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Transcriptome analysis of grain-filling caryopses reveals the potential formation mechanism of the rice *sugary* mutant☆

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ABSTRACT

A *sugary* mutant with low total starch and high sugar contents was compared with its wild type Sindongjin for grain-filling caryopses. In the present study, developing seeds of Sindongjin and *sugary* mutant from the 11th day after flowering (DAF) were subjected to RNA sequencing (RNA-Seq). A total of 30,385 and 32,243 genes were identified in Sindongjin and *sugary* mutant. Transcriptomic change analysis showed that 7713 differentially expressed genes (DEGs) ($\log_2$ fold change ≥ 1, false discovery rate (FDR) ≤ 0.001) were identified based on our RNA-Seq data, with 7239 genes up-regulated and 474 down-regulated in the *sugary* mutant. A large number of DEGs were found related to metabolic, biosynthesis of secondary metabolites, plant-pathogen interaction, plant hormone signal transduction and starch/sugar metabolism. Detailed pathway dissection and quantitative real time PCR (qRT-PCR) demonstrated that most genes involved in sucrose to starch synthesis are up-regulated, whereas the expression of the ADP-glucose pyrophosphorylase small subunit (*OsAGPS2b*) catalyzing the first committed step of starch biosynthesis was specifically inhibited during the grain-filling stage in *sugary* mutant. Further analysis suggested that the *OsAGPS2b* is a considerable candidate gene responsible for phenotype of *sugary* mutant.

1. Introduction

Rice (*Oryza sativa* L.) is one member of Poaceae and this family also contains economically important cereal crops such as barley, wheat, maize, and sorghum supporting the global food supply (Zhao et al., 2013). Rice is also a representative model system for monocots, because of its various advantages as an experimental plant including a small genome size and a known genome sequence (Sasaki and Burr, 2000). Consistent with other angiosperm species, seed development in rice is initiated by double fertilization and asymmetric zygote cell division, which produces a small apical cell that ultimately becomes the embryo and a large basal cell that develops into the endosperm. The classification of gene expression patterns associated with the specific stages of seed development and a functional characterization of the encoded genes are critical for understanding the molecular and biochemical events associated with endosperm development. Seed development is a major item of plant growth and development research, but most of the molecular mechanisms regulating this developmental process are still enigmatic.

Starch is the major storage substance that accounts for over 80% of the total dry mass in rice grains and is stored as energy reserves in the sink tissues such as endosperm (Hoshikawa, 1968; Liu et al., 2010). Starch in rice endosperm is composed of relatively unbranched amylose (linear α-1, 4-polyglucans) and highly branched amylopectin (α-1, 6-branched polyglucans) and both starches are synthesized by adding glucose-1-phosphate (Glc-1-P) to the non-reducing ends of the α-glucan acceptor molecules catalyzed by ADP glucose pyrophosphorylase (AGPase). Subsequent elongation reactions for the α-1,4-chains of amylose and amylopectin are distinctively catalyzed by a

Abbreviations: RNA-Seq, RNA sequencing; FDR, false discovery rate; DAF, day after flowering; DEGs, differentially expressed genes; *OsAGPS*, *Oryza sativa* ADP-glucose pyrophosphorylase small subunit; *OsAGPL*, *Oryza sativa* ADP-glucose pyrophosphorylase large subunit; AGPase, ADP glucose pyrophosphorylase; GBSS, granule-bound starch synthase; SS, starch synthase; SBE, starch branching enzyme; DBE, debranching enzyme; DPE, disproportionating enzyme; PHO, phosphorylase; *ISA1*, isoamylase I; SAGE, serial analysis of gene expression; qRT-PCR, quantitative real-time PCR; DP, degree of polymerization; RPKM, reads per kb per million reads; FC, fold change; SSRGs, synthesis starch-related genes; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; *eEF1a*, eukaryotic elongation factor 1-alpha; *bt2*, *Brittle2*; *sh2*, *Shrunken*; PPDK, Pyruvate orthophosphate dikinase; SAGE, Serial analysis of gene expression.

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starch granule-bound form of starch synthase (GBSS) and a soluble form of starch synthase (SS), respectively. Amylopectin has a much more defined structure called 'tandem-cluster structure' than glycogen because it is composed of tandem-linked clusters (approximately 9–10 nm each in length) where linear α-1,4-glucan chains are regularly branched via α-1,6-glucosidic linkages (Ohdan et al., 2005). AGPase and GBSS synthesize amylose, whereas amylopectin is synthesized by the coordinated actions of AGPase, SS, starch branching enzyme (SBE), and starch debranching enzyme (DBE) (Fig. 1). Disproportionating enzyme (DPE) and phosphorylase (PHO) are involved in starch degradation, but several studies suggested that they may also play possible role(s) in starch biosynthesis (Ball and Morell, 2003; Colleoni et al., 1999; Lu and Park, 2012a; Tetlow et al., 2004). The α-1,4- and α-1,6-glucosidic linkages of amylopectin are formed by multiple types of SS (SSI, SSII, SSIII, and SSIV), SBE (SBEI and SBEII), and DBE (isoamylase and pullulanase) (Fig. 1). All these isoforms of starch-synthesizing enzymes coordinate a network that regulates starch synthesis in the rice endosperm, which finally affects flavor and taste of grain cooking. However, the detailed molecular mechanisms of starch synthesis remain largely unknown.

With the availability of complete genome sequences (Sakai et al., 2013), critical materials such as mutants have been used to study gene function and genetic variations. For example, mutations in the *waxy* gene (encoding granule-bound sucrose synthase, or *GBSSI*) and its regulators *du1* (encoding mRNA splicing factor) and *du3* (encoding cap-binding protein 20-kDa subunit) resulted in low amylose content (≤2%) and whole opaque endosperm (Dung et al., 2000; Isshiki et al., 2000, 2008). The *amylose-extender* mutation reduced activity of starch branching enzyme II (SBEIIb) and was culminated in the structural alterations of amylopectin (Nishi et al., 2001). The *flo-2* and *flo-5* floury endosperm mutations affected the activities of rice starch branching enzyme I (SBEI) and starch synthesis enzyme III (SSIIIa), respectively (Kawasaki et al., 1996; Ryoo et al., 2007). The *floury endosperm-4* mutant and the *sugary-1* mutant are defective in the activity of pyruvate orthophosphate dikinase (PPDK) and debranching enzyme isoamylase I (ISA1) (Kang et al., 2005; Nakamura et al., 1997). Similar with rice, several caryopsis-related mutations were described in maize. For example, maize *sugary*-1 and *sugary*-2 were defective in ISA1 and SSIIa (Kang et al., 2005; Nakamura et al., 1997; Zhang et al., 2004). In addition, *brittle-2* (*bt2*) and *shrunken-2* (*sh2*) were resulted from the mutations in the small or large subunits of AGPase (Bhave et al., 1990; Hannah et al., 2001).

In plants, the major cytosolic AGPase activity is prerequisite for normal starch synthesis in the seed endosperm among barley, maize and rice (Greene and Hannah, 1998; James et al., 2003; Johnson et al., 2003; Lee et al., 2007a). AGPase catalyzes the first committed step of starch biosynthesis and regulates the production of ADPglc and pyrophosphate (PPi) from glucose-1-phosphate (Glc-1-P) and adenosine 5′ triphosphate (ATP) (Lee et al., 2007b; Lu and Park, 2012b). The resulting ADPglc serves as an activated glucosyl donor during α-1,4-glucan synthesis (Lee et al., 2007b). Whereas the prokaryotic AGP is a homotetrameric structure composed of four identical subunits (α4) (Haugen et al., 1976; Lee et al., 2007a), the AGPases in higher plants exist as a heterotetramer (α2β2) containing two large and two small subunits with slightly different molecular weight (Okita et al., 1990; Smith-White and Preiss, 1992; Villand et al., 1993). Rice contains six AGPase genes; two of them encode small subunits OsAGPS1 and OsAGPS2 and remained four encode large subunits OsAGPL1, OsAGPL2, OsAGPL3, and OsAGPL4. The *AGPS2* gene encodes the transcripts for *AGPS2a* and *AGPS2b*, which differ only in their first exons (the first exon of AGPS2a serves as the first intron of AGPS2b) and are either processed from the common pre-mRNA by alternative splicing or from different promoters. Previously reported gene expression results have also indicated that while *OsAGPS2b* is largely present in seed endosperm, *OsAGPS2a* is expressed in leaves (Akihiro et al., 2005; Hirose et al., 2006; Ohdan et al., 2005). Lee et al. suggested the complex formation of OsAGPS2a and OsAGPL3 during transitory starch in rice leaves (Lee et al., 2007a). In rice developing endosperm, at an early stage, the amyloplast-targeted OsAGPS1/OsAGPL1 heterotetramer has the main functional role and the cytosolic OsAGPS2b/OsAGPL2 complex plays a relatively minor role due to its low levels. As the endosperm matures, the cytosolic OsAGPS2b/OsAGPL2 complex confers the dominant enzyme activity in starch synthesis (Lee et al., 2007a). In maize and rice, mutations in *AGPS2b* and *AGPL2* resulted in the *bt2* and *sh2* phenotypes due to the significant reduction of starch synthesis in grains (Bhave et al., 1990; Greene and Hannah, 1998; Lee et al., 2007a).

Massively parallel sequencing technology is more sensitive for detection of transcripts expressed at low levels than traditional methods

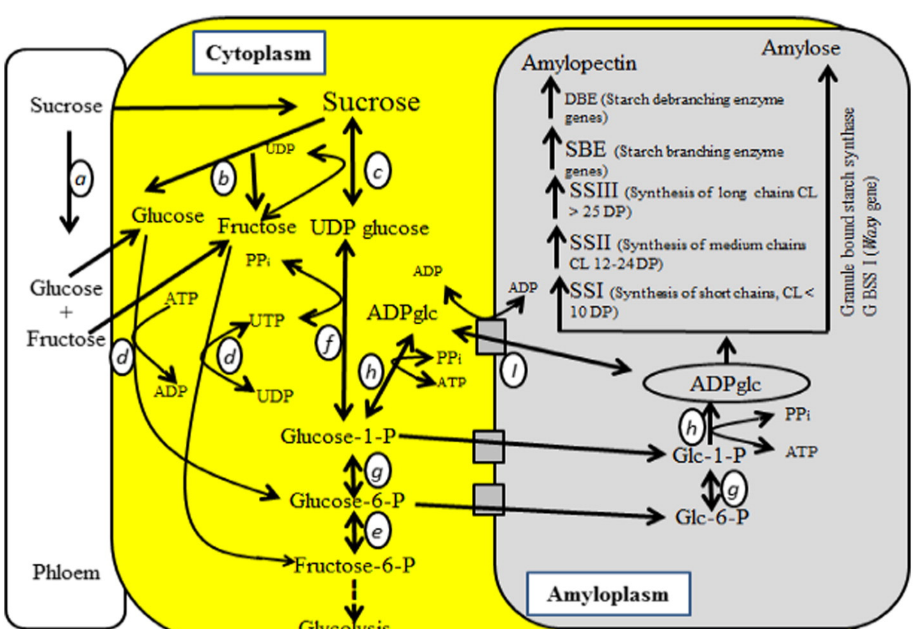

Fig. 1. A simplified metabolic pathway from sucrose to starch in rice caryopsis. a, cell wall invertase; b, cytoplasmic invertase; c, sucrose synthase; d, hexokinase; e, phosphoglucose isomerase; f, UGPase; g, cytoplasmic and plastidial phosphoglucomutase; h, cytoplasmic and plastidial AGPase; i, ADPglc transporter.

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