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Starch Biosynthesis and Engineering Starch Yield and Properties in Cassava

Youzhi Li
State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Guangxi Research Center for Microbial and Enzyme Engineering Technology, College of Life Science and Technology, Guangxi University, Nanning, 530004, Guangxi, China
Corresponding email: dyz@gxu.edu.cn

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Abstract Cassava (*Manihot esculenta* Crantz), a perennial shrub but a root crop in Euphorbiaceae, produces a bulk of starch in the storage roots and serves as a staple food for millions of people in tropical and subtropical regions. Additionally, cassava starch is widely used in food processing and industrial sectors due to its unique physicochemical properties of swelling and solubility, gelatinization, retrogradation, pasting, and viscoelasticity. Up to now, the starch biosynthesis and improvement have been well reviewed by a large number of literatures at different layers and aspects in other plant species/crops but the understanding is limited in cassava. Therefore, how to increase starch yield and improve starch properties has received great attention. This article briefly reviews plant starch biosynthesis, and complexity of starch biosynthesis, cases of engineering-based improvement of starch yield and properties, quantitative trait loci controlling starch yield and properties, challenges of breeding and engineering, and opportunities and future prospects in cassava.

Keywords Cassava (*Manihot esculenta* Crantz); Starch biosynthesis; Engineering starch; Starch yield; Starch properties

1 Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial shrub but a root crop in Euphorbiaceae. This crop serves as a staple food for millions of people in tropical and subtropical regions because the dry matter in its storage roots contains more than 80% starch (Alves, 2002; El-Sharkawy, 2004). In addition, cassava can grow on barren and drought land where other crops fail due to its stronger tolerance to stressful environments (Alves, 2002). Beyond its role in human nutrition, cassava starch is also a versatile material with a wide range of applications in food processing and industrial sectors (Tappiban et al., 2019) due to its unique physicochemical properties of swelling and solubility, gelatinization, retrogradation, pasting, and viscoelasticity (Chisenga et al., 2019). On these ground, the roles of cassava starch in economic and nutritional value cannot be overstated. Therefore, understanding starch biosynthesis of cassava is very necessary for tailoring it to specific needs.

Up to now, a large number of in-depth reviews at different layers and aspects have been conducted on the starch biosynthesis in plants (Martin and Smith, 1995; Hannah and James, 2008; Orzechowski, 2008; Jeon et al., 2010; Kötting et al., 2010; Stitt and Zeeman, 2012; Bahaji et al., 2014; Saripalli and Gupta, 2015; Tappiban et al., 2019; Tetlow and Bertoft, 2020; Huang et al., 2021; Li et al., 2021). But, integrative understanding of cassava starch biosynthesis is relatively limited. Fortunately, the completion of cassava genome sequencing (Wang et al., 2014) provides an excellent opportunity and foundation for doing this.

Based on our understanding, this brief review is to focus on the biochemical pathways of starch synthesis in and implications for breeding programs of cassava, only making a start.

2 Outline of Starch Biosynthesis in Plants

Starch biosynthesis in plants usually occurs in the chloroplast of leaves during the day (Martin and Smith, 1995; Orzechowski, 2008; Stitt and Zeeman, 2012). However, this process is involving multi-step catalytic processes by multiple enzymes and further complicated due to the transient synthesis that is also present in other organs such as meristems and root cap cells (Martin and Smith, 1995). No matter how, the key enzymes for starch biosynthesis are, but not limited to, adenosine 5'-diphosphate glucose pyrophosphorylase (AGPase) responsible for the

synthesis of ADP glucose, comprising two large (ApL) and two small (ApS) catalytic subunits; starch branching enzyme (SBE) to produce branches connected by a-1,6-glycoside bonds, including SBEI (SBE1), SBEII, and SBEIII; starch debranching enzyme (DBE) hydrolyzing α-(1,6)-linkages, with three isoforms of isoamylase-type DBE and one pullulanase-type DBE; soluble starch synthase (SS) catalyzing the transfer of glucose from ADP-glucose to an acceptor glucan chain and involving solely in amylopectin synthesis, with 4 classes of SSI to SSIV; granule bound starch synthase (GBSS) involving amylose biosynthesis, with GBSSI and GBSSII; phosphoglucoisomerase converting fructose 6-phosphate to glucose 6-phosphate; phosphoglucomutase converting glucose 6-phosphate to glucose 1-phosphate; and starch phosphorylase responsible for glucan-elongation reactions (Orzechowski, 2008; Keeling and Myers, 2010; Tetlow and Emes, 2014; Li and Gilbert, 2016; Huang et al., 2021). In these enzymes, AGPase is considered as a rate-limiting enzyme responsible for the synthesis of ADP-glucose in the first and key step of starch biosynthesis (Ihemere et al., 2006).

3 Complexity of Cassava Starch Biosynthesis

According to current research in other plants, each enzyme has multiple isoforms (Tappiban et al., 2019), and the enzyme activity and function of the isormors are not entirely the same (Ohdan et al., 2005; Keeling and Myers, 2010; Kötting et al., 2010; Li and Gilbert, 2016; Huang et al., 2021). The expression of some starch biosynthesis genes such as AGPases have been found in both source (leaves) and sink (seeds) organs of rice, and the gene expression modes are tissue and developmental stage-specific (Ohdan et al., 2005). The enzymes' function depends on the formation of protein complexes (Keeling and Myers, 2010; Cho and Kang, 2020), showing protein–protein interactions.

Cassava is considered one of orphan crops that are also known as underutilized crops, lost crops, neglected crops, or crops for the future (Tadele, 2019; Zambrano et al., 2022). The research on genetic background and molecular mechanisms controlling many traits in cassava is still insufficient. The investigation of the mechanism of cassava starch synthesis is currently only in the age of enlightenment. The 98 known wild species of the New World genus *Manihot* have been found, which are extremely heterogeneous for any particular genotype. Cassava is an outbreeding species (2*n*=36 chromosomes) and considered to be an amphidiploid or sequential allopolyploids, and asexually propagated by mature woody stem cuttings (El-Sharkawy, 2004). Recently, a total of 45 genes participating in starch biosynthesis in cassava (Tappiban et al., 2019), including AGPase, GBSS, SS, SBE, DBE, and glucan, water dikinase (GWD). The starch synthesis of cassava may be much more complex than expected and may also have its unique characteristics. With 6 field-grown cultivars and 1 wild species, we have found that starch synthesis-related enzymes have multiple active isoforms in cassava. The types of the active isoforms varied depending on the cultivars. The same active isoforms varied greatly with the roots, stems, and leaves of the same and different cultivars with the growth stage. What is even more confusing was that it was hard to associate these corresponding changes with the starch accumulation (unpublished). These factors together will undoubtedly make cassava starch biosynthesis processes more complex than existing paradigms/frameworks proposed in other plant species.

4 Cases of Engineering Cassava Starch

Efforts have been made to improve cassava starch yield and alter starch properties by regulating the expression of starch biosynthesis-related genes through gene engineering, with several cases. Expressing *AGPase* genes usually enhances starch production of plants including cassava in most cases but were found to have no impacts on starch production in rare cases, and even generated unexpected results with respect to yield components including starch content (Tuncel and Okita, 2013). For example, transgenic cassava expressing AGPase-encoding *glgC* gene of bacterial *Escherichia coli* showed slight decrease in root starch contents (mg per gram fresh weight) (Ihemere et al., 2006), 151 for wild type cassava and 143 (149 and 138) for transgenic cassava. Overexpressing *AGPase* gene in cereals increased starch yield, and meantime, resulted in increases in seed number and plant biomass (Tuncel and Okita, 2013). Suppression of *GBSSI* gene expression caused the reduced amylose content but increased values for clarity, peak viscosity, gel breakdown, and swelling index (Zhao et al., 2011). CRISPR-Cas9-mediated targeted mutagenesis of *PROTEIN TARGETING TO STARCH* or *GBSS* gene reduced or eliminated amylose content in root starch of cassava (Bull et al., 2018). Silencing expression of *SBE1* and *SBE2* by short interfering RNAs-mediated

RNAi produced starch containing up to 50% amylose (Zhou et al., 2020). Three mutants with long fragment deletions in the second exon of *SBE2* showed higher amylose (up to 56% in apparent amylose content) and resistant starch (up to 35%), and also resulted in starch viscosity with a higher pasting temperature and peak time (Luo et al., 2022). Simultaneous suppression of both *SBE1* and *SBE2* endowed cassava with a reduced degree of polymerization of 6–13 chains in amylopectin (Utsumi et al., 2022b). *GWD1*-RNAi cassava plants not only showed both retarded plant and storage root growth, had excess starch accumulation in leaves, and also led to changes in physico-chemical properties of transient and storage starch (Zhou et al., 2017). *MeSSII*-RNAi cassava had an increase in amylose content and presented alterations in starch physicochemical properties in the storage roots (He et al., 2022). In fact, engineering cassava as well as testing in the field are still in its infancy (Koehorst-van Putten et al., 2012; Zambrano et al., 2022).

5 Quantitative Trait Loci (QTL) Controlling Starch Yield and Properties

Starch yield and properties are very close but different traits, which are associated with QTLs. The QTLs could be used for identification of key target genes of interest and for selection of cassava germplasms of desirable traits for breeding. So far, research on QTL controlling starch yield and properties has not been as extensive as one might think. Fifteen QTLs associated with starch pasting viscosity were identified by using 100 lines of an F1 mapping population from a cross between two cassava cultivars Huay Bong 60 and Hanatee (Thanyasiriwat et al., 2014). Total 115 QTLs controlling starch yield and properties on starch content, amylose content, pasting temperature, thermal and retrogradation, and textural property were reported from 2005-2018 (Tappiban et al., 2019), with candidate genes. Five QTLs for starch content were identified with 2 cassava cultivars of CI-732 (high dry matter content and starch content) and MNga-1 (low dry matter content and starch content) by simple interval mapping (Prasannakumari et al., 2021). With a panel of 276 cassava genotypes by using the genome-wide association study (GWAS), 21 starch pasting property-related QTLs were recently found (Phumichai et al., 2022).

6 Challenges in Engineering Cassava Starch Yield and Starch Properties

Cassava improvement either through conventional cross-breeding or by engineering biotechnologies faces more rigorous challenges (Otun et al., 2023). After entering the era of omics, many new and powerful genetic engineering technologies have emerged and are constantly being improved, such as CRISPR/Cas9 for gene editing, and RNAi and virus-induced gene silencing (VIGS) for suppressing gene expression. Each technology has its own pros and cons. For all these technologies, the basic principle and requirement is high specificity and precision (Senthil-Kumar and Mysore, 2011; Ma et al., 2014; Rössner et al., 2022). However, although not all, unexpected off-target phenomena and non-specific events are also commonly reported. The engineering strategies based on Agrobacterium-mediated overexpression (Utsumi et al., 2022a), CRISPR/Cas9, RNAi, and VIGS have been used for cassava improvement and gene function identification research. The challenges are, but not limited to, as follows.

It is currently not very clear about the chromosomal ploidy and heterozygosity for the vast majority of cassava cultivars. Cassava materials resulting from natural outcrosses are preferentially retained in the long-term production and breeding process because larger and much more vigorous cassava materials from outcrosses are more favored by farmers. Therefore, it can be speculated that most of the cultivars/elite variety should be heterozygous polyploids. However, such heterozygosity results in wide and unpredictable diversity of phenotypes that breeders are interested in but farmers dislike in propagation (Ceballos et al., 2004).

The heterozygosity makes it very likely that some key starch biosynthesis genes are in a heterozygous state. For *MeSSI* gene, there are 5 heterozygous loci in coding regions in 44 cassava accessions, and 1 heterozygous locus is in non-coding region in 44 cassava accessions (Vasconcelos et al., 2016). With regard to *MeGBSS1* gene, only one copy is in cassava genome (Tappiban et al., 2019), however, there existed 1 heterozygous locus in coding regions in 87 cassava accessions, and 5 heterozygous loci were present in non-coding regions in 84 cassava accessions (Vasconcelos et al., 2016). The *MeSBE* gene had 1 heterozygous locus in non-coding regions in 280 cassava accessions (Vasconcelos et al., 2016). In addition, expression of genes encoding starch biosynthesis enzymes, such as *AGPase*s, shows changes with tissues and growth stages of cassava (Tappiban et al., 2019). All these factors

will be bound to bring great difficulties to selection in hybrid breeding, and also cause instability of traits of cassava which is engineered but vegetatively propagated.

In some cases of improving cassava starch yield and properties by key starch synthesis gene, in addition to expected changes, additional unexpected traits or characteristic changes have also emerged as mentioned above, indicating that precision and targeting are still problematic. Even though engineered cassava that has been obtained, it is unclear whether the traits have genetic stability in propagation and production through stem cutting in the field. Additionally, there seem to be very few cases of cassava germplasm selection/screening with the help of the currently obtained QTLs. The core functional genes in the QTLs have not been identified yet, expression regulation mechanisms of which are still unknown.

7 Opportunities and Future Prospects

(1) With regard to cassava materials for engineering cassava and conventional crossing breeding, it is much more important to develop homozygous, heterozygous, and possibly chimeric lines containing a spectrum of different starch contents and properties. In this regard, Bull and his colleagues have done an excellent job through targeted mutagenesis of *GBSS* or *PROTEIN TARGETING TO STARCH1* genes (Bull et al., 2018).

(2) As for enzymes, it is very necessary to conduct identifications of isoforms, active enzyme species and their activity profiles with tissues and growth stages due to the lack of holographic information in these aspects.

(3) Developing new starch-related QTLs, and integrating QTLs and GWAS data to address expression regulation of the key genes in QTLs and further understand the functional role of both genotype and phenotype-associated variations in cassava. These include splicing QTL which is a genetic variant regulating alternative splicing as one of the major causal mechanisms in GWAS loci (Yamaguchi et al., 2022), and expression quantitative trait loci which are namely the discovery of genetic variants that explain variation in gene expression levels (Nica and Dermitzakis, 2013; Joehanes et al., 2017).

(4) Utilizing informative and accurate access maps for engineering cassava. A remarkable research is that the genome-based reconstruction of starch biosynthesis pathway has been established in the form of an informative map with all important information of the pathway to investigate the dynamic regulation of starch biosynthesis in cassava roots, which is available at the Systems Biology and Bioinformatics Research Group's website (http://sbi.pdti.kmutt.ac.th/?page_id=33) (Saithong et al., 2013).

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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