indirectly (via synthesis of amylase, amylopectin and grain packing) contributes to the variation in the granular structure of starch grain through modulations in their RS content (Fig. 2).

A link between starch synthesis genes and starch digestion properties is well established. GBSSI (waxy) is primarily responsible for the linear chains of glucose molecules i.e amylose content. A number of SNPs in the rice waxy gene are found to impact starch cooking quality and starch grain texture (Kharabian-Masouleh *et al.,* 2012). There are two types of cultivated Asian rice-*indica* and *japonica*. Grains of *indica* rice generally contain higher amylose than *japonica* and this makes a good basis for distinguishing them from each other. The type of Waxy gene that sets them apart is, *indica* rice has the fully active wild-type allele (Wxa), whereas *japonica* rice has the intermediate one (Wxb). Zhou *et al*. (2016) found that the RS level is different between *indica* and *japonica* and such difference depends on their Wx alleles. Some studies also reported that higher amylose content increases RS level and thus it is not surprising for the Wxb allele in *japonica* to cause lower RS content relative to that in *indica* rice. According to Fujita *et al*. (2007) ssIIIa mutation causes 1.4- to 1.8-fold increase in GBSS1 and 1.3- fold increase in the amylose content of *japonica* rice, which is good in terms of being able to increase RS in *japonica*. Crofts *et al*. (2012) introduced the *indica*Wx allele into *japonica* rice carrying either SSIIIa or ssIIIa. The amount of GBSS1 has been found to be increased in the *japonica* rice containing SSIIIa allele. In *japonica* rice containing ssIIIa allele, the amylose content was higher but not much difference in Wx expression, probably because the GBSS1 level was already maximal. Zhou *et al*. (2016) suggested that the interaction between SSIIIa and GBSS1 is likely to occur at the post translational stage. Considering the nutraceutical properties of phenolics present in pigmented rice varieties, three QTLs viz., qPC1.1, qPC11.1, and qPC11.2 were associated with phenolic content (PC) of brown rice (Qin *et al*. 2009). Zhong *et al.* (2011) reported two consistent QTLs for PC in milled rice as qPr1 and qPr7 on chromosome 1 and 7 respectively. Rc and Rd locus regulates pigmentation where Rc encodes for a regulatory BHLH protein that allows accumulation of proanthocyanidins (Sweeney *et al.,* 2006; Furukawa *et al.,* 2007); Rd codes for DFR (Dihydroflavanol reductase) which encodes anthocyanins. Understanding of the role of these genes led to manipulating this complex pathway for better quality starch.

Manipulation of starch metabolism for improving RS content – Breeding to genome editing

Until now, classical breeding had a significant impact on improving the starch quality of rice cultivars by making crosses, back crosses and selection of high amylose or high RS cultivars. For identifying this desired property, two general approaches have been followed. Either directly screening the phenotype, which requires a rapid screening strategy to identify variants or through identifying mutations in the genes that are known to influence RS content. The amount of amylose content (AC), waxy haplotype and the digestibility of rice has been significantly correlated (Kharabian-Masouleh *et al.,* 2012) and hence an initial step in this direction is to screen the variation in AC content. Analyses of the ACs of a set of germplasm collected by the International Rice Research Institute showed that AC in wild and cultivated rice ranges from 0 to 30% depending on the rice variety (Butardo *et al.,* 2008). Apparent AC is primarily controlled by the Waxy gene, which codes for GBSS (Chen *et al*. 2008a). Even though GBSSI synthesizes amylose and high amylose content is correlated to RS, over-production of GBSS-I by mutations did not result with high RS. GBSS has also known to contribute towards starch retrogradation properties, which yield type 3RS. Other genes that have reported to contribute in retrogradation were glucose-6-phosphate translocator 1, SSI, SBEI and SSIIIa. Chen *et al* (2008a, b) reported that the waxy gene showed 4 haplotypes - viz., In1T-Ex6A, In1GEx6C, In1G-Ex6A and In1T-Ex6C used for the classification of AC in rice. Angwara *et al.* (2014) characterized 26 Thai rice varieties for RAG and Waxy haplotype (In1-Ex6) as glycemic index indicators. Zeng *et al.,* 2016 identified QTLs of RS for rice (qRS7-1, qRS7-2) on chromosome 7. Other than altering amylose content, it is also possible that alterations in amylopectin structure, for example the production of highly branched molecules that inhibit the access of alpha amylase to its 1, 4-linked substrate might increase RS but this has not yet been demonstrated. Many mutants with elevated RS content have been identified in rice, including Goami 2, RS111,and Jiangtangdao 1 (Yang *et al.,* 2012). Discovery of QTLs associated with RS will assist for future fine mapping or pinpointing more functional genes, which can be manipulated using reverse genetic approaches.

Lower activity of ADP-Glc pyro phosphorylase and SBEs, and higher activity of SS and SDBEs has observed in high-RS rice, which might be responsible for the formation of small, irregular starch granules with large spaces (Shu and Rasmussen, 2014). However, morphologically different, starches have been identified in some rice mutants due to gradual decrease in enzymatic expression of SBE responsible for the formation of heterogenous starch granules (Wang *et al.,* 2018).Considering the immense role of starch biosynthetic enzymes; they have been manipulated for increasing RS content using various genomic strategies. SSI mutants, produced by the insertion of the Tos-17 retro transposon into the gene for rice SSI has been described in rice. Mutants with an altered structure where the proportion of short chains (DP6–7) and long chains (DP16–19) were increased and the chains of DP8–DP15 were reduced have been observed. A map based cloning of a RS locus in *indica* rice identified a defective soluble starch synthase gene (SSIIIa) responsible for RS production in b10 mutant (Zhou *et al.,* 2016). Teqing resistant starch (TRS) is another high amylose and RS transgenic line developed by modifying antisense RNA inhibition for SBE in rice. In high-amylose TRS rice, the C-type starch, which might result from the combination of both A-type and B-type starch, was observed and subsequently confirmed by multiple physical techniques, including X-ray powder diffraction, solid-state nuclear magnetic resonance and fourier transform infrared.

Induced mutations in SBEII were phenotyped with high amylose and with increased RS content (Hazard *et al.,* 2012). In rice, high amylose sbeIIb mutants were generated by means of chemical treatment or radiation (Shu *et al.,* 2006), or through hairpin RNA (hp-RNA) mediated RNA interference (RNAi) (Butardo *et al.,* 2011). Putative gene sbe3-rs for RS mutated from SBE3 for starch branching enzyme in Rice (*Oryza sativa* L.) has been reported as a marker gene for RS content (Yang *et al.,* 2012). In rice, both the sugary1 (sug1) locus and the ISA1 gene are located on chromosome 8 (Fujita *et al.,* 1999), while the PUL gene is located on chromosome 4. The reduction of ISA1 activity to about only 6% by using antisense technology resulted in modified amylopectin with more abundant short side chains and increased accumulation of soluble a-glucans (Fujita *et al.,* 2003). In transgenic rice generated by the introduction of the wheat ISA1 gene into sug1 rice, phytoglycogen synthesis was substantially replaced by starch synthesis in the endosperm (Kubo *et al.,* 2005). These reports strongly suggest that sugary1 mutations in maize and rice are caused by ISA1 deficiency and ISA1 plays a crucial role in amylopectin biosynthesis. Generation of high-amylose rice through CRISPR/Cas9-mediated targeted mutagenesis of SBEI and SBEIIb reported with 25% increase in AC and 9.8% RS content (Sun *et al.,* 2017). Recently a novel class

of regulators of starch metabolism have been described. These proteins, known as water di-kinases, are involved in starch degradation through control of phosphorylation of C3 and C6 positions of glucose in the leaves of the model plant Arabidopsis and lead to the starch excess phenotype in leaves (Ritte *et al.,* 2004). Modulation of granule micro-structure achieved by decreasing starch branching and increasing starch-bound phosphate content in the barley caryopsis starch by RNAi suppression of all three Starch Branching Enzyme (SBE) isoforms or over expression of potato GlucanWater Dikinase (GWD) resulted Amylose-Only (AO) and Hyper Phosphorylated (HP) starch chemotypes. Such AO lines explained how re-direction of carbon partitioning occurs in starch grain (Shaik *et al.,* 2016).

Wild pigmented rice has been found to be effective in improving abnormal glucose metabolism by having higher proportion of digestion RS as well as due to the presence of antioxidants like anthocyanins (Han and Huang, 2013). Previous studies have demonstrated that food derived active ingredients such as phenolic compounds can control blood glucose level (Thondre *et al.,* 2013). In addition, fortification with anthocyanins from plant sources effectively reduced the digestion rate of bread (Sui *et al.,* 2016). Bae *et al.,* (2017) reported the role of anthocyanins in inhibiting digestive enzymes and thus suppressing starch hydrolysis under *in vitro* conditions. Anti-glucosidase activity of anthocyanins has been reported (McDougall *et al.,* 2005). Yao *et al*. (2010) found that anthocyanins isolated from black rice inhibited alpha-glucosidase. In addition, in an animal model of T2DM, treatment with an anthocyanin (cyanidin 3-glucoside) significantly reduced blood glucose concentration and improved insulin sensitivity after an insulin tolerance test in male mice. Through classical genetic approaches, Yoshimura *et al* (1997) identified pb and pp loci on chromosome 4 and 1 for pericarp pigmentation in black rice. Further, by association mapping Shao *et al.,* 2011 reported RM339 and RM316 as common markers for antioxidants, flavonoids and phenolics. These can assist breeders in developing neutraceutically rich varieties with improved RS content.

Conclusion

Digestion-resistant starches are type of functional starch with immense health benefits but its process of synthesis and organization is still a mystery. Environmental and genetic factors that affect starch resistance in crops are being identified, including using biotechnology to control starch digestibility. A myriad of enzymes, during granule biogenesis as well as packing will assist in RS formation. Starch pathway engineering using classical tool to genome editing has revealed potential candidates. Future integrative research that addresses all of these issues will help expand the potential uses for digestion-RS in health promotion. Starch granule initiation and formation, enzyme complexes involved in starch metabolism and control of flux in starch synthesis critically contribute towards functional aspects of starch. Careful manoeuvring the key regulatory enzymes using genetic tools will help in developing the desired configurations of starch molecules.

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