Diversification of Genes Encoding Granule-Bound Starch Synthase in Monocots and Dicots Is Marked by Multiple Genome-Wide Duplication Events

Jun Cheng¹*, Muhammad Awais Khan²,⁴, Wen-Ming Qiu³, Jing Li³, Hui Zhou¹, Qiong Zhang¹, Wenwu Guo³, Tingting Zhu¹, Junhua Peng¹, Fengjie Sun⁴, Shaohua Li¹, Schuyler S. Korban²,⁴, Yuepeng Han¹⁺

¹ Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, Wuhan Botanical Garden of the Chinese Academy of Sciences, Wuhan, Hubei, China, ² Department of Natural Resources and Environmental Sciences, University of Illinois, Urbana, Illinois, United States of America, ³ Key Laboratory of Horticultural Plant Biology (Ministry of Education), Huazhong Agricultural University, Wuhan, Hubei, China, ⁴ School of Science and Technology, Georgia Gwinnett College, Lawrenceville, Georgia, United States of America

Abstract

Starch is one of the major components of cereals, tubers, and fruits. Genes encoding granule-bound starch synthase (GBSS), which is responsible for amylose synthesis, have been extensively studied in cereals but little is known about them in fruits. Due to their low copy gene number, GBSS genes have been used to study plant phylogenetic and evolutionary relationships. In this study, GBSS genes have been isolated and characterized in three fruit trees, including apple, peach, and orange. Moreover, a comprehensive evolutionary study of GBSS genes has also been conducted between both monocots and eudicots. Results have revealed that genomic structures of GBSS genes in plants are conserved, suggesting they all have evolved from a common ancestor. In addition, the GBSS gene in an ancestral angiosperm must have undergone genome duplication ~251 million years ago (MYA) to generate two families, GBSSI and GBSSII. Both GBSSI and GBSSII are found in monocots; however, GBSSI is absent in eudicots. The ancestral GBSSII must have undergone further diversification when monocots and eudicots split ~165 MYA. This is consistent with expression profiles of GBSS genes, wherein these profiles are more similar to those of GBSSII in eudicots than to those of GBSSI genes in monocots. In dicots, GBSSII must have undergone further divergence when rosids and asterids split from each other ~126 MYA. Taken together, these findings suggest that it is GBSSII rather than GBSSI of monocots that have orthologous relationships with GBSS genes of eudicots. Moreover, diversification of GBSS genes is mainly associated with genome-wide duplication events throughout the evolutionary course of history of monocots and eudicots.

Citation: Cheng J, Khan MA, Qiu W-M, Li J, Zhou H, et al. (2012) Diversification of Genes Encoding Granule-Bound Starch Synthase in Monocots and Dicots Is Marked by Multiple Genome-Wide Duplication Events. PLoS ONE 7(1): e30088. doi:10.1371/journal.pone.0030088

Editor: Debashish Bhattacharya, Rutgers University, United States of America

Received September 7, 2011; Accepted December 13, 2011; Published January 23, 2012

Copyright: © 2012 Cheng et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This research was jointly supported by National Program on Key Basic Research Project of China (973 Program, grant no. 2011CB100600), the CAS Strategic Priority Research Program (grant no. XDA01020304), and the National 948 Project from the Ministry of Agriculture of China. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: yphan@wbgcas.cn (YH); korban@illinois.edu (SK)

† These authors contributed equally to this work.

Introduction

Plant starch consists of a mixture of two different components, amylose (20-30%) and amylpectin (70-80%). Amylose is a linear polymer of glucose (Glc) residues joined together by α-1,4-glucosidic bonds, while amylpectin is a highly branched glucose polymer with α-1,6-glucosidic bonds linking linear chains. Amylose synthesis is relatively simple, and it is mainly catalyzed by granule-bound starch synthase (GBSS), which is encoded by the waxy or the GBSS gene. In contrast, the synthesis of amylpectin is rather complex and involves coordinated activities of different classes of enzymes, including soluble starch synthases (SSs), starch branching enzymes (SBEs), and starch debranching enzymes (DBEs) [1,2]. Of these enzymes, SBEs introduce α-1,6-glucosidic linkages into polyglucans, while DBEs hydrolyze α-1,6-glucosidic linkages and play an important role in determining starch structure and granule characteristics during starch biosynthesis [2]. SSs catalyze the transfer of Glc from ADP-Glucose (ADP-Glu) to non-reducing ends of glucan chains via an α-1,4-glucosidic linkage.

Genes encoding GBSS have been well characterized in starch crops as amylose content has a significant impact on physico-chemical properties of starch [3]. GBSS differs from other SS isoforms due to its localization in granules and its unique functional role in starch synthesis. Not only it can transfer glucosyl residues from ADP-Glu to glucan substrates to produce relatively long-chain amylose molecules, but it also acts on side chains of amylpectin to form long chains of amylpectin [2]. The latter activity may have been the original function of GBSS, early in its evolutionary path. Moreover, amylose synthesis may be responsible for starch density, and ultimately improving the efficiency of carbon storage, thus justifying the conservation of GBSS in higher plants [2].

In cereals, GBSS consists of two isoforms, GBSSI (also known as waxy protein) and GBSSII. The GBSSI gene is exclusively expressed in storage tissues such as endosperms and embryos of
seeds, while the GBSSI gene is expressed in non-storage tissues such as leaf, stem, root, and pericarp [4,5]. Interestingly, the ratio of amylose to amylpectin in pericarps is different from that in endosperms as endosperm starch consists of both large and small granules, while pericarp starch granules are small and relatively uniform in size [6]. Thus, it is unclear if GBSSI and GBSSII genes have diverged in their functions, thereby playing different roles in amylose synthesis. Besides cereals, different isoforms of GBSS have also been reported in eudicots. For example, a pea GBSSI consists of two isoforms, GBSSIA and GBSSIB [7]. The GBSSIA gene is expressed predominantly in embryos with transcripts detected in leaves as well [8]. The GBSSIB gene is highly expressed in leaves, but with transcripts also detectable in embryos. Therefore, the expression profiles of both GBSSIA and GBSSIB genes are different from those of GBSSI genes in cereals.

GBSS genes have been widely used in many plant species to study phylogenetic and evolutionary relationships as they are of either single- or low-copy nuclear genes. For example, Evans et al. [9] used GBSS genes to investigate the early origins of the subfamily Maloideae, belonging to the Rosaceae family, and contrary to an earlier proposed hypothesis of wide hybridization between ancestors of two other subfamilies, they proposed its aneuploidy (x = 17) origin. They also suggested that duplication of the GBSSI gene must have occurred prior to diversification of Rosaceae. Moreover, GBSS genes have also been used to study the molecular taxonomy of Rosaceae revealing that the Pyreae tribe has originated either by autopolyploidization or by hybridization between two sister taxa, followed by diploidization, and then subsequent aneuploidization [10].

Starch is not only the main component of cereal grains, legumes, and tubers, but it is also an important component of many fruits, such as apple and banana. For example, in apple, starch begins to rapidly accumulate in young fruitlets as they reach 20 mm in diameter, and starch content can be as high as 15% before ripening [11]. Similarly, banana fruit contain about 25% starch, and may serve as raw material for producing starch [12]. Moreover, fruit starch content has a strong influence on both fruit texture and processing quality [13,14]. For example, apples with low starch content taste sweet, and some varieties, such as Granny Smith, that contain high starch content are deemed good cooking apples. However, high starch content is a problem for processing of apple juice as it contributes to cloudiness of the apple juice. In order to degrade and eliminate insoluble starch, amylases that catalyze the hydrolysis of α-1,4-glucosidic linkages are widely used during processing of apple juice production. Although starch is one of the main components of fruits, and has a high influence on fruit quality, little is known about those genes involved in fruit starch synthesis. To date, only genes encoding SBEs have been isolated and characterized in apple [15,16].

Recently, whole genome sequences have been released for several fruit trees such as apple [10], peach (http://www.rosaceae.org/peach/genome), and citrus (http://www.citrusgenomedb.org/). These genome sequence databases, together with integrated physical and genetic maps of the apple [17], provide unique opportunities for genome-wide investigation of genes involved in fruit starch synthesis. Identifying and characterizing starch synthesis genes, such as GBSS, would also afford an opportunity to pursue fundamental evolutionary studies underlying differences between monocots and eudicots. As endosperms are usually absent in mature seeds of eudicots and starch accumulates in cotyledons, it is unclear as to whether or not endosperm-specific GBSSI genes in monocots have diverged from GBSS genes in eudicots. In this study, identification and characterization of GBSS genes in apple, peach, and orange have been conducted, and these have been used to perform a comprehensive study to assess the evolutionary pathway of GBSS genes in monocots and eudicots.

**Materials and Methods**

**Plant material**

Young leaves, flowers, and fruits from three fruit crops, including apple (*Malus* × *domestica* Borkh.), peach (*Prunus persica* (L.) Batsch.), and orange (*Citrus sinensis* (L.) Osbeck.) were collected in spring, while orange leaves were collected in summer. Flower buds were collected at the balloon stage. Fruits were collected at early, middle, and mature stages of development. Briefly, the early stages of peach, apple, and orange fruits were 30, 42, and 40 days after pollination (DAP), respectively. The middle stages of peach, apple, and orange fruits were 60, 105, and 150 DAP, respectively. The late stages of peach, apple, and orange fruits were 90, 168, and 220 DAP, respectively. Whole fruits were used for gene expression analysis. Both apple, belonging to the subfamily Maloideae, and peach, belonging to subfamily Amygdaloideae, are members of the Rosaceae family; while orange, belongs to the family Rutaceae. Both Rosaceae and Rutaceae families belong to rosids, and hence they are eudicots.

**Isolation of genes encoding GBSS in apple**

The coding DNA sequence of an *Arabidopsis* GBSS gene (GenBank accession no. NM_103023) was BLASTed against an apple expression sequence tag (EST) database (http://titan.bioc. iuie.edu/apple/), and three homologous EST sequences (GenBank accession nos. CN489540, CO9093202, and CN496889) were identified. Primer pairs were then designed based on the three EST sequences, and used to screen an apple BAC library (cv. GoldRush) according to a PCR-based screening protocol [15]. The three primer pairs were designated as AW×1F/AW×1R, AW×2F/AW×2R, and AW×3F/AW×3R, and their sequences were as follows: 5’-GGCCTTGGATGTTCTTGGG-3’/5’-CCTGGACACGCAAGCGTAAG-3’, 5’-TGAGACCTTGGTGATGTTCTTGGG-3’/5’-CTAGACCCGGCTGGCAGATAGAGAG-3’, and 5’-CTGCTTGTTGAGCCCTGAAGTTG-3’/5’-GGTTCGCCACAGGGAAGTTAG-3’. The PCR program consisted of 34 cycles of 30 s at 94°C, 30 s at 60°C, 60 s at 72°C, and a final extension for 5 min at 72°C.

The primer pair AW×2F/AW×2R amplified two different size bands, and two positive BAC clones containing the two different bands, respectively, were selected for subcloning. Each of the other two primer pairs amplified only a single band, and a single positive BAC clone derived from each primer pair was subjected to subcloning. BAC DNA was extracted from a 300 ml culture using the Plasmid Midi kit (QIAGEN, Valencia, CA, USA), and BAC DNA subcloning was carried out according to Han et al. [15]. Subsequently, positive subclones were sequenced using a primer-walking strategy, and genomic DNA sequences were recovered.

**Identification of genes encoding GBSS in peach and orange**

Coding sequences of genes encoding GBSS in peach and orange were downloaded from the Genome Database for Rosaceae (http://www.rosaceae.org/peach/genome) and the Citrus Genome Database (http://www.citrusgenomedb.org/), respectively. The coding sequences were then BLASTed against whole genome sequence databases to recover their corresponding genomic DNA sequences. Exon lengths were calculated by alignment of genomic DNA sequences with cDNA sequences, and introns were determined according to the “GC-AG” rule [18].
Expression profiles of GBSS genes in fruit trees using real-time PCR

Total RNA was extracted according to the protocol described by Gasic et al. [19]. Approximately 3 µg of total RNA per sample was treated with DNase I (Invitrogen Life Science), and then used for cDNA synthesis. A SYBR Green-based real-time PCR assay was carried out in a total volume of 25 µL reaction mixture containing 12.5 µL of 23 SYBR Green I Master Mix (Applied Biosystems), 0.2 µM of each primer, and 100 ng template cDNA. An actin gene was used as a constitutive control.

Amplifications were performed using a 7300 Real-Time PCR System (Applied Biosystems). The amplification program consisted of one cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescent products were detected in the last step of each cycle. Melting curve analysis was performed at the end of 40 cycles to ensure proper amplification of target fragments. Fluorescence readings were consecutively collected during the melting process from 60°C to 90°C at a heating rate of 0.5°C s⁻¹. Reaction mixes without cDNA templates were also run as negative controls to evaluate the specificity of the real-time PCR. All analyses were repeated three times using biological run as negative controls to evaluate the specificity of the real-time PCR. All analyses were repeated three times using biological replicates. Differences in cycle threshold (Ct) values were used to compare expression levels of target genes.

Table 1. Primers for qPCR analysis of GBSS genes in apple, peach, and orange

| Species | Gene name | Forward primer | Reverse primer |
|---------|-----------|----------------|----------------|
| Apple   | MdGBSSI-1 | 5'-CTGCCGTTCTCTTGGTACCAGTG-3' | 5'-GCAACAGCTCTCTTGGACAGTAGT-3' |
|         | MdGBSSI-2 | 5'-GCCCAATGAGAAGCAGCTTTA-3' | 5'-CGCAGTGTTGACCGACCAATG-3' |
|         | MdGBSSI-3 | 5'-GGTGTAGGTCACTCCTTGGTTG-3' | 5'-CTCTTGTACCTGAGGTG-3' |
| MdActin | 5'-CTCAACAGTTCTCAGCTCAGCTAT-3' | 5'-TGGAGCATGTGAGAGATTT-3' |
| Peach   | PpGBSSI-1 | 5'-TATGGCAAGCTCTTGGTACCAGTG-3' | 5'-CATCTGTGGGCCTACAATG-3' |
|         | PpGBSSI-2 | 5'-ATGGCGTTACGGAACTGTGC-3' | 5'-TGGAGCATGTGAGAGATTT-3' |
|         | PpActin  | 5'-GCTTGTAGGTCACTCCTTGGTTG-3' | 5'-GCAACAGCTCTCTTGGACAGTAGT-3' |
| Citrus  | CSGBSSI-1 | 5'-GCAAGATTCTCTTGGTGCTAG-3' | 5'-AAAGCCCCCTGAAATCATC-3' |
|         | CSGBSSI-2 | 5'-GGGAGCTTTGTTGCTAGTCG-3' | 5'-TCATGACAGCTGGGCCTAATC-3' |
|         | CaActin  | 5'-CCAAGCAGCATGAAATGAAA-3' | 5'-ATGCTGGAAGGAGTGCTAGA-3' |

Results

Genomic sequences of GBSS genes in apple, peach, and orange

In apple, three genes encoding GBSS, designated as MdGBSSI-1, MdGBSSI-2, and MdGBSSI-3 with GenBank accession nos. JN187080, JN187081, and JN187082, respectively, were isolated and sequenced in this study. Both MdGBSSI-1 and MdGBSSI-3 are very closely related and share 92% identity in coding DNA sequences. Both MdGBSSI-1 and MdGBSSI-3 show 74% identity in coding DNA sequences with MdGBSSI-2. These three genes are conserved in their genomic structures (Table 2). Both MdGBSSI-1 and MdGBSSI-3 contain 13 exons (Fig. 1A), with all exons, except for exon 9, having identical lengths. MdGBSSI-2 consists of 12 exons, with exons 2 to 5, 8, and 10 to 13 being of identical lengths to those of either MdGBSSI-1 or MdGBSSI-3. However, exon 6 of MdGBSSI-2 is equal to the combined lengths of exons 6 and 7 of GBSS genes of both eudicots and monocots, thus indicating that an intron loss event must have occurred during the evolution of the apple MdGBSSI-2 gene. Moreover, a pseudogene, designated as MdGBSSI-4, has also been isolated in this study. This MdGBSSI-4 gene consists of a GBSS-like fragment, a repetitive sequence, and a typical poly (A) tail at its 3’ end (Fig. 1A). The GBSS-like fragment of 804 bp in size shows a high nucleotide sequence identity (96%) with seven combined exons of the MdGBSSI-2 gene, including a partial sequence of exon 1, exons 2 to 6, and a partial sequence of exon 7, while it has only 75% nucleotide sequence identity with coding sequences of either MdGBSSI-1 or MdGBSSI-3. Therefore, MdGBSSI-4 may have originated from MdGBSSI-2 via a retroposition mechanism (Fig. 1B) [24]. In addition, DNA sequences of MdGBSSI-1, MdGBSSI-2, and MdGBSSI-3 were BLASTed against the apple genome sequence database, and a GBSS pseudogene, designated MdGBSSI-5, was identified. The MdGBSSI-5 contains frame shifts and stop codons in exon 6.

In both peach and orange genomes, there are two genes encoding GBSS. The peach PpGBSSI-1 and PpGBSSI-2 share 74% identity in coding DNA sequences. Each of PpGBSSI-1 and
Table 2. Exon lengths (bp) of genes encoding GBSS in plants*

| No. | Apple | Citrus* | Maize | Rice* | Potato | Arabidopsis |
|-----|-------|---------|-------|-------|--------|-------------|
|     | MdGBSSII-1 | MdGBSSII-2 | MdGBSSII-3 | ZmGBSSII | ZmGBSSII | OsGBSSI |
| 1   | 351   | 357     | 366   | 399   | 307    | 333        |
| 2   | 8     | 18      | 18    | 18    | 18     | 18         |
| 3   | 9     | 99      | 99    | 99    | 99     | 99         |
| 4   | 92    | 90      | 90    | 90    | 90     | 90         |
| 5   | 94    | 94      | 94    | 94    | 94     | 94         |
| 6   | 64    | 64      | 64    | 64    | 64     | 64         |
| 7   | 101   | 101     | 101   | 101   | 101    | 101        |
| 8   | 180   | 177     | 177   | 177   | 177    | 177        |
| 9   | 184   | 174     | 174   | 174   | 174    | 174        |
| 10  | 192   | 192     | 192   | 192   | 192    | 192        |
| 11  | 87    | 87      | 87    | 87    | 87     | 87         |
| 12  | 129   | 129     | 129   | 129   | 129    | 129        |
| 13  | 117   | 117     | 117   | 117   | 117    | 117        |
|     |       |         |       |       |       |             |
| 1   | 351   | 351     | 351   | 351   | 351    | 351        |
| 2   | 8     | 8       | 8     | 8     | 8      | 8          |
| 3   | 9     | 9       | 9     | 9     | 9      | 9          |
| 4   | 92    | 92      | 92    | 92    | 92     | 92         |
| 5   | 94    | 94      | 94    | 94    | 94     | 94         |
| 6   | 64    | 64      | 64    | 64    | 64     | 64         |
| 7   | 101   | 101     | 101   | 101   | 101    | 101        |
| 8   | 180   | 177     | 177   | 177   | 177    | 177        |
| 9   | 184   | 174     | 174   | 174   | 174    | 174        |
| 10  | 192   | 192     | 192   | 192   | 192    | 192        |
| 11  | 87    | 87      | 87    | 87    | 87     | 87         |
| 12  | 129   | 129     | 129   | 129   | 129    | 129        |
| 13  | 117   | 117     | 117   | 117   | 117    | 117        |

*The first and last exons are represented as the encoding sequence, and GenBank accession nos. are the same as those used in Fig. 3. Those exons that are derived from two combined exons are highlighted in bold.

Expression profiles of GBSS genes in apple, peach, and orange

Real-time PCR results of GBSS genes of apple cv. Golden Delicious revealed that the three GBSS genes, MdGBSSII-1 to MdGBSSII-3, were expressed in all analyzed tissues, including young leaves, flowers, and fruits (Fig. 2). Overall, MdGBSSII-3 transcripts were the lowest in all analyzed tissues when compared to those of both MdGBSSII-1 and MdGBSSII-2. Moreover, expression levels of MdGBSSII-2 in both leaves and flowers were higher than those of MdGBSSII-1 and MdGBSSII-3. Whereas, expression levels of MdGBSSII-1 in apple fruit at different stages of development were higher than those of either MdGBSSII-2 or MdGBSSII-3. Throughout apple fruit development, levels of both MdGBSSII-1 and MdGBSSII-2 transcripts were relatively low at the young fruitlet stage, but then increased as fruit continued to develop and reached maturity.

When transcripts of GBSS were investigated in orange cv. Honganlucheng, expression levels of CsGBSSII-1 were too low for detection in all analyzed tissues, including leaves, flowers, and fruits. In contrast, CsGBSSII-2 was expressed in all analyzed tissues. The expression level of CsGBSSII-2 in leaves was higher than those in either flowers or fruits (Fig. 2).

Analysis of GBSS expression in peach cv. Huyou 018 revealed that transcripts of PpGBSSI-1 and PpGBSSI-2 were detected in all analyzed tissues, and these were higher in fruits than in either leaves or flowers (Fig. 2). PpGBSSI-2 was primarily expressed in leaves and young fruitlets; however, its levels of expression in both leaves and fruits were lower than those of PpGBSSI-1. Transcripts of PpGBSSI-2 were relatively high at the young fruitlet stage, but then decreased as fruit continued to develop and reached maturity. PpGBSSI-1 transcripts were relatively low in fruit at middle development stage, during the period of endocarp hardening, as compared to early and mature stages of development.

Genomic structure of GBSS genes in monocots and eudicots

Genomic sequences of genes encoding GBSSI and GBSSII have been isolated from both eudicots, such as Arabidopsis and potato, as well as monocots, such as rice, maize, wheat, and barley. All these GBSS genes, including rice OsGBSSI and OsGBSSII, maize ZmGBSSI and ZmGBSSII, potato StGBSSI, apple MdGBSSII-1 and MdGBSSII-3, peach PpGBSSI-1 and PpGBSSI-2, and orange CsGBSSII-2, contain 13 exons, and their exon profiles are strikingly similar, with exons 2 through 7 and 10 through 13 being of identical lengths (Table 2). Whereas, wheat TaGBSSI, barley HeGBSSI, and Arabidopsis AtGBSS have either one or two less exons compared with GBSSI or GBSSII genes in other plants such as rice, maize, and peach. Exon 4 of GBSSI in both wheat and barley is equal in length to the two combined exons 4 and 5 of GBSSI or GBSSII in rice and maize. Likewise, exon 6 of GBSSI in wheat and barley is equal in length to the two combined exons 4 and 5 of GBSSI or GBSSII.
exons 7 and 8 of GBSSI or GBSSII in rice and maize. Thus, two introns must have been lost during the evolutionary process of GBSSI genes in wheat and barley. Moreover, exon 10 of Arabidopsis AtGBSS is equal in length to the two combined exons 10 and 11 of GBSSI or GBSSII in other plants, suggesting that a single intron must have been lost during the evolutionary path of AtGBSS genes. Taken together, these results indicate that GBSS genes in both eudicots and monocots are conserved in genomic structure, and they have all evolved from a common ancestor.

Timeline of GBSS evolution in higher plants

The evolutionary history of GBSS genes was assessed across monocots and eudicots, revealing multiple duplication events (Fig. 3). The ancestral GBSS gene in early monocots and dicots must have undergone duplication, ∼251 million years ago (MYA), to generate the two gene families GBSSI and GBSSII. The next major divergence of GBSS must have occurred when monocots and dicots split ∼165 MYA. As monocots have not undergone any major events until speciation of grasses, beginning ∼47–54 MYA, this must have contributed to recovery of various orthologs of GBSSI and GBSSII. Subsequently in dicots, GBSS once again must have diverged when rosids and asterids split from one another ∼126 MYA. While GBSS in asterids must have retained isoforms of GBSS genes resulting from that divergence, a major duplication event that has occurred ∼119 MYA must have also generated two isoforms of GBSS. Any further divergence has represented speciation events. These results suggest that there are no new additional isoforms of GBSS that have evolved following the well-known pome fruit origin event that has occurred ∼27 to 35 MYA. However in apple, a duplication event that has occurred ∼18 MYA has generated paralogs of GBSS. Meanwhile, one of the dicot-specific GBSS isoforms has been lost in Arabidopsis.

In eudicots, multiple GBSS genes within the same species exhibit relatively high sequence divergence although they still have higher identities with each other than with either GBSSI or GBSSII genes from monocots. For example, apple MdGBSSI-1 and MdGBSSI-2 share 74% identity in amino acid sequences, while they show 59 to 68% identity in amino acid sequences with either GBSSI or GBSSII of monocots. Also, peach PpGBSSI-1 and PpGBSSI-2 share 76% identity in amino acid sequences, but show 60 to 66% identity in amino acid sequences with GBSSI or GBSSII of monocots. Again, orange CsGBSSI-1 and CsGBSSI-2 share 71% identity in amino acid sequences, while they show 59 to 66% identity in amino acid sequences with GBSSI or GBSSII of monocots. Finally, pea PsGBSSIa and PsGBSSIb share 68% identity in amino acid sequences, yet they have only 55 to 64% identity in amino acid sequences with GBSSI or GBSSII from monocots.

Overall, these findings demonstrate that genes encoding GBSS in plants are grouped into two clusters, GBSSI and GBSSII, and that genes encoding GBSSI are exclusive to monocots. Moreover, GBSS genes in eudicots share a common ancestor with GBSSII rather than with GBSSI of monocots.
Diversification of GBSS genes in monocots and eudicots is mainly associated with genome duplication events.

It has been well documented that a whole-genome duplication (WGD) event must have occurred in an angiosperm ancestor 150 to 270 MYA [25]. Thus, duplication of the ancestral GBSS gene must have likely been the result of the WGD of the angiosperm ancestor. Based on findings in this study, the GBSS gene must have subsequently undergone duplication into the two families of GBSSI and GBSSII ~251 MYA (Fig. 3). In monocots, GBSSI and GBSSII have been isolated from many plants, including rice, wheat, barley, maize, sorghum, foxtail millet, and miscanthus [26,27,28,29,30,31]. In this study, divergence of GBSSI and GBSSII in monocots must have occurred 47 to 54 MYA. This result is consistent with a previous report suggesting that radiation of grasses has occurred 50 to 70 MYA [32]. Moreover, findings in this study have revealed that all GBSS genes in eudicots belong to

Figure 2. Analysis of expression profiles of genes encoding GBSS in apple, peach, and orange using real-time PCR. L, young leaves; FW, flowers; FTI, young fruitlets; FTI, fruits at mid-developmental stage; and FTII, fruits at maturity.
doi:10.1371/journal.pone.0030088.g002

Discussion

Diversification of GBSS genes in monocots and eudicots is mainly associated with genome duplication events.

It has been well documented that a whole-genome duplication (WGD) event must have occurred in an angiosperm ancestor 150 to 270 MYA [25]. Thus, duplication of the ancestral GBSS gene must have likely been the result of the WGD of the angiosperm ancestor. Based on findings in this study, the GBSS gene must have subsequently undergone duplication into the two families of GBSSI and GBSSII ~251 MYA (Fig. 3). In monocots, GBSSI and GBSSII have been isolated from many plants, including rice, wheat, barley, maize, sorghum, foxtail millet, and miscanthus [26,27,28,29,30,31]. In this study, divergence of GBSSI and GBSSII in monocots must have occurred 47 to 54 MYA. This result is consistent with a previous report suggesting that radiation of grasses has occurred 50 to 70 MYA [32]. Moreover, findings in this study have revealed that all GBSS genes in eudicots belong to
the GBSSI family. As GBSSI is absent in eudicots, this suggests the GBSSI subfamily has been lost during the evolutionary process of eudicots.

It has been reported that WGD must have also occurred in an ancestral eudicot, 125 MYA [25]. Based on this study, the GBSS gene in rosids must have undergone duplication into two isoforms, 119 MYA. For example, there are two copies of GBSS genes in genomes of both peach and orange. Moreover, Edwards et al. [7] have confirmed presence of two isoforms of GBSS in pea. Therefore, duplication of the GBSS gene in rosids must have also resulted from the WGD event. Thus, duplication of the GBSS gene has likely occurred prior to divergence of rosids and asterids. The Arabidopsis has undergone WGD [33], and thus the key model eudicot plant species A. thaliana should also contain two copies of GBSS genes. Upon search, only a single GBSS gene has been identified in the Arabidopsis genome (http://www.arabidopsis.org/). In a previous study, we have reported that a starch branching enzyme encoding gene, SBEI, has been lost in the Arabidopsis genome [15]. Thus, it is quite likely that a GBSS gene has also been lost during the speciation of Arabidopsis as silencing of a GBSS gene is not lethal.

It is worth noting that there are more than two copies of the GBSS gene found in apple. DNA sequences of three genes, MdGBSSII-1 to MdGBSSII-3, have been BLASTed against the apple genome sequence database, and a GBSS pseudogene MdGBSSII-5 has been identified on chromosome 16. The MdGBSSII-3 shares 92% identity in genomic DNA sequence with

---

**Figure 3. Estimated divergence time between GBSS genes in plants based on aligned nucleotide sequences using Bayesian MCMC analysis.** A relaxed molecular clock approach, uncorrected lognormal distribution model, Yule model of speciation process, and the HKY model of nucleotide substitution were used. The Beast 1.5.4. was run for four independent times, each with 20,000,000 MCMC steps, and sampled once every 1000 generation. The molecular clock was calibrated using the divergence of rice-maize (31 ± 6.0 MYA) and apple-orange (106 ± 4.0 MYA). Numbers at each node represent estimated time in million years (MYA). The gray circle shows the duplication event in the ancestors of both monocots and dicots resulting in 2 copies of GBSS gene while divergence between monocots and dicots and rosids and asterids are represented by double-headed arrows. Clades shaded in green and grey correspond to GBSSI and GBSSII genes in monocots, respectively. Yellow shade indicates GBSS gene duplication in the apple genome. GBSS genes identified in this study are highlighted in red. The GenBank accession numbers of previously reported GBSS genes are listed as follows: Manihot esculenta GBSS (X74160), Arabidopsis thaliana GBSS (AY123983), Antirrhinum majus GBSS (AJ006293), Ipomoea batatas GBSS (IBU44126), Solanum tuberosum GBSS (EU403426), Phaseolus vulgaris GBSS (AB029546), Pismum sativum GBSSIa (X88799), Pismum sativum GBSSIIb (AJ345045), Triticum aestivum GBSSI (AF109395), Triticum aestivum GBSSII (AB019624), Hordeum vulgare GBSSI (AK368223), Hordeum vulgare GBSSII (AF474373), Zea mays GBSSI (NM_001111531), Zea mays GBSSII (NM_001112569), Sorghum bicolor GBSSI (EF472254), Sorghum bicolor GBSSII (SBU23945), Oryza sativa GBSSI (AY069940), Oryza sativa GBSSII (AF141955), Miscanthus japonicus GBSSI (AF446083), Setaria italica GBSSI (AB089141), Secale cereale GBSSI (FJ491377), Citrus sinensis CsGBSSI-1 (JN936858), Citrus sinensis CsGBSSII-2 (JN936859), Prunus persica PpGBSSI-1 (JN936860), Prunus persica PpGBSSI-2 (JN936862), and Chlamydomonas reinhardtii GBSS (AF026420).

doi:10.1371/journal.pone.0030088.g003
Genes encoding GBSS in eudicots have an orthologous relationship with GBSSI rather than GBSSII genes in monocots

Comparisons of coding sequences and expression profiles of GBSS genes between species of monocots and eudicots strongly suggest that GBSSI rather than GBSSII genes in monocots have orthologous relationships with GBSS genes in eudicots. GBSSI and GBSSII genes in monocots have diverged greatly, sharing ~63% identity in nucleotide sequences. GBSSI genes in monocots and GBSSII genes in eudicots share 59–67% identity in coding DNA sequences. Whereas, coding sequences of GBSSI genes in monocots share 67–70% identity with those of GBSS genes in eudicots. Thus, GBSS genes in eudicots show slightly higher identity in nucleotide sequences with GBSSI genes than with GBSSII genes of monocots.

In monocots, GBSSI genes are exclusively expressed in reproductive tissues such as endosperm and pollen; whereas, GBSSII genes are expressed in both vegetative and reproductive tissues including leaf, stem and pericarp [4,5]. In this study, GBSSII genes in apple, peach, and orange are expressed in both vegetative and reproductive tissues, including leaves, flowers, and fruits, and thus are similar to GBSSI genes in monocots. Moreover, while the pea GBSSII gene is expressed in all tissues such as leaves, pods, roots, embryos, stigmas, and flowers, the pea GBSSI gene is expressed in leaves, pod, roots, and embryos, but not in flowers and stigmas [36]. Overall, expression profiles of GBSSII genes in eudicots are more similar to those of GBSSI than to GBSSI genes of monocots. Based on divergence time estimation, GBSSII genes in eudicots are also more closely related to GBSSI genes than to GBSSI genes of monocots (Fig. 5).

Expression of GBSS genes is likely related to starch accumulation in fruit

In this study, it is observed that expression of GBSS genes is likely related to starch content in fruit, and that high levels of expression of GBSS genes contribute to starch accumulation in apple fruit. As starch accumulates in apple fruit throughout development, it is a major component of apple fruit [11]. In contrast, sugars are the main carbohydrates in both peach and orange fruits [37,38]. In apple fruit, all three GBSS genes, MdGBSSII-1 to MdGBSSII-3, are highly expressed during all developmental stages. In peach fruit, the PpGBSSIII-1 gene is highly expressed during early and late stages of development; while, the PpGBSSIII-2 gene is expressed only in early developing fruit, but its transcripts are not detectable during later stages of fruit development. In orange fruit, the CsGBSSII-2 gene is only weakly expressed throughout fruit development, while the CsGBSSII-1 transcript is not detected at any stage of fruit development. These findings suggest that different GBSS genes in eudicot fruit species, belonging to Rosaceae (apple and peach) and Rutaceae (orange) families, contribute to starch accumulation in fruit, but at different stages of development and at varying levels.

Divergence analysis has revealed that the orange CsGBSSII-2 and the pea PgGBSSI genes must have shared a common ancestor ~82 MYA (Fig. 3). Both genes are predominately expressed in leaves [7]. However, the orange CsGBSSII-1 gene shares higher nucleotide sequence identity with the pea PgGBSSI gene, which is predominately expressed in embryos [8], than with the pea PgGBSSI gene. In this study, when expression profiles of CsGBSSII-1 are investigated in two citrus cultivars, Hongan-cheng and Clementine, a tangerine, transcripts of CsGBSSII-1 are either non-detected or very weak, respectively. Moreover, when the coding sequence of CsGBSSII-1 is blasted against the citrus EST database at NCBI, no matching ESTs are identified. Given the fact that citrus fruits accumulate little amount of starch, it is reasonable to suggest that throughout the evolutionary history of citrus, the orange CsGBSSII-1 gene has lost its function due to absence of selection pressure. It is likely that this loss of function of the GBSS gene has also occurred in potato as well.

Retropseudogenes derived from mRNA encoding GBSS in apple

In this study, we have reported for the first time on the presence of retropseudogenes in the apple genome. Retropseudogenes, also known as processed pseudogenes, are generated by reverse transcription of processed mRNAs that randomly insert the resulting cDNAs into the genome [39]. These retropseudogenes contain several distinguished features, including Poly-(A) tails at their 3’ ends, lack of promoters and introns, and presence of flanking short direct repeats [40]. To date, retropseudogenes have been extensively investigated in the human genome [41,42,43] with only few reports on retropseudogenes in plants.

The apple MdGBSSII-4 lacks introns and possesses a Poly-(A) tail at its 3’ end. It shares 75%, 96%, 75%, and 92% identity in nucleotide sequences with MdGBSSII-1, MdGBSSII-2, MdGBSSII-3, and MdGBSSII-5, respectively. Thus, it is apparent that MdGBSSII-4 shows higher nucleotide sequence identity with MdGBSSII-2 than with MdGBSSII-1, MdGBSSII-3, or with the pseudogene MdGBSSII-5. Therefore, MdGBSSII-4 represents a retropseudogene found in the apple genome, and it must have been derived from mRNA of the functional gene MdGBSSII-2 through the mechanism of retroposition (Fig. 1B) [44]. When the DNA sequence of the retropseudogene MdGBSSII-4 is blasted against the apple genome sequence database, two additional homologues of MdGBSSII-4 are detected in the apple genome. MdGBSSII-4 and its two homologues share 95% identity in nucleotide sequences, and they are all located on chromosome 9.

The two homologues are clustered within a 26 kb region, and they are ~14 Mb apart from MdGBSSII-4. Multiple copies of the retropseudogene found on the same chromosome may be attributed to the following two factors. First, sequences of MdGBSSII-4 have been compared with the apple EST database, identifying an EST (GenBank accession number EG631210) that is almost identical with one of the MdGBSSII-4 homologues. The expressed MdGBSSII-4 homologue may be located downstream of a promoter region, and its mRNA transcript can thus be transcribed and subsequently incorporated back into the apple genome, leading to expansion of retropseudogenes. Second,
GBSSI and SBEIIb genes may represent unique evolutionary processes of the starch biosynthetic pathway in grasses

Findings in this study as well as those reported in previous studies suggest that GBSSI and SBEIIb genes, exclusively expressed in endosperms of monocots, may play critical roles in the evolution of the starch biosynthesis pathway in cereal grains. Earlier, it has been reported that there is an evolutionary disparity in SBEII genes between monocots and eudicots [16]. In monocots, the SBEIIb gene has duplicated into SBEIIa and SBEIIb prior to the radiation of grasses (Poaceae); while in eudicots, the duplication of SBEIIb genes has followed the process of speciation. SBEIIa in monocots has shown orthologous relationships with SBEII genes in eudicots; moreover, SBEIIb in monocots is likely derived from the duplication of SBEIIa. However, SBEIIb may have diverged in its function from SBEIIa, wherein SBEIIb plays an important role in branching short chains during starch biosynthesis in endosperms, while SBEIIa is responsible for starch-branching activity in leaves [45]. As reported in this study, GBSSI genes in monocots and GBSSI genes in eudicots have orthologous relationships, suggesting these are derived from the common ancestor of monocots and eudicots. Our finding that GBSSI is absent in eudicots may be due to the fact endosperms are usually absent in mature eudicot seeds. Thus, GBSSI has remained specific to monocots, and leading to a separate evolutionary path for GBSSI genes in eudicots. GBSSI and GBSSI genes in monocots may have also diverged in function as well. GBSSI transcripts are predominantly expressed in endosperm and pollen, while GBSSI transcripts are expressed in non-storage tissues, including leaf, stem, and pericarp [4]. As stated above, endosperms are usually absent in mature seeds of eudicots and starch accumulates in cotyledons as seeds develop. This evolutionary path can be attributed to the fact that amylase synthesis can contribute to increased density of starch, and thereby leading to increased efficiency in carbon storage [2].

Besides GBSSI and SBE genes, SS and DBE genes are also involved in starch synthesis. Based on the phylogenetic analysis of SS and DBE genes conducted in this study, there are no differences in duplication patterns between monocots and eudicots (data not shown). For example, DBE in plants can be divided into two families, pullulanase (PULL) and isoamylase (ISA); and the latter has three ISA isoforms, including ISA1, ISA2, and ISA3 [46,47]. Although the four types of DBE genes, including PULL, ISA1, ISA2, and ISA3, are present in both eudicots and monocots, DBE genes of the same type, from both monocots and eudicots, are clustered together. It is likely that SS has four isoforms in plants, including SSI, SSII, SSIII, and SSIV. SS genes encoding the same isoform from monocots and eudicots are also clustered together. Therefore, GBSSI and SBEIIb genes may correspond to unique evolutionary processes undertaken in the starch biosynthetic pathway in grasses (Poaceae). It may be feasible to produce novel starch by transforming GBSSI and/or SBEIIb genes of monocots into eudicot crops, such as those of cassava and potato. In addition, ISA1 and ISA2 proteins function together in a complex in tuber, leaf, and cotyledon of dicots [48], while both homomeric ISA1 and heteromeric ISA1/ISA2 complexes have been reported in endosperm of monocots [49]. This result suggests that ISA1 gene may also have diverged in functionality between monocots and dicots.

Acknowledgments

We would like to thank Bo Yang and Junjun Ma for their assistance with peach material collection.

Author Contributions

Conceived and designed the experiments: YH. Performed the experiments: JC MK. W-MQ, HZ QZ. Analyzed the data: JC MK TZ. Contributed reagents/materials/analysis tools: MK JP WG FS SL SK. Wrote the paper: JC MK SK YH.

References

1. Nakamura Y (2002) Towards a better understanding of the metabolic system for amylase starch biosynthesis in plants: rice endosperm as a model tissue. Plant Cell Physiol 43: 718–725.
2. Zeeman SC, Kossmann J, Smith AM (2010) Starch: Its metabolism, evolution, and biotechnological modification in plants. Annu Rev Plant Biol 61: 209–234.
3. Takahiro N, Nishiba Y, Sato T, Suda I (2003) Properties of starches from several low-amylase rice cultivars. Cereal Chem 80: 193–197.
4. Vinrent PL, Nakamura T (2000) Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. Plant Physiol 122: 255–264.
5. Dian W, Jiang H, Chen Q, Liu F, Wu P (2003) Cloning and characterization of the granule-bound starch synthase II gene in rice: gene expression is regulated by the nitrogen level, sugar and circadian rhythm. Planta 218: 261–268.
6. Nakamura T, Yamamori M, Hirano H, Hidaka S, Nagamine T (1995) Production of waxy (amylose-free) wheats. Mol Gen Genet 248: 253–259.
7. Edwards A, Vincken JP, Suurs LC, Visser RG, Zeeman S, et al. (2002) Discrete forms of amylose are synthesized by isoforms of GBSSI in pea. Plant Cell 14: 1767–1783.
8. Denyer K, Barber LM, Edwards EA, Smith AM, Wang TL (1997) Two isoforms of the GBSSI class of granule-bound starch synthase are differentially expressed in the pea plant (Pisum sativum L.). Plant Cell Environ 20: 1566–1572.
9. Evans RC, Alice LA, Campbell CS, Kellogg EA, Dickinson TA (2000) The granule-bound starch synthase (GBSSI) gene in the Rosaceae: multiple loci and phylogenetic utility. Mol Phylogenet Evol 17: 388–400.
10. Velasco R, Zakharkin A, Aliouret J, Dhingra A, Cestaro A, et al. (2010) The genome of the domesticated apple (Malus xdomestica Borkh.). Nat Genet 42: 833–839.
11. Magen H, Leurquin D (2000) Changes in amylose, amylopectin and total starch content in Jonagold apple fruit during growth and maturation. Acta Horticult 517: 47–55.
12. Bello-Pérez LA, Agama-Acevedo E, Sánchez-Hernández L, Paredes-López O (1999) Isolation and partial characterization of banana starches. J Agric Food Chem 47: 854–857.
13. Carrin ME, Ceci LN, Lozano JE (2004) Characterization of starch in apple juice matrix (by amylograph: candy texture, crust texture and degradation by amylases). Food Chem 87: 175–178.
14. Stevenson DG, Domote PA, Jane J (2006) Structures and functional properties of apple (Malus domestica Borkh.) fruit starch. Carbohydr Polym 63: 432–441.
15. Han Y, Gasic K, Sun F, Xu M, Korban SS (2007) A gene encoding starch branching enzyme I (SBEI) in apple (Malus xdomestica, Rosaceae) and its phylogenetic relationship to Sbe genes from other angiosperms. Mol Phylogenet Evol 43: 832–863.
16. Han Y, Bendik F, Sun FJ, Gasic K, Korban SS (2007b) Genomic isolation of genes encoding starch branching enzyme II (SBEII) in apple: toward characterization of evolutionary disparity in SbeII genes between monocots and eudicots. Planta 226: 1263–1276.
17. Han Y, Zheng D, Vimalmangakang S, Khan MA, Bever JE, et al. (2011) Integration of physical and genetic maps in apple confirms whole-genome and segmental duplications in the apple genome. J Exp Bot: doi:10.1093/jxb/err215.
18. Breathnach R, Chambon P (1981) Organization and expression of eukaryotic split genes coding for proteins. Annu Rev Biochem 50: 349–383.
19. Gasic K, Hernandez A, Korban SS (2008) RNA Extraction from different apple tissues rich in polyphenols and polysaccharides for cDNA library construction. Plant Mol Biol Rep 22: 437–438.
20. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1797–1800.
21. Drummond AJ, Rambaut A (2007) BEAST: bayesian evolutionary analysis sampling trees. BMC Evol Biol 7: 214.
22. Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22: 160–174.
23. Rambaut A, Drummond AJ (2011) Tracer v1.5 [computer program]. Available: http://beast.bio.ed.ac.uk/tracer. Accessed 2011 July 11.
24. Pavlicek A, Gentles AJ, Paces J, Paces V, Jurka J (2005) Retroposition of processed pseudogenes: the impact of RNA stability and translational control. Trends Genet 22: 69–73.
25. Pavlicek A, Gentles AJ, Paces J, Paces V, Jurka J (2005) Retroposition of processed pseudogenes: the impact of RNA stability and translational control. Trends Genet 22: 69–73.
26. Yao J, Wickett NJ, Ayasmalayam S, Chandler AB, Landherr L, et al. (2011) Ancestral polyplody in seed plants and angiosperms. Nature 473: 97–100.
26. Wessler SR, Varagona MJ (1985) Molecular basis of mutations at the waxy locus of maize: correlation with the fine structure genetic map. Proc Natl Acad Sci USA 82: 4177–4181.

27. Wang Z, Zheng F, Shen G, Gao J, Sunstad DP, et al. (1995) The amylose content in rice endosperm is related to the post-transcriptional regulation of the waxy gene. Plant J 7: 613–622.

28. Hylton CM, Denyer K, Keeling PL, Chang MT, Smith AM (1996) The effect of waxy mutations on the granule-bound starch synthases of barley and maize endosperms. Planta 198: 230–237.

29. McIntyre CL, Drenth J, Gonzalez N, Henzell RG, Jordan DR (2000) Molecular characterization of the waxy locus in sorghum. Genome 51: 524–533.

30. Sattler SE, Singh J, Haas EJ, Guo L, Sarath G, et al. (2009) Two distinct waxy alleles impact the granule-bound starch synthase in sorghum. Mol Breed 24: 349–359.

31. Matsumoto T, Tanaka T, Sakai H, Amano N, Kanamori H, et al. (2011) Comprehensive sequence analysis of 12 clone libraries. Plant Physiol 156: 20–28.

32. Kellogg EA (2001) Evolutionary history of the grasses. Plant Physiol 125: 1198–1205.

33. Ermolaeva MD, Wu M, Eisen JA, Salzberg SL (2003) The age of the Arabidopsis thaliana genome duplication. Plant Mol Biol 51: 859–866.

34. Korban SS, Tartarini S (2009) Apple structural genomics. In: Folta K, Gardiner S, eds. Genetics and Genomics of Rosaceae, Springer, Dordrecht, The Netherlands. pp. 85–119.

35. Wolfe J, Wehr WJA (1988) Rosaceous Chamaebatiaria-like foliage from the Paleogene of western North America. Aliso 12: 177–200.

36. Dry I, Smith A, Edwards A, Bhattacharrya M, Dunn P, et al. (1992) Characterization of cDNAs encoding two isoforms of granule-bound starch synthase which show differential expression in developing storage organs of pea and potato. Plant J 2: 193–202.

37. Lo Bianco R, Rieger M (2002) Partitioning of sorbitol and sucrose catabolism within peach fruit. J Am Soc Hortic Sci 127: 115–121.

38. Kelebek H, Selli S (2011) Determination of volatile, phenolic, organic acid and sugar components in a Turkish cv. Dortyol (Citrus sinensis L. Osbeck) orange juice. J Sci Food Agric 91: 1855–1862.

39. Vanin EF (1985) Processed pseudogenes: Characteristics and evolution. Annu Rev Genet 19: 253–272.

40. Long M (2001) Evolution of novel genes. Curr Opin Genet Dev 11: 673–680.

41. Graur D, Shuda Y, Li W (1989) Deletions in processed pseudogenes accumulate faster in rodents than in humans. J Mol Evol 28: 279–285.

42. Ophir R, Graur D (1997) Patterns and rates of indel evolution in processed pseudogenes from humans and marmots. Gene 203: 191–202.

43. Gonçalves I, Duret L, Menchirou D (2000) Nature and structure of human genes that generate retropseudogenes. Genome Res 10: 672–678.

44. Kaessmann H, Vinsckenbosch N, Long M (2009) RNA-based gene duplication: mechanistic and evolutionary insights. Nature Rev Genet 10: 19–31.

45. Blauth SL, Yao Y, Khúcinec JD, Shannon JC, Thompson DR, et al. (2001) Identification of mutator insertional mutants of starch-branching enzyme 2a in corn. Plant Physiol 125: 1396–1405.

46. Kubo A, Fujita N, Harada K, Matsuda T, Satab H, et al. (1999) The starch-debranching enzymes isoamylase and pullulanase are both involved in amylopectin biosynthesis in rice endosperm. Plant Physiol 121: 399–409.

47. Burton RA, Jenner H, Carrangis L, Fahy B, Fincher GB, et al. (2002) Starch granule initiation and growth are altered in barley mutants that lack isoamylase activity. Plant J 31: 97–112.

48. Waterbled F, Dong Y, Dumez S, Delvalle D, Planchot V, et al. (2005) Mutants of Arabidopsis lacking a chloroplastic isoamylase accumulate phytylglycogen and an abnormal form of amylpectin. Plant Physiol 138: 194–195.

49. Kubo A, Colleoni C, Dingés JR, Liu Q, Lappe RR, et al. (2010) Functions of heteromeric and homomeric isoamylose-type starch-debranching enzymes in developing maize endosperm. Plant Physiol 153: 956–969.