Soluble Starch Synthase III-1 in Amylopectin Metabolism of Banana Fruit: Characterization, Expression, Enzyme Activity, and Functional Analyses

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OPEN ACCESS

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Specialty section:
This article was submitted to Crop Science and Horticulture, a section of the journal Frontiers in Plant Science

Received: 25 August 2016
Accepted: 15 March 2017
Published: 30 March 2017

Citation:
Miao H, Sun P, Liu Q, Jia C, Liu J, Hu W, Jin Z and Xu B (2017) Soluble Starch Synthase III-1 in Amylopectin Metabolism of Banana Fruit: Characterization, Expression, Enzyme Activity, and Functional Analyses. Front. Plant Sci. 8:454, doi: 10.3389/fpls.2017.00454

INTRODUCTION

Starch is the most widespread carbohydrate storage molecule in plants and plays a vital role in human nutrition, food industry and chemical manufacturing. The physicochemical properties of starch are strongly affected by its two key components, amylose and amylopectin (Zeeman et al., 2010; Bischof et al., 2013). In higher plants, there are at least six classes of enzymes that are involved in amylose and amylopectin biosynthesis: ADP-Glc...
pyrophosphorylase (AGPase), granule-bound starch synthase (GBSS), soluble starch synthase (SS), starch branching enzyme (SBE), starch debranching enzyme (DBE), and starch phosphorylase (SP) (Fujita et al., 2008; Szydlowski et al., 2009; Jeon et al., 2010; Subasinghe et al., 2014).

Starch synthase uses ADP-glucose for chain elongation via α-1,4-glycosidic linkages, and directly catalyzes the amylopectin biosynthesis (Szydlowski et al., 2009, 2011). A plant typically carries at least four SS subfamilies, termed as SSI, II, III, and IV, respectively (Dian et al., 2005). Comparison of the deduced amino acid sequences showed that these four subfamilies are highly similar in a span of approximately 450 amino acid residues in the C-terminus that comprises the catalytic and starch-binding domains (Schwarte et al., 2013). The sizes of SSI, II, and III proteins were estimated to be approximately 67, 81, and 112 kDa, respectively, in Arabidopsis leaves (Delvallè et al., 2005; Zhang et al., 2008; Gámez-Arjona et al., 2014). SS gene expression was recently profiled in a numbers of plants including rice (Crofts et al., 2015), maize (Liu et al., 2015; Huang et al., 2016), potato (Edwards et al., 1999), Arabidopsis (Schwarte et al., 2013), wheat and taro (Li et al., 2000; Lin and Jeang, 2005). However, different SSs may predominate in storage organs of different species e.g., SSI in rice endosperm, SSSI in wheat (Li et al., 2000; Crofts et al., 2015). In common wheat, SSSI expression was detected from very early to the middle stages of endosperm development (Li et al., 2000). Rice SSSI-2 and SSV-1 showed the highest expression level at the middle and late development stages of rice endosperm, respectively (Dian et al., 2005).

Amylopectin chain length distribution and amylopectin/amylase ratio affected starch functionality (Dian et al., 2005; Zhang et al., 2005). Biochemical and gene overexpression experiments revealed that SSI, II, and III are involved in the elongation of short (dp 8–12) (Fujita et al., 2008), medium (dp 13–25), and long (dp > 30) starch chains (Szydlowski et al., 2011; Zhu et al., 2014), respectively. SSIIV is closely related to SSSI; both have similar structures (Leterrier et al., 2008). Further reverse genetic studies showed that impaired SSI expression was reported to result in structurally altered amylopectin in Arabidopsis leaves (Delvallè et al., 2005) and rice endosperm (Takekomo-Kuno et al., 2006; Fujita et al., 2008). Inhibition of SSSI gene expression gave rise to lower pasting temperature as the result of alterations in amylopectin structure (Edwards et al., 1999). Loss of SSSI expression reduced the proportion of amylopectin with very long chains and affected the amylopectin/amylase ratio in maize (Zhu et al., 2014). Arabidopsis (Zhang et al., 2005), and rice (Dian et al., 2005). A defective mutant of SSIIV displayed a severely compromised growth phenotype with fewer but larger starch granules within the plastid in Arabidopsis (Roldán et al., 2007). However, Zhang et al. (2008) reported that some SSs, such as SSI and SSSI, may overlap in amylopectin biosynthesis in Arabidopsis. In addition, GBSS from other SS isoforms also contributed to amylopectin synthesis in rice and Chlamydomonas reinhardtii (Ral et al., 2006; Hanashiro et al., 2008). These evidences suggested the important roles of the SS genes in regulating plant amylopectin metabolism.

Banana (Musa spp.) is not only one of the most highly consumed fruits in the world but also widely used as a staple food in the tropical and subtropical regions (D’Hont et al., 2012). The typical starch content of a green dessert banana fruit accounts for 20–25% of fresh weight or 60–75% of dry weight (DW), and the starch granules are relatively large (8–48 µm in diameter) compared to those of cereals (Hubbard et al., 1990; de Barros Mesquita et al., 2016). Hence banana is also a potential excellent model plant for studying fresh fruit starch metabolism. Current research on banana starch has mainly focused on granule structure (Low et al., 2015) and antioxidant capacities (Sarawong et al., 2014), as well as physicochemical properties of starch (Zhang and Hamaker, 2012; Utrilla-Coello et al., 2014). Several key genes involved in starch biosynthesis or conversion of starch to sucrose have been isolated and characterized in banana, including MaGBSSI (Miao et al., 2014), DBE (Bierhals et al., 2004), SUCROSE PHOSPHATE SYNTHASE (SPS), SUCROSE SYNTHASE (SuSy), and INVERTASE (Hubbard et al., 1990). However, to our knowledge, the function of SS genes has not been characterized in banana.

Amylopectin is the major component of immature banana fruit (Torre-Gutiérrez et al., 2008), which has a number of remarkable features, including higher retrogradation and lower pasting temperature (Sarawong et al., 2014), relative to those in rice (Syahariza et al., 2013), maize (Huang et al., 2016), and potato (Wikman et al., 2014). Due to the important role of SS in amylopectin metabolism, there is a need to investigate the function of SS in banana. In this study, we revealed that MaSSIIV-1 is a key gene expressed in banana fruit and responsible for the active amylopectin biosynthesis by expression, enzyme activity, and functional analyses. This result could be used in the genetic manipulation of banana fruit for genetic improvement of its nutritional values as well as value-added industrial applications, such as high starch foods, higher retrogradation and lower pasting temperature raw processing materials.

MATERIALS AND METHODS

Plant Materials and Treatments

Banana (M. acuminata L. AAA group, cv. ‘Dwarf Cavendish’; ITC 0002) seedlings were obtained from the banana tissue culture center (Chinese Academy of Tropical Agricultural Sciences, Danzhou, China) and were grown at 28°C with 70% humidity, 200 µmol·m−2·s−1 light intensity, and long day condition (16 h light/8 h dark cycle). When banana seedlings produced five leaves, they were planted in the Institute of Tropical Bioscience and Biotechnology banana plantation (Chengmai, Hainan, 20N, 110E) until harvest. Roots, stems, leaves, bracts, flowers, peels, and pulps at 60 days after emergence from the pseudostem (DAF) were collected separately using tweezers, frozen immediately in liquid nitrogen, and stored at −80°C until expression analysis in different tissues. For each biological replicate, two banana hands having a similar developmental stage from two plants were selected and six fingers from the hands were obtained. Banana pulps at 0, 10, 20, 30, 40, 50, and 60 DAF were collected, immediately frozen in liquid nitrogen, and stored at −80°C until starch/amylopectin quantification, mRNA and protein expression analysis.
Banana hands (60 DAF) were separated into individual fingers representing the same developmental stage. For natural ripening treatment, banana samples were kept at 22°C and allowed to ripen in open air. In accordance with the previously published banana ripening stages (Miao et al., 2014), fruits stored for 0, 5, 10, 15, 20, 25, and 30 days period of post-harvest (DPH) were frozen in liquid nitrogen and stored at −80°C until starch/amylopectin quantification, mRNA and protein expression analysis.

**Scanning Electron Microscopy (SEM) Observation**

Pulp samples were fixed in stubs using double-faced tape and coated with a 10 nm thick platinum layer using a Bal-tec MED 020 Coating system (Kettleshulme, UK) before analysis with a FEI Quanta 600 FEG Scanning Electron Microscope (FEI Company, Hillsboro, OR, USA). SEM observations were performed using the secondary electron mode operating at 15 kV.

**Determination of Total Starch Content, Amylopectin Content, and SS Activity**

Banana pulp was immersed in 0.5% (w/v) sodium bisulfite solution for 10 min to prevent browning, and then dried at 40°C for 20~24 h. The dried pulp was milled to powder and suspended in 5 mL 80% (w/v) Ca(NO₃)₂, placed in a boiling water bath for 10 min, and centrifuged for 4 min at low speed (3,800 g). The supernatant was then transferred to a 20 mL volumetric flask. The total starch, amylose, and amylopectin contents of the extract were determined following Yang et al. (1992) and Miao et al. (2014). The amylopectin content was calculated by subtracting the amylose content from the total starch content. Enzymatic hydrolysis for 10 min, and centrifugation for 5 min at 3,800 g. The supernatant was then transferred to a 20 mL volumetric flask. The total starch, amylose, and amylopectin contents of the extract were determined following Yang et al. (1992) and Miao et al. (2014). The amylopectin content was calculated by subtracting the amylose content from the total starch content. Enzymatic hydrolysis was performed for 10 min at 40°C and centrifugation for 5 min at 3,800 g. The supernatant was then quantified by qRT-PCR using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA) with the SYBR ExScript RT-PCR Kit (TaKaRa, Dalian, China). ACTIN or GAPDH that were verified to be constitutive in expression and hence suitable to be used as internal controls were used as reference genes to normalize transcriptional levels of each MaSS gene and SSIII-1 (Supplementary Table S1). Relative expression levels of four MaSS genes were analyzed in three technical replicates and calculated using the 2^{−ΔΔCT} method (Livak and Schmittgen, 2001). Each sample contains three biological replicates.

**Detection of SSIII-1 Protein Levels by Western Blot Analysis**

Pulp samples (0.5 g) were homogenized with a set of mortar and pestle in an equal volume of solution, which consisted of 50 mM HEPES-NaOH (pH 7.4), 2 mM MgCl₂, 50 mM β-mercaptoethanol, and 12.5% (v/v) glycerol (Nishi et al., 2001). A 20 μL aliquot of the supernatant was incubated with 36 μL of solution 1 consisting of 50 mM HEPES-NaOH (pH 7.4), 1.6 mM adenosine diphosphate glucose (ADPG), 0.7 mg amylopectin, and 15 mM DL-dithiothreitol (DTT) for 20 min at 30°C. Following the reaction termination at 100°C for 30 s, 20 μL solution II was added, which consisted of 50 mM HEPES-NaOH, 4 mM phosphoenolpyruvate (PEP), 200 mM KCl, 10 mM MgCl₂, and 1.2 U pyruvate kinase, and incubation on ice for 5 min. The reaction was incubated at 30°C for 20 min before it was terminated at 100°C for 30 s. A 60 μL aliquot of the supernatant was mixed with 43 μL of solution III (50 mM HEPES-NaOH, 10 mM glucose, 20 mM MgCl₂, 2 mM nicotinamide adenine dinucleotide phosphate, 1.4 U hexokinase, 0.35 U glucose-6-phosphate dehydrogenase) and incubated at 30°C for 10 min. The supernatant (SS enzyme solution) was used to Western blot analysis.

For Western blot analysis, 30 μL of the SS enzyme solution from banana pulps at different developmental stages extracted by the above methods was loaded on each lane and separated on a 12% polyacrylamide gel. Proteins were then transferred onto Hybond™-N+ membranes (Amersham Biosciences, Buckinghamshire, UK). Membranes were probed with rabbit anti-MaSSIII-1 polyclonal antibody (Abmart, Shanghai, China) and banana actin antibody (control) in 1:1,000 dilution in PBS-Tween 20 plus 3% BSA, respectively, followed by alkaline

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1http://banana-genome.cirad.fr/
phosphatase-conjugated anti-rabbit IgG secondary antibody (Sigma, St. Louis, MO, USA) in 1:1,000 dilution. Positive signals on the membranes were detected with a 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) solution (Amresco, Solon, OH, USA).

**Plant Transformation, Generation and Southern Blot Analysis of MaSSIII-1 Transgenic Plants**

The entire MaSSIII-1 coding region was inserted into the pCAMBIA-1302 vector under the transcriptional control of CaMV 35S promoter following a double digestion with Nco I and Spe I. The pCAMBIA-1302-MaSSIII-1 was transferred into *A. tumefaciens* strain LBA4404. Transgenic tomato (*Solanum lycopersicum* L.) plants were generated using the Agrobacterium-mediated transformation as previously described (Arshad et al., 2014). Kanamycin-resistant transgenic tomato lines were selected and the transgene integration was determined by Southern blot analysis. Genomic DNA (10 µg per sample) from transgenic tomato leaves was isolated using a CTAB method (Li et al., 2015) and digested with EcoRI overnight at 37°C, separated on a 0.8% (w/v) agarose gel and transferred onto Hybond-N nylon membranes (Hybond N+, Amersham, UK) (Li et al., 2015). Probes were prepared from the PCR product amplified using the primers (5′-gagagagaagagttgagcacg-3′ and 5′-gagagcagcagacagctg-3′) and labeled with DIG-dUTP according to the manufacturer’s instructions (Roche Applied Science, Mannheim, Germany). Fruits from two single-copy transgenic plants L4 and L11 were used for expression analysis and functional investigation of MaSSIII-1 in comparison to wild-type.

**Statistical Analysis**

Three biological replicates were performed for each sample, unless specified otherwise. Statistical analyses were performed using Microsoft Excel and SPSS (Chicago, IL, USA). Analysis of variance was used to compare the statistical difference based on Student’s t-tests, at significant levels of *p* < 0.05 (*), and *p* < 0.01 (**).

**RESULTS**

**Change in Starch Granules, Total Starch Content, Amylopectin Content, and SS Enzyme Activity at Different Developmental and Storage Stages of Banana Fruit**

During banana fruit development the fruit size gradually increases and it becomes curved in a crescent shape as fruit develops (Figure 1A). As observed using SEM, starch granules in banana pulp were barely detectable at the initial sampling time, i.e., 0 DAF, but the oval-shaped starch granules become clearly visible and were significantly increased in size as the fruit developed from 10 (6.0−8.2 µm) to 60 DAF (28.0−35.7 µm).

Morphological comparison of starch granules during this period of time indicated that the shape and number of starch granules remained consistent, but the size was significantly increased and maximized at 60 DAF (Figure 1B).

Starch granules in mature banana pulp were also observed using SEM at the 30 DPH during storage at 22°C, at 5 days’ intervals. Compared to the first time point (0 DPH), the quantity of starch granules per unit volume decreased rapidly over time. After 15 DPH of storage, starch granules were hardly detectable (Figure 1C).

The total starch content and amylopectin content in pulp increased as fruit developed and peaked at 50 DAF (these two polymers reached to 67 and 44% DW, respectively) before decline at the 60 DAF time point and continued to decrease gradually during storage (Figures 1D,E), implying that after maturity starch and amylopectin may have experienced a rapid degradation process. However, the SS enzyme activity increased later than starch and amylopectin accumulation at maturity and had an abrupt decline at the very beginning of storage (Figure 1F).

**Nucleotide Sequence Characteristics, Chromosomal Localization, and Phylogenetic Analysis of Banana MaSS Genes**

Full-length cDNAs encoding *MaSSI*, *MaSSII*, *MaSSIII-1*, and *MaSSIII-2* were 2,076 bp, 1,851 bp, 2,397 bp, and 3,267 bp, respectively. All these sequences were deposited at GenBank and their accession numbers were listed in Figure 2. BLAST analysis against the banana DH-Pang (AA group) genome sequence database revealed that *MaSSI*, *MaSSII*, *MaSSIII-1*, and *MaSSIII-2* are located on chromosome 3, 6, 11, and 5, respectively (Supplementary Figure S1). The sequence analysis also revealed markedly different primary structures as *MaSSI*, *MaSSII*, *MaSSIII-1*, and *MaSSIII-2* contain 25, 6, 5, and 9 exons, respectively. The stop codon usage is also different as TAA was used by *MaSSI* and *MaSSIII-1*, but TGA was used by *MaSSII* and *MaSSIII-2* (Supplementary Figure S1).

The deduced amino acid sequences of the *MaSSI* and *MaSSII* shared three conserved regions, referred to as Domain I, II, and III (Supplementary Table S2), as previously identified in SS enzymes from amaranth (Park et al., 2012). The *MaSSIII-1* and *MaSSIII-2* amino acid sequences contained four distinct regions: a transit peptide region, a variable repeat region, a SSIII specific region, and a C-terminal catalytic domain (Supplementary Table S3), as previously identified in SSIII enzymes from wheat (Li et al., 2000). According to pl/MW software analysis, the predicted molecular weights of the *MaSSI*, *MaSSII*, *MaSSIII-1*, and *MaSSIII-2* proteins were 76.69, 69.85, 90.69, and 119.26 kDa, respectively, and their theoretical pIs were 5.24, 5.36, 6.44, and 7.35, respectively.

To elucidate phylogenetic relationship of banana MaSS genes, the four deduced polypeptide sequences were aligned

2http://banana-genome.cirad.fr/
3http://expasy.org/computepl/
FIGURE 1 | Changes in starch granules, total starch content, amylopectin content, and SS activity in banana pulp at different stages of development and during storage. (A) Banana fruit at different development stages (0, 10, 20, 30, 40, 50, and 60 DAF) and following storage for varying amounts of time (0, 5, 10, 15, 20, 25, and 30 DPH), (B) SEM of starch granules in pulp during banana fruit development, (C) SEM of starch granules in pulp during banana fruit storage, (D) Total starch content in banana pulp at different stages of development and during storage, (E) Amylopectin content in banana pulp at different stages of development and during storage, (F) SS activity in banana pulp at different stages of development and during storage. DAF: days after emergence from the pseudostem; DPH: days of post-harvest. The vertical bars represent the mean ± SD of three replicates. Asterisks indicate significant difference from 0 DAF and 0 DPH vs. the following days (*p < 0.05; **p < 0.01). Scale bar = 20 μm.
with orthologous SS sequences and a NJ tree was constructed (Figure 2). MaSSIII-1 and MaSSIII-2 are aligned next to each other, forming a side branch in association with other SSIII sequences from monocot species. Similarly, MaSSI and MaSSII are clustered together with their respective orthologs from other species. This may also indicate that the divergence of the SSI, SSII and SSIII occurred prior to the speciation of these plant species, but the separation of SSIII-1 and SSIII-2 might be of a more recent history likely through a gene duplication and subsequent divergence.

**Spatially and Temporally Differential Expression of Four MaSS Genes**

The spatial expression patterns of the four MaSS genes were analyzed by qRT-PCR in seven different tissues, including...
FIGURE 3 | Expression of MaSSI, MaSSII, MaSSIII-1, and MaSSIII-2 genes in different banana tissues (A) and in fruit at different stages of development and after various storage times (B). The y-axis represents the relative fold-difference in mRNA level, which was calculated using the $2^{-\Delta\Delta Ct}$ formula with MaActin and MaGAPDH as internal controls. The vertical bars represent the mean ± SD of three replicates. Asterisks indicate significant difference from 0 DAF and 0 DPH vs. the following days (*$p < 0.05$; **$p < 0.01$).

FIGURE 4 | Western blot analyses of the MaSSIII-1 protein. (A) Western blot analyses of MaSSIII-1 proteins in banana pulp at different stages of development, (B) Western blot analyses of MaSSIII-1 proteins in banana pulp at different stages of storage. DAF: days after emergence from the pseudostem; DPH: days of post-harvest.

roots, stems, leaves, bracts, flowers, peels, and pulp (60 DAF) (Figure 3A). MaSSI was expressed at low levels in all tissues examined with the highest expression in pulps. MaSSII was also found to express low in all the vegetative tissues, including roots, stems, leaves, and bracts, and particularly low in flowers and fruit tissues. The expression patterns of MaSSIII-1 and MaSSIII-2 were very similar, with highest expression in pulps and leaves (starch content in leaves was approximately 22.67 ± 1.32%), but that MaSSIII-1 had the highest levels of expression.

The temporal expression of MaSSI, MaSSII, MaSSIII-1, and MaSSIII-2 during banana fruit development was also determined by qRT-PCR (Figure 3B). Despite of being highly variable, the expression levels of MaSSI and MaSSII genes were found to be generally low throughout the entire banana pulp development. Both MaSSIII-1 and MaSSIII-2 showed a sharp increase of expression during the fruit development, the increase in expression occurred earlier in MaSSIII-1 than in MaSSIII-2, with the highest expression detected at the last sampling stage when banana had reached its maturity. However, the expression of MaSSIII-1 was about 10∼20-fold higher than that of MaSSIII-2 during the fruit development.

When the naturally ripen banana fruits were subjected to varying period of storage, the MaSS genes were also found to be differentially expressed (Figure 3B). MaSSI showed consistently low levels of expression throughout the storage period, but the MaSSI expression was slightly increased during storage. The seemingly negative association between MaSSI expression and starch degradation needs to be further investigated. Although there can be a lack of congruency between transcript and activity, it is possible that there can be some starch biosynthesis occurring even during net degradation (Luengwilai and Beckles, 2009). In contrast, a drastic reduction of expression was detected at 5 days after storage, as observed in both MaSSIII-1 and MaSSIII-2.

Western Blot Analyses of MaSSIII-1 Protein

Western blot analysis with rabbit anti-MaSSIII-1 polyclonal antibody as a probe indicated that the size of MaSSIII-1
is approximately 90.0 kDa, which is consistent with the molecular weight (90.69 kDa) as predicted by the PeptideMass program. The expression of MaSSIII-1 protein was gradually increased during banana fruit development, but drastically reduced from 0 to 30 DPH of storage (Figures 4A,B). The Western blot results was consistent with changes in starch granules (Figures 1B,C), amylopectin content (Figure 1E) and SS activity (Figure 1F) during banana development and ripening, suggesting the expression of MaSSIII-1 protein might play an important role in regulating amylopectin metabolism in banana fruit during development and ripening.

**Overexpression of MaSSIII-1 in Tomato Changes the Morphology of Starch Granules and Increases the Amylopectin Content and SS Activity**

To further examine the function of MaSSIII-1 during fruit development and ripening, MaSSIII-1 was introduced into a
The SS isoforms comprise at least four families (namely SSI, SSII, SSIII, and SSIV) in plants and play important roles in starch and amylopectin metabolism (Zhu et al., 2014). Such an observation is consistent with previous studies in rice, wheat, and Arabidopsis (Li et al., 2000; Dian et al., 2005; Busi et al., 2008; Valdez et al., 2009). Taken together, it is proposed that the four MaSS genes cloned from banana fruit may play divergent roles, with MaSSI and MaSSII being house-keeping, but MaSSIII-1 and MaSSIII-2 expression at transcription level clearly occurs at later stages of fruit development, they’re not involved in amylopectin synthesis during early phase of fruit development.

SSIII protein as a catalytic factor plays an important role in amylopectin metabolism (Zhu et al., 2014; Huang et al., 2016). SSIII contributes the major activity (80% of the total) in potato tubers (Abel et al., 1996). In Arabidopsis, loss of both SSII and SSIII caused slower plant growth and dramatically reduced starch content (Zhang et al., 2008). Defective SSIII mutations resulted in change of starch structure into large clusters with more singly branched building blocks in amylopectin in maize (Zhu et al., 2014). Inhibition of SSIII expression resulted in a drastic drop in the overall rate of starch accumulation and the proportion of amylopectin with very long chains in potato (Abel et al., 1996; Edwards et al., 1999). SSIII activity was found to affect starch phosphorylation in potato tubers (Carpenter et al., 2015). In fresh fruits, whether starch metabolism was affected by SS, as far as we know there has not been reported. In this study, MaSSIII-1 transcript in both transgenic lines was 100-fold higher than WT. SS activity, total starch content, and amylopectin content

**DISCUSSION**

Despite of the extensive studies on cereal starch, little information is available regarding the dynamics of starch accumulation (net result of starch synthesis and degradation), SS activity, and amylopectin metabolism during fruit development and storage in fresh starchy fruits, such as banana. In tomato, starch is transiently accumulated during fruit development and degraded within the lifecycle of that organ, while starch synthesis and degradation are occurring simultaneously (Luengwilai and Beckles, 2009). However, change characteristics of SS activity are unclear. In this study, a temporal modulation in starch content was found concomitant to banana fruit development and storage. The result was consistent with the report of tomato (Luengwilai and Beckles, 2009). SS activity increased as the starch granule expanded in size and amylopectin content increased during fruit development, but decreased significantly along with the degradation of amylopectin and starch granule during storage. Moreover, we found that SS activity increased later than starch and amylopectin accumulation, the same was true for transcript and protein analyses, implying SS act at later stages of fruit development while other enzymes may act in the early phases.

The SS isoforms comprise at least four families (namely SSI, SSII, SSIII, and SSIV) in plants and play important roles in amylopectin biosynthesis (Park et al., 2012). GBSS as a separate from the other SS isoforms also influences biosynthesis of extra-long unit chains of amylopectin in rice (Hanashiro et al., 2008). Six GBSS genes have been cloned and identified in banana (Miao et al., 2014). In this report, we characterized the function of MaSSIII-1 in amylopectin metabolism of banana fruit and described the putative role of the other MaSS family members. Four MaSSs could be categorized into three classes, including MaSSI, MaSSII, and MaSSIIIs (MaSSIII-1 and MaSSIII-2). This scenario seems to apply in some lower green algae species where the gene distribution of SSIV is patchy (Descamps et al., 2008). Moreover, in Arabidopsis the role of SSIV in starch granule seeding can be replaced, in part, by the phylogenetically related SSIII (Szydlowski et al., 2011). Sequence analysis indicated that the MaSSI, II, III-1, and III-2 genes showed characteristics of a typical plant SS gene (Li et al., 2000; Park et al., 2012). The MaSSI and MaSSII amino acid sequences shared three conserved regions encoding Domain I, II, and III, respectively (Park et al., 2012). Domain I is a putative N-terminal transit peptide, Domains II and III are the C-terminal catalytic domain (Park et al., 2012). The amino acid sequences of MaSSIII-1 and MaSSII-2 contained four distinct regions: a transit peptide region, a variable repeat region, a SSIII specific region, and a C-terminal catalytic domain. The SSIII domain organization was reported in wheat (Li et al., 2000).

Starch synthase paralogs show varying spatial-temporal expression (Senoura et al., 2004; Dian et al., 2005). MaSSI and MaSSII were found to express in low levels in vegetative organs, flower and banana fruits, similar to their homologs in kidney bean (Senoura et al., 2004) and rice (Jiang et al., 2004). In comparison, MaSSIII-1 and MaSSIII-2 were abundantly expressed in the late phase of developing fruit pulp, with the expression level of MaSSIII-1 being significantly higher (Figure 3A). Such an observation is consistent with the report of tomato (Luengwilai and Beckles, 2009). SSIII transcript in both transgenic lines was 100-fold higher than WT. SS activity, total starch content, and amylopectin content
were enhanced significantly by overexpressing *MaSSIII-1* gene in tomato transgenic plants at MG stage (**Figure 5**), suggesting these parameter increases may be attributed to *MaSSIII* because of increased transcript and polypeptide. Similar results were reported in wheat (Li et al., 2000) and rice (Dian et al., 2005), but this is the first report in a fresh fruit species. In addition, we found that overexpression of *MaSSIII-1* in tomato fruit showed severe cracks in the surface of starch granules and changed the starch granules morphology. This study suggests that *MaSSIII-1* is a key gene in the amylpectin biosynthesis and therefore it could be used as a useful tool for marker assisted molecular breeding in banana.

**CONCLUSION**

Starch synthase activity increased along with the amylpectin accumulation at later stages of banana fruit development, but declined during storage. Four SS genes encoding *MaSSI*, *MaSSII*, *MaSSIII-I*, and *MaSSIII-II* were cloned and characterized. Expression pattern of only *MaSSIII-I* was highly consistent with dynamic changes in starch granules, amylpectin content, and SS activity. *MaSSIII-I* transgenic lines distinctly changed the morphology of starch granules. Overexpression of *MaSSIII-I* in tomato plants significantly increased the amylpectin accumulation and SS activity in comparison to WT. This is the first report about the *MaSSIII-I* gene involved in amylpectin metabolism in a fresh fruit species. These findings establish a solid foundation to further regulate the amylpectin metabolism in banana fruits or other fresh fruits using the *MaSSIII-I* or its homologous genes.

**AUTHOR CONTRIBUTIONS**

ZJ and BX conceived and designed the experiments. HM, PS, QL, CJ, JL, and WH performed the experiments and carried out the analysis. HM, PS, and QL designed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

**ACKNOWLEDGMENTS**

This work was supported by the National Natural Science Foundation of China (31401843 and 31071788), the Modern Agro-industry Technology Research System (CARS-32), the 973 Projects (2014CB160314), and the Natural Science Foundation of Hainan Province (314100).

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00454/full

**FIGURE S1** | Sequence motifs and chromosomal localization of four *MaSS* genes in banana. (A) Structural organization of banana *MaSS* genes, solid boxes indicate exons, and bold lines represent introns, (B) Chromosomal localization of banana *MaSS* genes.

**FIGURE S2** | Expression of *SSIII-I* at different stages of development in tomato fruit. IG: immature green; MG: mature-green; BR: orange-breaker; RR: red ripening stage. The y-axis represents the relative fold-difference in mRNA level, which was calculated using the 2⁻ΔΔCt formula. The vertical bars represent the mean ± SD of three replicates. Asterisks indicate significant difference from IG stage vs. the following stages (*p < 0.05; **p < 0.01).

**TABLE S1** | Primers used in this study.

**TABLE S2** | Comparison of the deduced amino acid sequences of banana fruit *MaSSi* and *MaSSii*. The three consensus regions (Domain I, II, and III) for both starch synthases are highlighted in bold and box.

**TABLE S3** | Comparison of the deduced amino acid sequences of banana fruit *MaSSIII-I* and *MaSSIII-II*. The four consensus regions (Transit peptide region, Variable repeat region, SSIII specific region, and Catalytic domain) for both starch synthases are highlighted in bold and box.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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