

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, SSSII, 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*, *SSSII* 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 amylopectin—at a ratio of approximately 1:3. Amylose is a lightly branched linear molecule with low polymerization, whereas amylopectin 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, SSSII, SSSIII, and SSSIV (Smith et al., 1997; Hirose and Terao, 2004; Huang and Brûlé-Babel, 2012; Bansal et al.,

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Abbreviations: adenosine diphosphate (ADP); branching enzymes (Bes); day after pollination (DAP); debranching enzymes (DBEs); granule-bound starch synthase (GBSS); glucose (Glc); neighbor joining (NJ); quantitative real-time PCR (qRT-PCR); semi-quantitative RT-PCR (sqRT-PCR); soluble starch synthases (SSSs)

2012). In addition, expression of the starch synthase gene family has been comprehensively analyzed in tissues of different types and at different developmental stages (Hirose and Terao, 2004; Smith et al., 2004; Ohdan et al., 2005).

Amaranth was first used as a grain crop more than 6,000 years ago in Central America (Sauer, 1967). All *Amaranthus* are drought-resistant C4 photosynthetic plants that can grow well in saline, alkaline, acidic, or poor soil (Saunders and Becker, 1984). Amaranth grains still used to this day include the three species *Amaranthus caudatus*, *Amaranthus cruentus*, and *Amaranthus hypochondriacus*. This crop was first introduced into Japan during the 1980s from the Rodale Research Institute in the United States. A new grain amaranth variety, "New Aztec", was developed in 2001 using the "Mexico line", which is a high-yielding semi-dwarf line selected in Japan from the introduced genetic resources of grain amaranth (*A. cruentus*) originating from Mexico (Katsuta et al., 2001). Currently, cultivation of this new grain, mostly *A. cruentus*, is gradually increasing in some areas in Japan, including Iwate, Akita, and Nagano Prefectures (Park et al., 2012).

Grain amaranths have agronomic potential because of the high protein content of the leaves and seeds, and the high levels of the essential amino acid, lysine, in the protein. (Brenner et al., 2000; Kauffman and Weber, 1990; Sauer, 1993). Because of its high nutritional content, the National Academy of Sciences has cited grain amaranth as one of the world's most promising foods. This crop, therefore, is one of the new world super grains and is 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 unusual 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 non-sense 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 d*Ct* 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 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. These four genes encode proteins predicted of 606, 649, 809, and 962 amino acids with calculated molecular weights of 67.3, 71.3, 89.8, and 108.4 kDa, respectively (Table 1). Based on the predicted amino acid sequences, the identities

Table 1. Sequence comparisons of amaranth starch synthase proteins

Protein	Size		Protein sequence identity (%)			
	a.a.	kDa	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 SSSII, 15.8% between GBSSI and SBE, 34.5% between SSSI and SSSII, 27.9% between SSSI and SBE, and 10.6% between SSSII and SBE (Table 1).

The deduced amino acid sequences of amaranth GBSSI, SSSI, and SSSII 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 KTGGGL, 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, PLIGFIGRL and PSRFEPCL, respectively. Within this region, the sequence PSRFEPCL 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 KTGGGL "look-alike" motif is so named because it resembles the N-terminal KTGGGL 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

glycogen synthase (Furukawa et al., 1993).

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) SSSIs, (III) SSSIIs, (IV) SSSIII, (V) SSSIVs, and (VI) SBEs. Each group contained starch synthases from monocots and dicots. The amaranth starch synthases GBSSI, SSSI, SSSII, and SBE were found in four of these groups, clustering with GBSSs, SSSIs, SSSIIs, and SBEs of dicots, respectively. Among these groups, the rice and wheat GBSSII from monocots grouped with GBSSIs 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 GBSSII proteins from monocot plants 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 SSSII protein sequences from diverse plants (Park et al., 2009, 2012). Therefore, there are significant differences in the protein sequences of starch synthases

GBSSI	METVTSSTHVSFN-FANTAMGSSDPKLTANN-----ALKSQNMSTHNGRLPLMSNIDMLR-----	54
SSSI	MESLFLQSFHMKGLCRSKLNFSSPLQIRGNQVG-----ISCLWQKRSRTWSNFSIKQTFSDG-----NEGRVSSSTDGS-----LVIES	77
SSSII	MASLNSLRFMIETRCSLHNSNGSHHLSYQRRKFPVNGGGFLKFMHDHSDNGRLSLYCVKSVSGCRRSSNMRYNLKAIIGDDLNEGEHSKEDKDENEALDAIEKSNKVSAMQKELLQIAE	120
GBSSI	-----LSNNPKSTTVELR-----	67
SSSI	Q-----RGGD-----EEKKG-----SLLGPVKDASGSIVGFELFS-----	107
SSSII	RKKLVSSIRNSVINSDDAPADKDYNSPQNDALGANQILVKNEYDISTGAARQSDSASSKLNPDQLQVPLSSLEEKSGQAKSALKMLNLDSSQPKELTKATETDSSISNGSPIVGKREMK	240
Motif I		
GBSSI	-----KERFHAPFIRSG-----MNVVFVGAEVAPWSKTGGLGDLVGLGLPALAARGHRVMTVSPR	122
SSSI	-----QSVGNNGN-----DVDEELEETVYEGQDKLLT-----KKKMSIIFVTSEAPYSKTGGLGDCVGLSPISLAERGHVMTVSPR	180
SSSII	TIGSLSEGNANGVDASKGDETRMLNPLSSTNKSSIMDKKEKELGESYFKGLSELVNDAAEDDKPPPLAQNVNMVILVAAECAPWCKTGGLGDLVAGALPKALARRGHVMTVSPR	360
GBSSI	YDQ-----YRDGWDTSVTVEFQVGNRTETVRYFHTYKRGVDRIFDVHPLFLARVWGITGSKLYGPKAGADYEDNQLRFSLLCQAALAPRVNLNNPNFSGPYGENVVFVFIANDWHTA	235
SSSI	YIHGTADKVIYAGAFDANCRIKVNCFGGPQEVAFHEHYKNGVDWVFDHLSYHRPGN-----PYGDSYG-AFGDNQFRFTLLCHAAEAPLVLPGLGTYTG-----KCMFVVDWVHAG	288
SSSII	YAE-----YADCQFTGVKRKYKQVAGQDNEVLYFHTYIDGVDFVFIEAPMLRNLG-----KDIYGGNRLDILKRMILFKVAVEVPHVPCGGVCYGDG-----NLVFIANDWHTA	460
GBSSI	LLPAYLKAIYQPKGIYNNAKVAFCIHNIVYQGRFALADYPRHLPEELRP---VFEFMDGYDRPIKGRKINWMKAGILQSDRVVTVSPYYAQELISGVERGVELDDVVR---QTGVTGIV	349
SSSI	LVPVLLAANYRYPYGVYKDARSVLVIHNLHQVEAALTYENLGLPPQWYGALEWVFPPEARAHALDKGEAVNLIKGAIVTSDRILTVSQGYSWEITT-VEGGYGLHDLTSSKFKVLNGII	407
SSSII	LLPVYLKAYYRDNGLMKYTRSLVLIHNIHAGRGVPVDDFTYVDLPHPYL-----DYFKLYDPVGEHFNIFAAGLKTADRILTVSHGYAWELKT-QEGGWGLHGIINDNDWKLRGIV	571
Motif II		
GBSSI	NGMDVQEWNPITDKYIGIN---FNITVTMTAKPLIKEALQAEVGLPVDRNIPPLIGFIGRLLEEKGSDILAEAIIPRIKENVQIVVLGTGKEVMEKQIEQLILEIPEKARGVTKFNSPLA	465
SSSI	NGINVNEWDPNDPLIAAP---YSVDLS-GKAECKAALQKELGLPIKPDCLPILFIGRLDYQKIGIDVIEAFQPLMEDDVQFVMLGSGDSRYESWMRAAEGACKDKFRGWVGFVSPTS	522
SSSII	NGIDVTEWNPELDLYLQSDGYANYSLKTPGKAQCKAALQNELGLPVREDVPLIGFIGRLDGQKGVDIIEAISIPWLSGQDVQLVMLGTGRPDLEQMLRECESRYPDKIRGWVGFVSPTA	691
Motif III		
Motif IV		
GBSSI	HMIVAGADFMLIPSRFEPCLLIQLYSMYRGTPVVAASKTGGLRDTVET-YNPFAVGPDTGTGTGWAFSPLTKESSLALSNAVRTYRDKESWAGLMKRGMQDDFTWDTAAQYEQVFEW	580
SSSI	HRITAGCDIILLMPSRFEPCLNQLYAMRYGTIPVHNKTGGLRDTVET-YNPFAVGPDTGTGTGWAFSPLTKESSLALSNAVRTYRDKESWAGLMKRGMQDDFTWDTAAQYEQVFEW	641
SSSII	HRITAGVDIILLMPSRFEPCLNQLYAMRYGTIPVHVAAGGLRDTVQF-FDPYNN-----TGYGWTFDRAENRLIDALGNCLLTYYQYKESWEGLOTRGMTQDLSWDHAAEIEYEEVLVA	804
GBSSI	LGVAGSRPGFECTESIPLATENIATP	606
SSSI	ALIDPPYC-----	649
SSSII	AKYQW-----	809

Fig. 1. Comparison of the deduced amino acid sequences of amaranth starch synthases, GBSSI, SSSI, and SSSII. 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.

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;

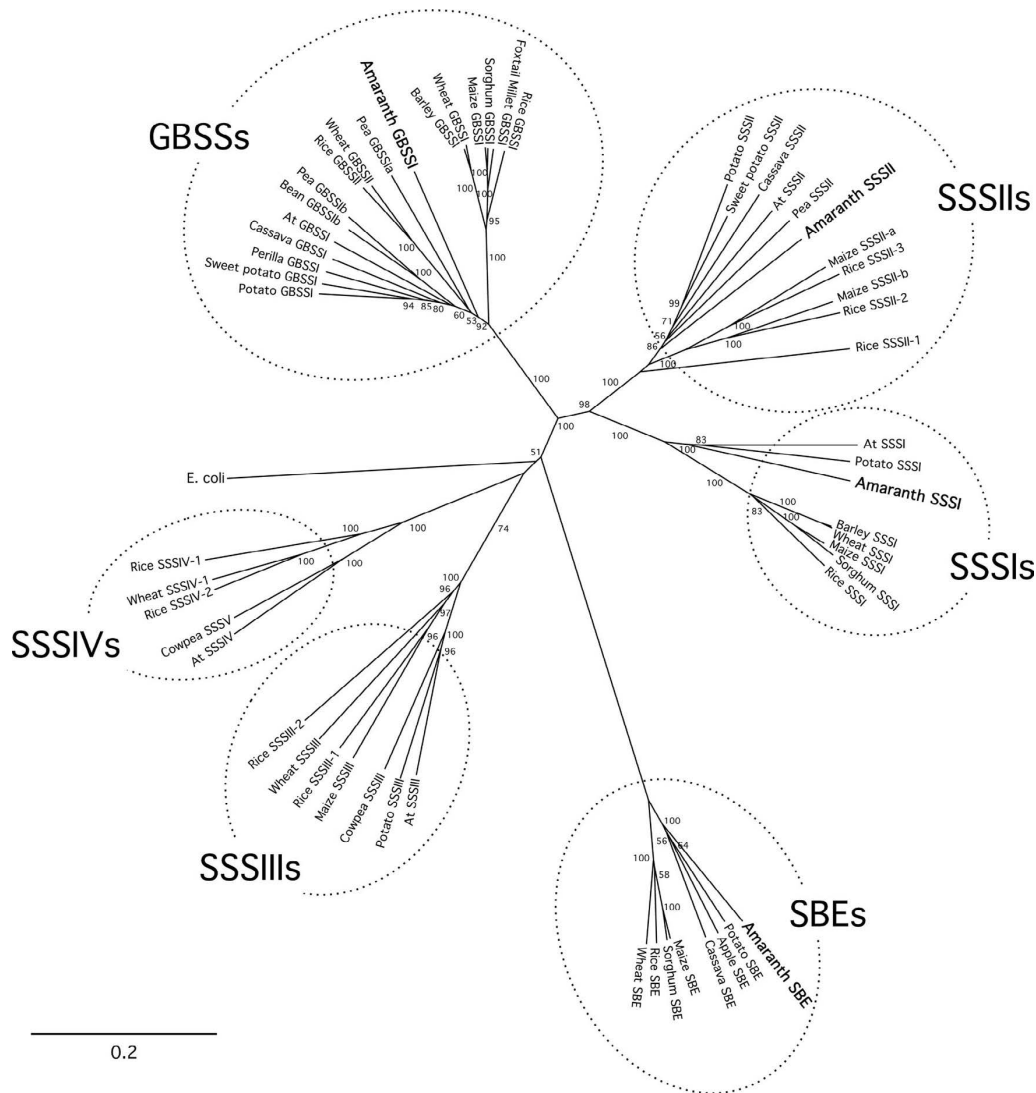


Fig. 2. Phylogenetic relationships among starch synthases of higher plants. Tree was constructed using the neighbor-joining method, and *E. coli* was used as an outgroup. Amaranth starch synthases are highlighted in bold. Scale bar represents evolutionary distance. Bootstrap support values from 1,000 replicates are shown at each node. The GenBank IDs for starch synthase genes are as follows: *Arabidopsis thaliana* At GBSSI, NM103023; AtSSSI, NM_122336; AtSSSII, NM_110984; AtSSSIII, NM_101044; AtSSSIV, NM_117934; Amaranth (*Amaranthus cruentus*) GBSSI, AB456685; SSSI AB626804; SSSII, DQ178026; 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; SSSII, AF173900; SBE, CAA54308; Cowpea (*Vigna unguiculata*) SSSIII, AJ225088; SSSV, AJ006752; Foxtail millet (*Setaria italica*) GBSSI, AB089141; Maize (*Zea mays*) GBSSI, X03935; SSSI, AF036891; SSSIIa, AF019296; SSSIIb, AF019297; SSSIII, AF023159; SBE, NP_001105370; Pea (*Pisum sativum*) GBSSIa, X99789; GBSSIb, AJ345045; SSSII X88790; Perilla (*Perilla frutescens*) GBSSI, AF210699; Potato (*Solanum tuberosum*) GBSSI, X58453; SSSI, Y10416; SSSII, X87988; SSSIII, X94400; SBE, CAA70038; Rice (*Oryza sativa*) GBSSI, X65183; GBSSII, AY069940; SSSI, D16202; SSSII-1, AF383878; SSSII-2, AF395537; SSSII-3, AF419099; SSSIII-1, AF432915; SSSIII-2, AY100469; SSSIV-1, AY100470; SSSIV-2, AY100471; SBE, BAA01584; Sorghum (*Sorghum bicolor*) GBSSI, Q43134; SSSI, AF168786; SBE, AAD50279; Sweet potato (*Ipomoea batatas*) GBSSI, AB071604; SSSII, AF068834; Wheat (*Triticum aestivum*) GBSSI, X57253; GBSSII, AF109395; SSSI, AF091803; SSSIII, AF258608; SSSIV, 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 gene family in amaranth seeds 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 develop-

ment (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*, *SSSII*, and *SBE* were very similar. All were already expressed at the initial developmental stage (st1), then transcript levels gradually increased to peak at the middle 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;

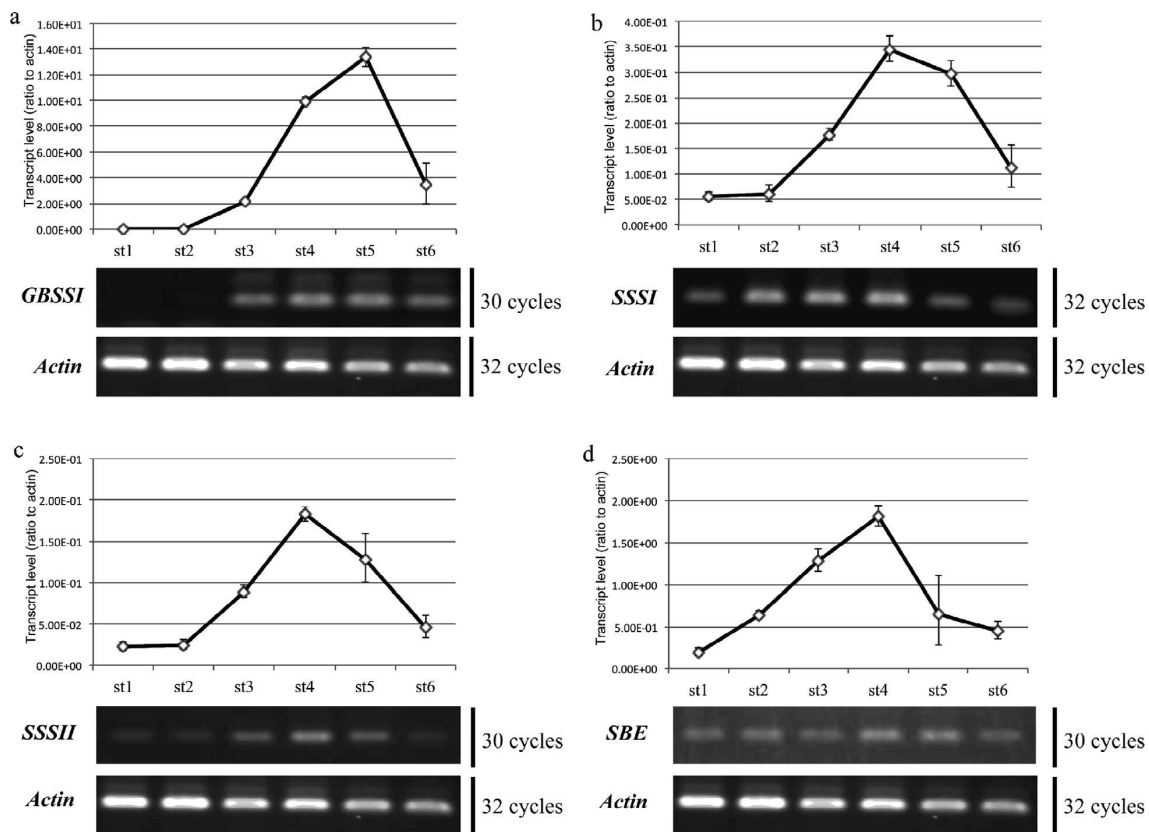


Fig. 3. Expression patterns of *GBSSI*, *SSSI*, *SSSII*, and *SBE* genes during seed development in *A. cruentus*. Total RNAs from amaranth seeds were extracted at different developmental stages (st1, initial; st2, early; st3, early late; st4, middle; st5, mid-late; st6, late). The expression patterns of (a) *GBSSI*, (b) *SSSI*, (c) *SSSII*, 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.

SSSIIa, Harn et al., 1998; *SBEI*, Gao et al., 1996), potato (*SSSI*, Kossmann et al., 1991; *SSSII*, Abel et al., 1996), rice (*SSSI*, *SSSII*, and *SBEIa*, Hirose and Terao, 2004; Dian et al., 2005; Ohdan et al., 2005), wheat (*SSSI*, Li et al., 1999a; *SSSII*, Li et al., 1999b; *SBEI*, Båga et al., 2000). According to previous reports, expression levels of mRNA transcripts of *SSSI*, *SSSII* and *SBE* appear to be steady in several plants during seed development. Thus, we suggested that *SSSI*, *SSSII*, and *SBE* genes could play important roles in the biosynthesis of storage starch during amaranth seed development. Therefore, the amaranth *SSSI*, *SSSII*, 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*, *SSSII* 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*, *SSSII* 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 synthe-

sis of storage starch: *SSSII-3*, *SSSIII-2*, *GBSSI*. Group II genes are associated with non-storage (or transient) starch synthesis: *SSSII-2*, *SBEIIb* and *GBSSII*. Group III genes are associated with both types of starch synthesis: *SSSI*, *SSSII-1*, *SSSIII-1*, *SSSIV-1*, *SSSIV-2*, *SBEI* and *SBEIIa* (Hirose and Terao, 2004; Dian et al., 2005; Ohdan et al., 2005). According to previous reports, *SSSI*, *SSSII* 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).

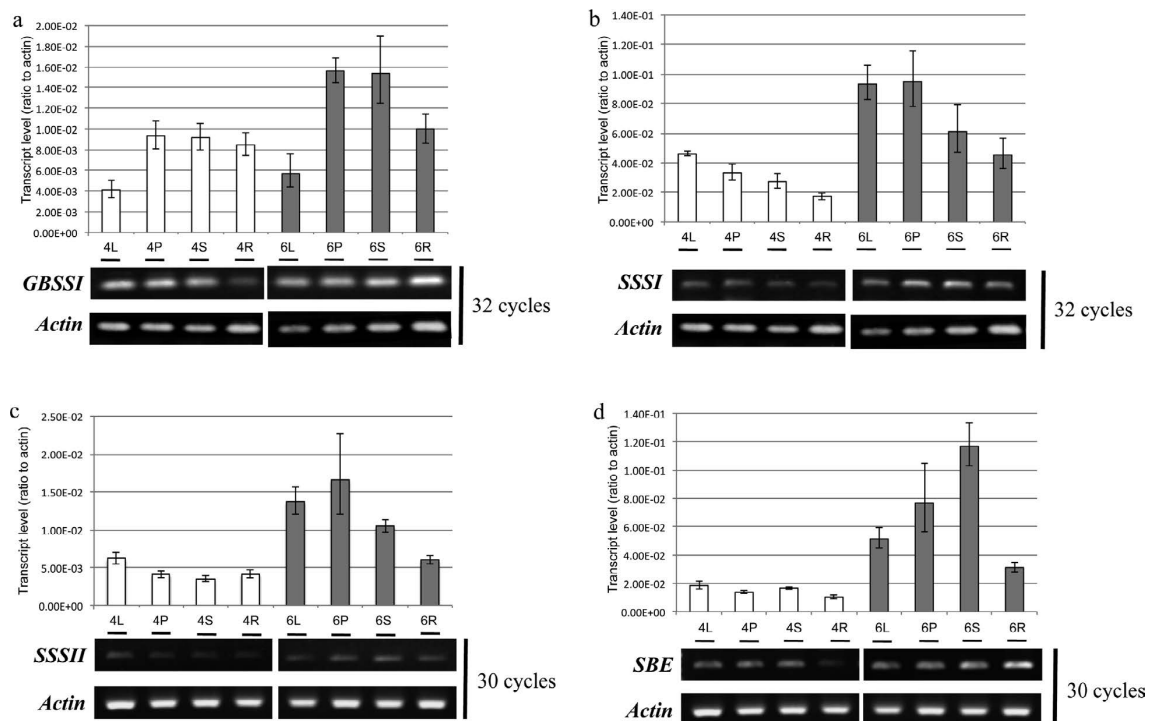


Fig. 4. Expression patterns of *GBSSI*, *SSSI*, *SSSII*, and *SBE* genes in different organs of *A. cruentus* during seedling development. Total RNAs from different organs (L, leaf; P, petiole; S, stem; R, root) were extracted at the four- and six-leaf stages. Expression patterns of (a) *GBSSI*, (b) *SSSI*, (c) *SSSII*, 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*, *SSSII*, 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 *SSSII* and *SBE* were detected in all non-storage tissues, as observed for their homologs in other plants, such as rice (*SSSII* and *SBE*) (Hirose and Terao, 2004; Ohdan et al., 2005), potato (*SSSII* 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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