Characterization and expression analysis of the starch synthase gene family in grain amaranth
(Amaranthus cruentus L.)

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Characterization and expression analysis of the starch synthase gene family was performed from grain amaranth. Extensive searches for sequence data for amaranth starch synthase genes were done in the literature and in GenBank, and identified four genes encoding GBSSI, SSSI, SSSII, and SBE. Alignment of predicted amino acid sequences showed that there were low levels of sequence identity among these four genes (10.6–34.5%). There was higher homology in the C-terminal region and lower homology in the N-terminal region of the amaranth starch synthases. Starch synthases in plants can be classified into six groups, and phylogenetic analyses showed that the starch synthases from amaranth were in four of these groups; GBSSs, SSSIs, SSSIIs, and SBEs of dicot plants. Interestingly, all amaranth starch synthases were sister to the subgroup consisting of the rest of the dicot plants, clustering more closely with dicots than with monocots. Analysis of the expression patterns of starch synthase genes in storage and non-storage tissues of amaranth revealed two types of expression patterns; (1) GBSSI was rapidly expressed at the middle or mid-late stages of seed development; and (2) SSSI, SSSI and SBE genes were expressed constitutively during seed maturation. These four genes were expressed in non-storage tissues as well as in storage tissue, suggesting that non-storage and storage tissues of amaranth share a common mechanism for expression of starch synthase genes. This summary of the basic characteristics of starch synthase genes will contribute further studies on amaranth starch.

Key words: amaranth, gene expression, qRT-PCR, starch, starch synthase

INTRODUCTION

In plants, the types and amount of starch are important factors contributing to grain yield and quality. Starch is used in both food and non-food industries. It can be chemically fractionated into two types of glucan polymer—essentially linear amylose, and highly branched amylpectin—at a ratio of approximately 1:3. Amylose is a lightly branched linear molecule with low polymerization, whereas amylpectin is a much larger molecule with extensive branches resulting from α-1,6 linkages (Smith et al., 1997). Amylose is synthesized by granule-bound starch synthase (GBSS) encoded by the Waxy gene, whereas amylopectin is synthesized by soluble starch synthases (SSSs), starch branching enzymes (BEs), and starch debranching enzymes (DBEs) (Park et al., 2012). Each type of polymer is elongated by starch synthase, which catalyzes the transfer of glucose (Glc) from adenosine diphosphate (ADP)-Glc to the growing glucan chains via an α-1,4-linkage (Martin and Smith, 1995). Improvements to grain quality, including increasing the economic value and functionality of starch, rely on understanding the action and effects of these enzymes and their encoding genes. There has been extensive research on isolation and characterization of the starch synthase gene family (Smith et al., 1997). Multiple forms of SSSs have been found in plant leaves and storage organs, and can be divided into at least six classes: GBSSI, GBSSII, SSSI, SSSI, SSSI and SSSIV (Smith et al., 1997; Hirose and Terao, 2004; Huang and Brulé-Babel, 2012; Bansal et al.,...
Amaranth, which first gained prominence as a food crop in Central America over 6,000 years ago (Sauer, 1967), is known for its drought-resistant C4 photosynthetic properties and ability to grow well in saline, alkaline, acidic, or poor soil (Saunders and Becker, 1984). It is capable of growing in diverse regions, including areas that are unsuitable for many other plants. Therefore, amaranth is gradually gaining favor among health-conscious consumers in many countries, including the USA and Japan.

Currently, there is a great deal of interest in basic and applied research on starch. Amaranth starch has many unique properties, including its extremely small granules, low amylose content, unique dodecahedral structure, stable viscosity, good clarity, and excellent moisture retention (Lorenz, 1981; Stone and Lorenz, 1984; Marcon, 2001; Park et al., 2012). It is likely to prove useful for applications in the food, plastics, cosmetics, and other industries. Thus, this starch and its derivatives have potential applications in both food and non-food industries. However, amaranth starches have been under-used in several industry sectors because of a lack of research and/or knowledge about their functional properties at the molecular level. Although there are many scientific and technical articles about the functional properties of starch at the molecular level, few have focused on amaranth starch. Recently, the GBSSI genes and their mutants from three species of grain amaranths were isolated and characterized (Park et al., 2009, 2010). In addition, genetic resources of three amaranth grains, collected from diverse regions, were used to examine allelic diversity in amaranth GBSSI genes that affect amylose content (Park et al., 2011, 2012). The results showed that the nonsense mutation and/or the frameshift were unique events in the evolution of the waxy allele in these crops. In particular, our research on the molecular genetics of genes encoding GBSS is the only investigations of this type among studies on starch biosynthesis for grain amaranth. Therefore, we still have an incomplete understanding of starch biosynthesis.

The aim of this research is to facilitate further studies on amaranth starch. For such studies, a thorough understanding of the genetic characteristics of starch synthases is required. In this study, we report the characterization of the starch synthase gene family in amaranth. We analyzed the evolutionary relationships between amaranth starch synthase genes and those in other plants. Also, we determined the expression patterns of starch synthases in amaranth tissues of different types and different developmental stages.

MATERIALS AND METHODS

Plant materials Amaranth plants (A. cruentus, accession no. PI 433228) were grown in the field in plastic pots for 90 days. This accession was obtained from the collection at the USDA-ARS, National Genetic Resources Program. We observed the development process of amaranth seeds from pollination of female flowers to mature seeds (Supplementary Fig. S1). The grains were harvested at several time points (day after pollination [DAP]) during maturation and divided into six developmental stages; namely initial (1-3 DAP; ca. 0.00003 g), early (3-5 DAP; ca.0.00012 g), early late (4-8 DAP; ca. 0.00026 g), middle (8-12 DAP; ca. 0.00042 g), mid-late (10-15 DAP; ca. 0.00054 g), and late (20 DAP; ca. 0.00065 g), based on their external morphology, fresh weight, and size. Fresh weight values are the average of 10 seeds. In order to study tissue/organ-specific gene expression patterns, the leaves, petioles, stems, and roots were collected from seedlings at the four- and six-leaf stages. The samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

RNA extraction and cDNA preparation Total RNA was isolated from the plant tissue samples using an RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. RNA was quantified by a Nanodrop ND-1000 spectrophotometer and the integrity of the RNA was checked on a 1.5% (w/v) agarose-formaldehyde gel, which was visualized under ultraviolet light after staining with ethidium bromide. Grain amaranth cDNA was synthesized from 50 ng of total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems) according to the manufacturer’s instructions. Quantitative real-time PCR analysis Quantitative
real-time PCR analysis (qRT-PCR) was carried out using an ABI Prism 7900HT sequence detection system and software version 2.2 (Applied Biosystems, USA). All primers used for SYBR real-time RT-PCR are listed in Supplementary Table S1. The gene-specific primers were designed based on the nucleotide sequences of A. cruentus GBSSI (GenBank ID: AB456685), SSSI (GenBank ID: AB626804), SSSII (GenBank ID: DQ178026), and SBE (Lu, 2006) genes. The A. tricolor Actin gene (GenBank ID: EF452618) was used as an internal constitutively expressed control (reference gene). All primers were designed using Primer Express 1.5 software (Applied Biosystems) following the supplier’s guidelines for primer design. Transcript abundance was determined using Power SYBR Green PCR Master Mix (Applied Biosystems) following the manufacturer’s recommendations. We used 1 μl cDNA as the template for PCR. The PCR cycling conditions consisted of an initial polymerase activation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 58°C for 30 s. For each sample, reactions were set up in triplicate to ensure reproducibility of the results. Each sample was normalized using values obtained for the level of Actin mRNA. Data were analyzed by using Sequence Detector Systems version 2.0 software (Applied Biosystems). The threshold cycle (Ct) value of the target genes and Actin mRNA in different samples were obtained after quantitative real-time PCR reaction. In brief, the normalizer Actin mRNA Ct value is subtracted from the gene of interest Ct (target genes) to produce the dCt value of the sample.

Semi-quantitative RT-PCR analysis The mRNA expression patterns of the amaranth starch synthase gene family were also analyzed by semi-quantitative RT-PCR (sqRT-PCR) analysis. We carried out sqRT-PCR using the primers listed in Supplementary Table S1. The gene-specific primers were designed based on the nucleotide sequences of A. cruentus GBSSI (GenBank ID: AB456685), SSSI (GenBank ID: AB626804), SSSII (GenBank ID: DQ178026), and SBE (Lu, 2006) genes. The A. tricolor Actin gene (GenBank ID: EF452618) was used as an internal constitutively expressed control (reference gene). All primers were designed using Primer3 software (Rozen and Skaletsky, 2000) (http://primer3.sourceforge.net/). PCR amplifications were carried out using ExTaq polymerase (0.5 U/μl), 2.5 mM dNTP, 10×PCR buffer (Takara, Japan) and primers at 10 μM. Annealing temperatures used for each primer combination are listed in Supplementary Table S1. Primers that amplify Actin (GenBank ID: EF452618) from A. tricolor were used as a control. The PCR cycling conditions were as follows: amplification for 30 or 32 cycles of denaturation at 98°C for 10 s, annealing at 55–60°C for 30 s, and extension at 72°C for 30 s. PCR products were separated on 1.5% agarose gels, and bands were visualized by ethidium bromide staining.

Alignment and phylogenetic analysis of starch synthases To identify members of the starch synthase gene family from diverse plant species, we searched the database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequence of glycogen synthase of Escherichia coli (GenBank ID: AAA23870) was used as an outgroup. Protein sequences were aligned using ClustalW (Thompson et al., 1997). Phylogenetic analyses on refined alignments were conducted using the neighbor joining (NJ) method (Saitou and Nei, 1987) with PAUP* 4.0 (Swofford, 1988). Bootstrap confidence values (Felsenstein, 1985) were obtained from 1,000 replicates.

RESULTS AND DISCUSSION Characterization of amaranth starch synthase gene family Improvements to grain quality, including increasing the economic value and functionality of starch, depend on understanding the actions and effects of the starch synthase gene family. Thus, a basic understanding of the amaranth starch synthase gene family underpins further studies on amaranth starch. To date, however, there is no basic information on the starch synthase gene family in amaranth.

In this study, we characterized the amaranth starch synthase gene family. We searched the literature and GenBank to determine whether any members of the starch synthase gene family in amaranth had been characterized previously. We found only four sequences for members of the starch synthase gene family in amaranth. Two starch synthase genes, GBSSI (GenBank ID: AB456685) and SSSI (GenBank ID: AB626804) were described in our previous studies (Park et al., 2009, 2012). The other two amaranth genes, SSSII (GenBank ID: DQ178026) and SBE (Lu, 2006), for which only the coding sequences were available, were found in GenBank and in the literature, respectively. The coding sequences of amaranth GBSSI, SSSI, SSSII, and SBE were 1821, 1950, 2430, and 2886 bp long, respectively (Table 1). Based on the predicted amino acid sequences, the identities

| Protein | Size | Protein sequence identity (%) |
|---------|------|------------------------------|
|         | a.a. | GBSSI | SSSI | SSSII | SBE  |
| GBSSI   | 606  | 67.3  | –     | 32.7  | 27.9  | 15.8  |
| SSSI    | 649  | 71.3  | –     | –     | 34.5  | 27.9  |
| SSSII   | 809  | 89.8  | –     | –     | –     | 10.6  |
| SBE     | 962  | 108.4 | –     | –     | –     | –     |
between pairs were as follows: 32.7% between GBSSI and SSSI, 27.9% between GBSSI and SBE, 34.5% between SSSI and SBE, 15.8% between GBSSI and SBE, 34.5% between SSSI and SSSII, 27.9% between SSSI and SBE, and 10.6% between SSSI and SBE (Table 1).

The deduced amino acid sequences of amaranth GBSSI, SSSI, and SSSI were aligned using ClustalW (Fig. 1). There was higher homology in the C-terminal region and lower homology in the N-terminal region. A motif analysis revealed four highly conserved regions. The first conserved region contains the consensus motif KTGGL, which is believed to be the ADP-Glc binding site of starch synthases (Furukawa et al., 1990). The second and third conserved regions each contain a conserved sequence, PLIRIFGKL and PSRFEPCGL. Within this region, the sequence PSRFEPCGL is identical between dicots, rice, and E. coli sequences and shares one conservative substitution compared with the other monocot plant sequences (Park et al., 2009). The fourth conserved region contains XGGL, the KXGGL ‘look-alike’ domain. The KTGGL “look-alike” motif is so named because it resembles the N-terminal KTGGL motif identified as a site of ADP/ADP glucose binding, although it lacks the lysine residue that is thought to interact with the polyphosphate group of ADPG and ADP in bacterial

The phylogenetic relationship of the amino acid sequences of plant starch synthases is shown in Fig. 2. The starch synthases from amaranth and other plants clustered into six branches: (I) GBSSs, (II) SSSIIs, (III) SSSIIs, (IV) SSSIIs, (V) SSSIs, and (VI) SBEs. Each group contained starch synthases from monocots and dicots. The amaranth starch synthases GBSSI, SSSI, SSSI, and SBE were found in four of these groups, clustering with GBSSs, SSSIIs, SSSIIs, and SBEs of dicots, respectively. Among these groups, the rice and wheat starch synthases from monocots grouped with GBSSs from dicots, especially the GBSSI of amaranth (see Fig. 2). Similar observations were reported in wheat and rice (Vrinten and Nakamura, 2000; Hirose and Terao, 2004). The deduced GBSSI proteins from monocot plant appear to be closely related to GBSSIs from several dicot plants, as has been previously observed. Interestingly, all amaranth starch synthases were sister to the subgroup consisting of the rest of the dicots, and were more closely related to dicots than monocots. Similar results have been reported in phylogenetic analyses of GBSSI, SSSI and SSSI protein sequences from diverse plants (Park et al., 2009, 2012). Therefore, there are significant differences in the protein sequences of starch synthases

Fig. 1. Comparison of the deduced amino acid sequences of amaranth starch synthases, GBSSI, SSSI, and SSSI. The four consensus regions for amaranth starch synthases are highlighted in bold. A bold bar indicates the N-terminal KXGGL motif involved in substrate binding and the C-terminal KXGGL ‘look-alike’ motif.
Characterization of amaranth starch synthase genes between amaranth and other dicots. This characteristic of sequence differences is probably a result of differences in the genetic backgrounds of the various crops. This study provides useful information to understand the characteristics and complexities of the starch synthase gene family in this crop.

Expression profiling separates the genes involved in storage starch synthesis into two groups

Starch is one of the major storage components in plants, and it is used in both food and non-food industries. Starch biosynthesis has been well studied by plant breeders and molecular biologists (Martin and Smith, 1995; Fig. 2. Phylogenetic relationships among starch synthases of higher plants. Tree was constructed using the neighbor-joining method, and E. coli was used as an outgroup. Amaranth starch synthases are highlighted in bold. Scale bar represents evolutionary distance. Bootstrap support values from 1,000 replicates are shown at each node. The GenBank IDs for starch synthase genes are as follows: Arabidopsis thaliana At GBSSI, NM1003023; AtSSSI, NM_122336; AtSSSII, NM_110984; AtSSSIII, NM_101044; AtSSSIV, NM_117934; Amaranth (Amaranthus cruentus) GBSSI, AB456685; SSSI, AB626804; SSSI, DQ175026; SBE, Lu 2006; Apple (Malus × domestica Borkh.) SBE, DQ115404; Barley (Hordeum vulgare) GBSSI, X07931; SSSI, AF234163; Bean (Phaseolus vulgaris); GBSSIb, AB110011; Cassava (Manihot esculenta) GBSSI, X74160; SSSI, AF173900; SBE, CA454308; Cowpea (Vigna unguiculata) SSSI, AJ225088; SSSV, AJ006752; Foxtail millet (Setaria italica) GBSSI, AB089141; Maize (Zea mays) GBSSI, X03933; SSSI, AF368891; SSSIa, AF192996; SSSIb, AF19297; SSSI, AF023159, SBE, NP_001055370; Pea (Pisum sativum) GBSSIa, X99789; GBSSIb, AJ345045; SSSI X8790; Perilla (Perilla frutescens) GBSSI, AF210899; Potato (Solanum tuberosum) GBSSI, X58453; SSSI, Y10416; SSSI, X87988; SSSI, X94400; SBE, CA454308; Rice (Oryza sativa) GBSSI, X65183; GBSSI, AY069940; SSSI, D16292; SSSI, AF39357; SSSI, AF39357; SSSI, AF419099; SSSI, AF432915; SSSI, AF39357; SSSI, AF39357; SSSI, AY100469; SSSI, AY100470; SSSI-2, AY100471; SBE, BAA01584; Sorghum (Sorghum bicolor) GBSSI, Q43134; SSSI, AF168786; SBE, ADA06279; Sweet potato (Ipomoea batatas) GBSSI, AB071604; SSSI, AF068834; Wheat (Triticum aestivum) GBSSI, X75253; GBSSI, AF109395; SSSI, AF091803; SSSI, AF258608; SSSI, AY044844; SBE, CAB40980.
Smith et al., 1997). In particular, there has been extensive research on isolation and characterization of the starch synthase gene family (Hirose and Terao, 2004; Smith et al., 2004; Ohdan et al., 2005). According to previous reports, the starch synthase gene family can be divided into at least six classes: GBSSI, GBSSII, SSI, SSII, SSIII, and SSSIV (Smith et al., 1997; Hirose and Terao, 2004). Also, members of this gene family can be classified into three types based on their expression pattern: (i) early expressers, (ii) late expressers, and (iii) steady expressers (Hirose and Terao, 2004; Dian et al., 2005; Ohdan et al., 2005).

A key aspect of understanding the mechanism of starch synthesis in amaranth perisperm is to understand the relationship between the expression patterns of the starch synthase genes and starch accumulation. In this study, we examined the expression patterns of the starch synthase genes detected at different developmental stages. The transcript levels of starch synthase genes detected at different seed developmental stages are shown in Fig. 3. Transcripts of GBSSI were not detected at the initial (st1) and/or early (st2) developmental stages. GBSSI transcripts rapidly increased from a low level at the early late stage of seed development (st3) to peak at the mid-late developmental stage (st5), and expression decreased rapidly thereafter. There have been several previous studies on the transcripts and protein of GBSSI in endosperm plants (Nakamura et al., 1998; Hirose and Terao, 2004; Dian et al., 2005; Ohdan et al., 2005). This gene was designated as a ‘late expresser’ because abundance of its transcripts increased from approximately 5 or 7 days after flowering (Hirose and Terao, 2004; Ohdan et al., 2005). Therefore, the amaranth GBSSI gene exhibits late expression in the perisperm, similar to the expression pattern of its homologs in endosperm plants. This finding is consistent with previous studies on the expression patterns of the GBSSI gene in amaranth (Park et al., 2011). In the other hand, the expression patterns of SSSI, SSII, and SBE were very similar. All were already expressed at the initial developmental stage (st1), then transcript levels gradually increased to peak at the mid developmental stage (st4), and then gradually declined at mid-late developmental stages (st5). However, all three genes were expressed at relatively high levels until seed maturation. This pattern was relatively constant during seed development, which is similar to the expression patterns of its homologs in maize (SSSI, Cao et al., 1999; SBE, 2005).
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SSSIa, Harn et al., 1998; SBEI, Gao et al., 1996), potato (SSSI, Kossmann et al., 1991; SSSII, Abel et al., 1996), rice (SSSI, SSSI, and SBEIa, Hirose and Terao, 2004; Dian et al., 2005; Ohdan et al., 2005), wheat (SSSI, Li et al., 1999a; SSSI, Li et al., 1999b; SBEII, Bagia et al., 2000). According to previous reports, expression levels of mRNA transcripts of SSSI, SSSI, and SBE genes could play important roles in the biosynthesis of storage starch during amaranth seed development. Therefore, the amaranth SSSI, SSSI, and SBE genes appear to be steady in several plants during seed development. Thus, we suggested that SSSI, SSSI, and SBE genes could play important roles in the biosynthesis of storage starch during amaranth seed development. Therefore, the amaranth SSSI, SSSI, and SBE genes are steady expressers, which are constitutively expressed during seed development.

In conclusion, based on their expression patterns during seed maturation, four amaranth genes involved in starch synthesis, GBSSI, SSSI, SSSI, and SBE, can be classed into two groups: (i) GBSSI is late expresser, which is rapidly expressed at the middle or mid-late stages of seed development; and (ii) SSSI, SSSI, and SBE are steady expressers, which are expressed constitutively throughout seed maturation.

Amaranth starch synthase genes are not specific to non-storage and/or storage tissues The starch synthesis genes in endosperm plants can be divided into three groups. Group I genes are associated with synthesis of storage starch: SSSI-3, SSSI-2, GBSSI. Group II genes are associated with non-storage (or transient) starch synthesis: SSSI-2, SBEII and GBSSI. Group III genes are associated with both types of starch synthesis: SSSI, SSSI-1, SSSI-1, SSSI-1, SSSI-2, SBEI and SBEII (Hirose and Terao, 2004; Dian et al., 2005; Ohdan et al., 2005). According to previous reports, SSSI, SSSI, and SBE genes are expressed in both storage and non-storage tissues, whereas GBSSI is mainly restricted to storage tissues. In the case of grain amaranth, the expression pattern of the GBSSI gene seems to differ from that in endosperm plants (Park et al., 2011), because it was expressed in various non-storage tissues as well as in storage tissue. A similar result also has been reported for other dicot plants such as Arabidopsis, potato, and sweet potato. That is, GBSSI was expressed in non-storage tissues (leaves and stems) and storage organs of the tuberous species (potato and sweet potato), and in Arabidopsis, it was expressed in leaves during diurnal cycles that followed the pattern of leaf starch accumulation (Wang et al., 1999; Smith et al., 2004). The amaranth SSSI gene was expressed strongly in non-storage tissues (leaf, petiole, stem, and root) and in storage tissue, which is similar to SSSI expression patterns in endosperm plants (Park et al., 2012). In contrast, the potato SSSI was expressed mainly in leaves and was almost undetectable in tubers (Kossmann et al., 1999).

Fig. 4. Expression patterns of GBSSI, SSSI, SSSI, and SBE genes in different organs of A. cruentus during seedling development. Total RNAs from different organs (L, leaf; P, petiole; S, stem; R, root) were extracted at the four- and six-leaf stages. Expression patterns of (a) GBSSI, (b) SSSI, (c) SSSI, and (d) SBE genes were analyzed by qRT-PCR and RT-PCR. Actin mRNA was used as an internal reference to normalize the RNA content of each sample.
These findings reveal that transcription of amaranth GBSSI typically corresponds to expression patterns of its homologs in dicots, and that of SSSI typically corresponds to expression patterns of its homologs in endosperm plants. In addition, the transcriptions of amaranth GBSSI and SSSI did not appear to be specific to non-storage and/or storage tissues (Park et al., 2011, 2012). Therefore, in amaranth, the storage and non-storage tissues might share a common mechanism for expression of starch synthase genes. This hypothesis is also well supported by the expression analysis results obtained in the present study. We examined the mRNA expression patterns of starch synthase genes (GBSSI, SSSI, SSSI, and SBE) in the leaves, petioles, stems, and roots of seedlings at the four- and six-leaf stages (Fig. 4). The GBSSI and SSSI genes were strongly expressed in all of the organs examined. Transcripts of amaranth SSSI and SBE were detected in all non-storage tissues, as observed for their homologs in other plants, such as rice (SSSI and SBE) (Hirose and Terao, 2004; Ohdan et al., 2005), potato (SSSI and SBE) (Kossmann et al., 1999; Jobling et al., 1999) and sweet potato (SBE) (Hamada et al., 2006). The mRNA levels of this gene family showed a tendency to increase rapidly during the four- to six-leaf stage of development. Therefore, we conclude that amaranth starch synthase genes are not specific to non-storage and/or storage tissues. In amaranth, it appears that these genes are associated with both the biosynthesis of storage starch in perisperm and transitory starch in non-storage organs. Our results suggest that the non-storage tissues share a common mechanism for expression of starch synthase genes during growth at the four- to six-leaf stages.

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