Molecular characterization of genes encoding isoamylase-type debranching enzyme in tuberous root of sweet potato, *Ipomoea batatas* (L.) Lam.

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Abstract  Isoamylase (ISA) is a starch debranching enzyme that removes α-1,6-glucosidic linkages in α-polyglucans such as amylopectin. From previous studies, plant isoamylases have been shown to play a crucial role in amylopectin biosynthesis; however, little is known about their function in storage root tissues of plants such as cassava, yam and sweet potato. In this study, we isolated cDNA clones and characterized the cDNA nucleotide sequences of three genes (*IbISA1*, *IbISA2*, *IbISA3*) encoding isoamylase from sweet potato (*Ipomoea batatas* (L.) cv. White Star). Deduced amino acid sequences of the three isolated *IbISAs* have the specific regions that are highly conserved among the α-amylase family members. The product of *IbISA2* is predicted to be enzymatically inactive, like other plant ISA2s, due to replacement of amino acid residues that are important for hydrolytic reaction. qRT-PCR analysis demonstrated that expression of *IbISA2* was higher than that of the other two *IbISAs* (*IbISA1* and *IbISA3*) in tuberous root at 109 days after planting, at which stage of tuberous root was at which stage tuberous roots were almost fully developed. This expression pattern observed in our experiments was different from that in other sink organs, such as seeds (endosperms), indicating that orchestration of ISA gene expression may depend on the differences in sink organ type between tuberous roots and seeds. The molecular characterization of three *IbISA* genes and their expression analysis in this study will contribute to further studies on starch biosynthesis in sweet potato, especially in storage root.

Key words: genes encoding isoamylase-type starch debranching enzymes, *Ipomoea batatas* (L.), tuberous root.

Plant starches, which are synthesized by photosynthesis, consist of two major types of glucose homopolymers: amylose and amylopectin (Smith et al. 1997). Amylose is essentially a linear molecule in which glucosyl monomers are joined via α-1,4 linkages, whereas amylopectin has a much more complex organization, in which linear α-1,4-glucan chains are regularly branched via α-1,6-glucosidic linkages (Dian et al. 2005; James et al. 2003). Many of the enzymes involved in starch metabolism have been investigated: at least four enzyme families have been found to participate in amylopectin biosynthesis, that is ADP-glucose pyrophosphorylase (AGPase), soluble starch synthase (SS, EC 2.4.1.21), starch branching enzyme (SBE, EC 2.4.1.18), and starch debranching enzyme (DBE, EC 3.2.1.70), whereas amylose is synthesized by ADP-glucose pyrophosphorylase (AGPase) and granule-bound starch synthase (GBSS, EC 2.4.1.21) (Ball and Morell 2003; Hwang et al. 2005; Jeon et al. 2010; Myers et al. 2000; Nakamura 2002; Smith et al. 1997). Among the four enzyme families involved in amylopectin biosynthesis, DBEs are involved in the removal of α-1,6-glucosidic linkages. In plants, DBEs can be classified into two types: isoamylase (ISA, EC 3.2.1.68) and pullulanase (PUL, EC 3.2.1.41) or limit dextrinase, EC 3.2.1.42 (Nakamura et al. 1996). Both ISA and PUL hydrolyze α-1,6-glucosidic linkages, but they differ in their substrate specificity: ISA catalyzes amylopectin, glycogen and phytoglycogen but scarcely attacks pullulan, whereas PUL can catalyze pullulan and amylopectin, but not glycogen and phytoglycogen (Nakamura et al. 1996). Furthermore, the ISA type enzymes can be divided into three isoforms.
designated ISA1, ISA2, and ISA3 (Hussain et al. 2003), whereas only one isoform of the PUL type is present (Dinges et al. 2003).

The importance of ISA for correct amylopectin biosynthesis has been suggested by several studies in maize (Beatty et al. 1999; James et al. 1995; Rahman et al. 1988), rice (Nakamura 1996; Nakamura et al. 1996; 1997), barley (Burton et al. 2002), Arabidopsis (Delat et al. 2005; Wattebled et al. 2005), potato (Hussain et al. 2003) and Chlamydomonas (Ball et al. 1996; Mouille et al. 1996). Current evidence suggests that plant ISA is active as a complex containing different isoforms (Dauvillée et al. 2001; Fujita et al. 1999; Hussain et al. 2003). In the synthesis of potato storage starch, StISA1 and StISA2 are active as hetero-oligomers (Hussain et al. 2003). In Arabidopsis leaves, a source organ, AtISA1 and AtISA2 are active as hetero-oligomers, as in potato tubers (Delat et al. 2005; Wattebled et al. 2005). In other sink organs, endosperms of rice and maize, both ISA1/ISA2 hetero-oligomer and ISA1 homo-oligomer were observed (Kubo et al. 2010; Utsumi and Nakamura 2006). When ISA1 and ISA2 form a hetero-oligomer, ISA1 has a direct catalytic role but the ISA2 subunit is likely to have a regulatory function because ISA2 is non-catalytic, due to substitutions of 6 out of 8 key amino acids within the active site (Hussain et al. 2003; Utsumi and Nakamura 2006). There are four regions (designated Region I, II, III and IV) that are highly conserved in the α-amylase family (GH13 family), and some amino acids in these conserved regions play an important role in activity of the enzyme (Lawson et al. 1994; Strokopytov et al. 1996). In particular, histidine (His) (H) in Region I, II, and IV (position His-137, His-269, and His-361 of Bacillus sp. strain TS-23 α-amylase) are important for correct catalytic activity (Chang et al. 2003). In contrast, ISA3 does not form a complex with ISA1 and ISA2, indicating that ISA3 functions as a monomer (Hishinuma et al. 2004; Takashima et al. 2007). However, in contrast to these findings, Arabidopsis AtISA2 was found to be co-expressed with AtISA3 in the absence of AtISA1 expression (Li et al. 2007).

In higher plants, there are specific sink organs, including seeds (endosperms), tuberous stems and tuberous roots. The expression of ISAs in relation to seed development has been investigated in several plant species, including rice, maize, barley, rye and amaranths (Kubo et al. 2010; Ohdan et al. 2005; Park et al. 2014; Sun et al. 1999; Zheng et al. 2013). However, in the case of tuberous root species, sweet potato (Convolvulaceae), cassava (Euphorbiaceae), and yam (Dioscoreaceae) (Scott et al. 2000), there are few reports of cloned and characterized ISAs (Beyene et al. 2010; Kim et al. 2005). Furthermore, the relationship between gene expression of ISAs and sink development is not well understood in tuberous root plants.

In the work reported here, we isolated the genes encoding IbISA1, IbISA2, and IbISA3 from one of the major tuberous root plant, sweet potato (Ipomoea batatas (L.)), and compared the amino acid sequences with those of other plant species with different types of sink (tubers and seed endosperms) and source (leaves). We concluded that IbISA2 does not have isoamylose enzyme activity, like ISA2 from other plant species. Furthermore, we determined the gene expression patterns of IbISAs by qRT-PCR, and examined their expression during tuberous root development. These three genes were spatiotemporally regulated in root (sink) and leaves (source) in sweet potato. Based on these results, we discuss the function of the three IbISAs in tuberous root hypertrophy.

Materials and methods

Plant Materials

Ipomoea batatas (L.), cultivar “White Star” plants were grown in the field. Mature leaves and storage roots were collected for cloning of genes for IbISAs. According to developmental stage, leaves and roots were also collected for qRT-PCR analysis.

Cloning and sequencing of genes encoding isoamylose-type starch debranching enzyme

Total RNAs were extracted from tuberous roots (ca. 2 g) using RNeasy Plant Mini Kit (Qiagen, Basel, Switzerland). From total RNAs, poly (A)+ RNAs were purified with Oligotex™-dT <Super> mRNA Purification Kit (TaKaRa Shuzo, Shiga, Japan) according to the manufacturer’s instructions. In order to isolate the full-length cDNA clones for genes encoding isoamylase-type starch debranching enzymes 1, 2, and 3 (IbISA1, IbISA2, and IbISA3), the RACE method was used. Briefly, first-strand cDNAs for 5’-RACE and 3’-RACE analyses were synthesized with SMARTer™ RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions. The 5’-RACE and 3’-RACE fragments for IbISA1, IbISA2, and IbISA3 genes were amplified with first-strand cDNA as a template according to the manufacturer’s instruction for the SMARTer™ RACE cDNA amplification kit (Clontech). Specific primer sets of each gene were used, as shown in Table S1. The PCR products of the RACE reaction were cloned in pTA2 vector (TOYOBO, Osaka, Japan). Inserts in the plasmid vector were sequenced by the dyeoxygen chain-termination method by using a model 3130xl DNA sequencer (Applied Biosystems, Foster City, CA, USA) with BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence data were analyzed by DNASIS for Windows (Hitachi Software Engineering, Yokohama, Japan).

On the basis of partial sequence cloned by the RACE method, additional primer sets were also designated to amplify the full-length cDNA of each IbISA gene. The nucleotide sequences of specific primer sets for each IbISA gene are shown in Table S1. The cloning and determination of the nucleotide
sequence the PCR products were performed as described above.

**Sequence analysis, sequence comparison and construction of phylogenetic trees**

Sequence analysis was performed by using DNASIS for Windows (Hitachi Software Engineering) and BLAST program (http://ncbi.nlm.nih.gov/BLAST/; Altschul et al. 1990). For the phylogenetic analysis, amino acid sequences of ISAs from several plant species were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed by the neighbor joining (NJ) method using GENETYX ver.11 (Software Development Co., Japan; Saitou and Nei 1987). Bootstrap values were calculated from 1,000 replications.

**Quantitative real-time PCR**

Total RNA and mRNA from leaves and tuberous roots were isolated as described above. Real-time PCR reactions were performed on a 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with KOD SYBR® qPCR Mix (TOYOBO, Osaka, Japan), according to the manufacturer’s instructions. Primers sets used in qRT-PCR are also described in Table S1. *IbCOX* encoding cytochrome c oxidase subunit Vc and *IbARF* encoding ADP-ribosylation factor genes were used as reference genes.

**Results**

**Isolation and characterization of *IbISA1*, *IbISA2*, and *IbISA3* cDNA clones expressed in tuberous roots**

cDNAs were reverse-transcribed from mRNA isolated from tuberous root of sweet potato, cultivar White Star. *IbISA1*, *IbISA2*, and *IbISA3* sequences were then amplified from the cDNAs. To design primer sequences for amplification of *IbISAs*, the partial and/or full-length amino acid sequences of *ISA1*, *ISA2*, and *ISA3* from several plant species (*Arabidopsis thaliana*: accession no. AEC09752, AEE27558, AEE82713, Zea mays: accession no. ACG43008, AAB97167, AAA91298, AAO17048, AAO17049, *Oryza sativa*: accession no. NP_001062271, BAA29041, ACY56088, ACY56099, BAC75533, *Solanum tuberosum*: accession no. AAN15317, AAN15318, AAN15319, *Hordeum vulgare*: accession no. AAM46866, *Triticum aestivum*: accession no. CAC82925, CAC41016, *Pisum sativum*: accession no. AAZ81835, AAZ81836, AAZ81837, *Phaseolus vulgaris*: accession no. BAF52941, BAF52943, BAF52942, *Medicago truncatula*: accession no. XP_003602623, XP_003602838, *Aegilops tauschii*: accession no. AAP44579, *Secale cereale*: accession no. ACM41701, and *Ipomoea batatas*: accession no. AAY84833) were obtained from public databases.

To determine the conserved amino acid sequences of *ISA1*, *ISA2*, and *ISA3*, deduced amino acid sequences were aligned. In a BLAST search (Alschul et al. 1990) using each ISA conserved consensus sequence as a query in public databases, *ISA1*, *ISA2*, and *ISA3* genes from *I. batatas* were identified as follows. In the case of *IbISA1*, a gene termed *Ibisa1* (DQ074643) had previously been isolated from sweet potato, using a different cultivar, Kokei 14, from that used in the present study (Kim et al. 2005). Furthermore, in the transcriptome analysis data deposition (Tao et al. 2012), TSA (transcriptome shotgun assembly) for *IbISA2* (accession number, JPI11226) and *IbISA3* (accession number, JPI04934) were identified. A phylogenetic tree was constructed using these three *I. batatas* genes identified in databases and other ISA genes from several plant species. These three *I. batatas* genes were classified into the corresponding *ISA1*, *ISA2*, and *ISA3* clusters, indicating that they are predicted to be functional genes. In the present study, from conserved sequences in three *I. batatas* ISA genes, degenerate primer sets (Table S1) were designed and used in PCR to obtain partial cDNA clones of *IbISAs* (*IbISA1*, *IbISA2*, and *IbISA3*) by 5′-RACE and 3′-RACE methods.

The nucleotide sequences of amplified fragments were determined and compared to ISA genes isolated from other plant species. This confirmed that the fragments amplified by 5′-RACE and 3′-RACE contained the partial sequence of *IbISA1*, *IbISA2*, and *IbISA3* genes (data not shown). Based on the nucleotide sequence from the 5′-RACE and 3′-RACE experiments, full-length cDNA clones for *IbISA1*, *IbISA2*, and *IbISA3* genes were amplified with primer sets as shown in Table S1. For each gene, the complete nucleotide sequences of eight independent clones were determined. Interestingly, for each gene, *IbISA1*, *IbISA2*, and *IbISA3*, the independent clone sequences were identical. The full-length cDNAs of *IbISA1*, *IbISA2* and *IbISA3* were 2,524 bp, 2,880 bp, and 2,919 bp long, respectively. The nucleotide sequence data for *IbISA1*, *IbISA2* and *IbISA3* genes have been deposited in the DDBJ, EMBL, and GenBank databases with accession numbers LC052789, LC052790, and LC052791, respectively.

In order to discover whether these clones code for isoamylase enzymes, amino acid sequences deduced from the *IbISA1*, *IbISA2* and *IbISA3* cDNA clones were analyzed. The deduced amino acid sequences of the *IbISAs* cDNA clones suggested that they encode polypeptides of 799, 865 and 768 amino acid residues, respectively. These deduced amino acid sequences were then aligned to ISAs from other plant species. *IbISA1*, *IbISA2* and *IbISA3* isolated from sweet potato in this experiment formed clusters with *ISA1*, *ISA2* and *ISA3* from other species, respectively (Figure 1A). The deduced amino acid sequence of *IbISA1* showed 95.1% similarity to *Ibisa1* (accession no. AAY84833), 81.3%
identity with StISA1 (accession no. AAN15317), 70.2% identity with AtISA1 (accession no. AEC09752) and 67.2% identity with ZmISA1 (accession no. EU970890) in the European Molecular Biology Open Software Suite (EMBOSS; Rice et al. 2000). The deduced amino acid sequence of IbISA2 showed 62.4% similarity with StISA2 (accession no. AAN15318), 52.1% identity with AtISA2 (accession no. AEE27558) and 43.6% identity with ZmISA2 (accession no. AAO17048). The deduced amino acid sequence of IbISA3 showed 74.7% similarity with StISA3 (accession no. AAN15319).

Alignment of the amino acid sequences of the three IbISAs revealed that they have four conserved regions (Region I to IV), as present in other α-amylase family (GH13 family) starch hydrolytic enzymes (Beatty et al. 1999; James et al. 1995; Jespersen et al. 1993). In particular, within these four conserved regions, eight amino acid residues are highly conserved in all active members of the α-amylase family, as in ISA1s and ISA3s. Other two sites in Region I and III indicated by (+) retained functionally similar amino acid residues, Asp (D) and Glu (E), in IbISA1 and IbISA3 but not in IbISA2, indicating that these four amino acids are sufficient for α-amylase enzyme activity. In contrast, at the other four positions, amino acid residues conserved in ISA1s and ISA3s were replaced in ISA2s by amino acids with different properties (Figure 1B). In particular, the replacement of His (H) by Asn (N) in Region IV is predicted to result in loss of function in hydrolytic enzymes, such as S-RNase (Royo et al. 1994).

Figure 1. Phylogenetic tree of ISAs based on deduced amino acid sequences and sequence alignment of ISAs in four conserved regions. (A) Phylogenetic tree of ISAs based on deduced amino acid sequences. The tree was constructed using the neighbor-joining method (Saitou and Nei 1987). Scale bar represents evolutionary distance. Bootstrap values were calculated from 1,000 replications. The deduced amino acid sequences of sweet potato IbISAs protein (in box) were derived in this study (IbISA1, accession LC052789; IbISA2, accession LC052790; IbISA3, accession LC052791). Accession numbers for genes encoding ISAs of other plant spieces are: Arabidopsis thaliana AtISA1, AEC09752; AtISA2, AEE27558; AtISA3, AEE82713; potato (Solanum tuberosum) StISA1, AAN15317; StISA2, AAN15318; StISA3, AAN15319; maize (Zea mays) ZmISA1, EU970890; ZmISA2, AAO17048; ZmISA3, AAO17049; rice (Oryza sativa) OsISA1, BAC75533; OsISA2, AAT93894; OsISA3, XP_450961; pea (Pisum sativum) PsISA1, AAZ81835; PsISA2, AAZ81836; PsISA3, AAZ81837; common bean (Phaseolus vulgaris) PvISA1, BAF52941; PvISA2, BAF52942; PvISA3, BAF52943; sweet potato (Ipomoea batatas) IbISA1, AAY84333; wheat (Triticum aestivum) TaISA3, AEV92948; cassava (Manihot esculenta) MaISA3, ADD10143. (B) Multiple sequence alignment of ISAs in four conserved regions. Amino acid sequences in Regions I to IV are highly conserved in the α-amylase family. In particular, eight amino acid residues (shaded dark gray) are highly conserved in all active members of the α-amylases, ISA1 and ISA3. Region IV of ISA2s shows replaced residues from H (His) to N (Asn).
of roots was 37 g at 60 DAP (Figure S1B). At 75 to 80 DAP, hypertrophy of roots was observed but there were individual differences within a plant of root size. The average fresh weight of roots was 133 g (Figure S1B). At 110 DAP, larger tuberous roots were present and the average fresh weight of roots was 659 g (Figure S1B). Therefore, we collected leaves (source) and tuberous roots (sink) for qRT-PCR analysis at approximately 10 days intervals from 48 DAP (that is at 48, 57, 67, 78, 90, 99 and 109 DAP).

Next, the optimum set of reference genes for qRT-PCR was determined. Generally, genes encoding β-actin (ACT) and/or α-tublin (TUB) are used as reference genes (Osaka et al. 2013), but there are no ideal references genes able to fulfill all experimental requirements, and so the selection of optimum reference genes is essential (Bustin et al. 2009; Park et al. 2012). In the case of sweet potato, according to variety and/or environmental conditions, expression of reference genes differed (Park et al. 2012). Thus, for this study, to determine suitable reference genes in the variety White Star, under non-stress condition, seven genes encoding β-actin (ACT), α-tubulin (TUB), cytochrome c oxidase subunit Vc (COX), ubiquitin extension protein (UBI), ADP-ribosylation factor (ARF), phospholipase D1α (PLD), and histone (H2B) were surveyed for gene expression stability in qRT-PCR. From this survey, COX and ARF genes were found to be expressed at constant levels, and their expression levels were stable in different organs, growth stages, and sampling times (data not shown). Thus, COX and ARF were selected as reference genes in this experiment. In addition to primer sets for these two reference genes, three sets of specific primers, discriminating the three genes (IbISA1, IbISA2, and IbISA3) were designed, as shown in Table S1. Expression levels of each IbISAs, shown in Figures 2 and S1, were designated as relative values compared to expression levels of ARF and COX designated at 100.

From examination of gene expression patterns in tuberous roots (sink), expression levels of the three ISA genes were constant until 99 DAP (Figure 2A). However, at 109 DAP, the IbISA2 expression level was significantly up-regulated to more than 2-fold and approximately 2.5-fold compared to that of IbISA1 and IbISA3, respectively (Figure 2A). In the leaves (source), the IbISA3 expression pattern was relatively constant at all seven stages (Figure 2B). The expression pattern of IbISA1 and IbISA2 in leaves was similar until 99 DAP, followed by a higher level of IbISA2 expression at 109 DAP, the mature stage (Figure 2B). From a comparison between tuberous root development (indicated by DAP) and the expression of IbISAs, the increase in tuberous root development correlated with the expression pattern of IbISA2, but not of IbISA1 and IbISA3 (Figure S1A). Thus, at least in the morphologically mature tuberous roots, the expression of the IbISA2 appears to be related to the accumulation of starch; that is, morphological maturation and gene function of IbISAs are linked during tuberous root development.
development in _I. batatas_. In contrasts, the expression patterns of _ISAs_ in seeds, another type of sink (Kubo et al. 2010; Ohdan et al. 2005; Park et al. 2014; Sun et al. 1999; Zheng et al. 2013), was different from our data in sweet potato, as discussed below.

**Discussion**

*Molecular characterization of isoamylase genes in sweet potato*

The genes encoding three isoforms of _ISA_ in plants have been isolated and characterized in various plant species, including rice, maize and potato, which store starch in seeds and stems (Fujita et al. 1999; Hussain et al. 2003). However, few studies have focused on tuberous root plants, such as cassava, sweet potato, and yam, which store their starch in roots (Beyene et al. 2010; Kim et al. 2005). In this study, we isolated three genes encoding _IbISA1_, _IbISA2_, and _IbISA3_ from one of the tuberous root plants, sweet potato (_Ipomoea batatas_ (L.)). Isolation of _Ibisa1_ (DQ074643) has been reported in a previous study, which used a different cultivar, Kokei 14 (Kim et al. 2005); however, the current study is the first to report cloning of genes encoding all three types of _ISA_ from sweet potato. The sequence diversity (4.9%) between the two _ISA1_ genes from different cultivars (_IbISA1_ and _Ibisa1_) could be due to the self-incompatibility trait in sweet potato (Martin 1965). In addition to this characteristics, the three _IbISA_ genes were more similar to corresponding _ISA_ genes of other dicotyledonous plants (_Solanum tuberosum_, _Arabidopsis thaliana_, _Phaseolus vulgaris_, _Pisum sativum_ and _Manihot esculenta_) than those of monocotyledonous plants (_Zea mays_ and _Oryza sativa_) in the phylogenetic tree (Figure 1A), suggesting the _ISA_ gene speciation occurred after diversification of dicotyledonous and monocotyledonous plants, as found in other functional genes (Nakayama et al. 2010). Furthermore, within dicotyledonous plants, the nucleotide sequences of _ISAs_ of sweet potato have higher similarity to those of potato and cassava _ISAs_ than those of other plant species. This high sequence similarity among these species may be related to the formation of starch storage organs.

From the sequence alignment of _ISAs_ from sweet potato and other species (Figure 1B), deduced amino acid sequences of the three _IbISA_ genes isolated in this study contain four highly conserved regions (Region I to IV), which are classified into the α-amylase enzyme family, as reported for other _ISAs_ from several plants (Jespersen et al. 1993; James et al. 1995; Beatty et al. 1999). In the case of _IbISA2_, six of eight residues thought to be important for α-amylase activity were replaced by other amino acid residues (Figure 1B; gray shading), indicating that _IbISA2_ has no catalytic function, as proposed for _ISA2s_ from other plant species (Hussain et al. 2003; Utsumi and Nakamura 2006). In particular, histidine residue (His) is important for the hydrolysis reaction catalyzed by several enzymes (Chang et al. 2003; Royo et al. 1994). When His was replaced by another amino acid residue, hydrolytic enzyme activity was lost in S-RNase (Royo et al. 1994) and α-amylase (Chang et al. 2003). In the enzymatically active _ISA1_ and _ISA3_, two His residues within Regions I and IV were conserved. In contrast, _ISA2_ had only one conserved histidine residue in Region I, but in Region IV, His was replaced by another residue (Figure 1B; Region IV box), suggesting that His in Region IV may be particularly important for hydrolysis of α-1,6-glucosidic linkages.

*Spatiotemporal expression of _IbISA_ genes during tuberous root development*

In a previous report, the expression pattern of sweet potato _ISA1_ gene was observed up to 60 DAP (Kim et al. 2005). However, tuber development continues after 60 DAP and expression of all three _ISAs_ in sweet potato are expected to play an important role in tuber maturation and starch storage after 60 DAP. The tuberous roots of sweet potato are harvested at around 90 DAP (Woolfe 1992), and the harvesting period varies from 90 to 120 DAP in different cultivars (Ravi et al. 2009). In the case of cultivar White Star used in this experiment, the dry weight of tuberous roots is maximum at about 105 to 119 DAP (La Bonte et al. 2000), and therefore at 109 DAP morphogenesis of roots would have reached the hypertrophy stage. In addition, the weight of the storage root was found to increase according to the accumulation of photosynthetic products such as starch (Wilson 1982), and the dry weight of sweet potato correlates with starch content of tuberous root at different plant development stages (Li and Liao 1983). As described by Noda et al. (1992), the percentage of amylose content against to dry weight does not increase according to growth of sweet potato tuberous root. In our experiment, the expression of _IbISA2_ gene was correlated to tuberous root development, indicating that the content of amylose and amylopectin should be precisely regulated by _ISA2_ and related genes in tuberous root development. From our gene expression data and the above information on root development, _IbISA2_ expression was highest in roots at the hypertrophy stage (around 109 DAP), and therefore _IbISA2_ is expected to function in amylopectin biosynthesis in the sink (roots) of sweet potato with tuberous roots.

The _IbISA2_ expression pattern in sweet potato was different from that of _ISAs_ found in rice, rye, barley, maize, and amaranths (Kubo et al. 2010; Ohdan et al. 2005; Park et al. 2014; Sun et al. 1999; Zheng et al. 2013). In rice, both _ISA2_ and _ISA3_ were expressed at a very low level, and _ISA1_ had the highest expression around the middle stage of seed developmental, indicating that in
the case of seeds as a sink, ISA1 is the main ISA active in amylopectin biosynthesis (Kubo et al. 2010). Thus the expression pattern of ISAs might be related to the pattern of the sink development. In the case of tuberous roots, sink size would be greater as roots develop. However, in the case of seeds (endosperms) of Poaceae, sink size is restricted by that of the lemma and palea. This difference in development patterns of sinks between seeds and tuberous roots may contribute to the different expression patterns of ISAs. In any case, orchestration of expression of the three ISAs genes, in both seeds and tuberous roots, is expected to be important for sink organ development.

As described above, IbISA2 is expected to be non-functional as an enzyme from the predicted amino acid sequence but showed a high level of expression at the mature stage of tuberous root development. This higher expression of non-enzymatically active ISA2 and low expression of enzymatically active ISA1 resembles the expression of cell-cell recognition genes, SLG and SRK, of the self-incompatibility system in *Brassica* species (Watanabe et al. 2012). The S-domain of SRK, the receptor for the pollen ligand SP11, is highly similar to SLG, and SRK interacts with SP11 but SLG does not. SLG expression is over 10 times higher than SRK. Interestingly, SLG is required for a full manifestation of the self-incompatibility response (Takasaki et al. 2000). Thus, like the SLG function in self-incompatibility in *Brassica* species, the actual function of ISA2 may be discovered by different approaches in future.

In conclusion, from our data and from other plant species, the three ISA genes appear to be regulated spatiotemporally in sinks (endosperms, tubers, or roots) and sources (leaves) for amylopectin biosynthesis in different plant species. From the present study, higher expression of *IbISA2* may play an important role in amylopectin biosynthesis during root hypertrophy in sweet potato. Furthermore, orchestration of ISAs gene expression was different the different sink organs, that is seeds and tuberous roots, indicating that ISAs may contribute to the differences in quality and quantity of amylopectin in seeds and tuberous roots.

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