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Cloning, Expression, and Characterization of Soluble Starch Synthase I cDNA from Taro (*Colocasia esculenta* Var. *esculenta*)

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Soluble starch synthase I (SSSI) cDNA was isolated from taro (*Colocasia esculenta* var. *esculenta*) by RT-PCR and rapid amplification of cDNA ends reaction. The transcript of this single-copy gene is 2340bp and encodes 642 amino acids protein containing a putative transit peptide of 54 residues. Recombinant SSSI protein displayed both primer-dependent and primer-independent activities of starch synthase. More SSSI transcript was expressed in taro leaves than in tubers, with no evident expression in petioles; and more transcript and protein were found in tubers of 597 ± 37 g of fresh weight than in smaller or larger ones. Two forms of SSSI, i.e., 72 and 66 kDa, exist in leaves, and only the 66 kDa form was found in tubers. The taro SSSI, proposed as a novel member, was located only in the soluble fraction of tuber extract, while SSSI from other sources exist in both soluble and granule-bound forms.

KEYWORDS: Starch; starch synthase; SSSI; taro; RT-PCR; RACE

INTRODUCTION

Starch, the major storage compound accumulated in chloroplasts of leaves or amyloplasts of storage organs, is used as a source of energy during periods of dormancy and growth. It remains the most important source of calories in the diet of both humans and animals and is utilized in many industrial processes. The structure and proportion of the two components, amylose and amylopectin, determine the physicochemical properties of starch, such as swelling, solubility, plasticity, viscosity, and retrogradation (*1*, *2*).

The dramatically increasing demand in industry for specialized starches has prompted efforts to tailor the quantity and quality of the polymers. A clarification of the mechanism of starch synthesis has enabled the genetic modification of crops in a rational manner to produce novel starches with improved functionality (*3*).

Starch synthase (SS, EC 2.4.1.21) catalyzes the elongation of α-1,4-glucosidic bonds on amylose and amylopectin by transferring glucose from ADP-glucose. Two forms have been reported, i.e., a soluble starch synthase (SSS) and a granule-bound starch synthase (GBSS) (*4*). The *waxy* mutants of some plant species containing little or no amylose exhibit less GBSS than normal plants (*5*, *6*), consistent with the findings that the *waxy* locus in plants is the structure gene encoding GBSS (*7*−*9*). Therefore, the lack of amylose in the starch of *waxy* mutants is related to the deficiency of GBSS, implicating that this enzyme is critical for amylose synthesis.

Multiple forms of SSS have been found in plant leaves and storage tissues (*10*). On the basis of the amino acid sequences, this synthase is classified into SSSI, SSSII, SSSIII, and SSSIV (*11*). SSSII is the major starch synthase in pea embryos, and its absence causes severe alterations of starch content and structure (*12*). SSSIII is the major starch synthase in potato tuber, contributing up to 80% of total SSS activity (*13*, *14*).

In contrast, the roles of SSSI in starch biosynthesis are still not well understood, partly because of the ill-defined nature of this enzyme. While it accounts for a low proportion of starch synthase activity in potato tuber (*15*), SSSI provides the major starch synthase activity in rice and maize endosperms (*16*, *17*). The failure to obtain mutants in these species suggests that the mutations are either lethal or result in no apparent morphological changes (*11*).

We herein report the isolation and expression in *Escherichia coli* of a novel SSSI cDNA from taro, an important food crop in Africa, Asia, and South America. The expression patterns of this soluble starch synthase gene in some taro tissues and during different developmental stages are also described.

MATERIALS AND METHODS

Plant Material. Taros (*Colocasia esculenta* var. *esculenta*) were grown in the field. The collected leaves, petioles, and tubers were frozen immediately in liquid nitrogen, lyophilized, and then stored at −20 °C until required.

RNA Isolation. RNA was prepared by modifying the method, described in "Current protocols in molecular biology" (*18*). In the procedure, RNA solution was added with 20% enthanol and 0.5 M potassium acetate to selectively remove polysaccharide contamination. And then chloroform was used to replace the phenol/chloroform for

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Table 1. Nucleotide Sequences of Degenerate Primers Designed According to the Alignment of SSSIs from Potato, Maize, Rice, and Wheat[a]

name[b]	amino acid sequence	nucleotide sequence	length	degeneracy
P1 (+)	234 YGDVYGA 240	GATATGGWGATAWTTATGGTGC	22	4
P2 (–)	510 VGFNVAIS 517	GGAAACTGGAACAYTAAATCCAA C	24	2
P3 (+)	132 EAAPYSK 138	GAAGCWKCTCCWTATKCWAAG	21	32

[a] W denotes A or T; Y denotes C or T; K denotes G or T. [b] "+" indicates the coding strand; "–" indicates the complementary strand.

removing the protein contamination. The quality and quantity of RNA were determined by spectrophotometric methods and by agarose gel electrophoresis (*19*). Finally, RNA was stored at −70 °C until required. In addition, poly(A) RNA was purified with Oligotex (Qiagen, Valencia, CA) following the manufacturer's protocol.

Taro SSSI cDNA Cloning. A schematic representation of cloning strategy is shown in **Figure 2A**. The first strand cDNA mixture was prepared by Superscript II RNaseH$^-$ reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and then was subjected to PCR with the degenerated primers (**Table 1**), P1 and P2 for the first RT-PCR and P3 and P4 (GCATGCCAATCATTGGCAA) for the second RT-PCR product. Based on the results of RT-PCR described above, information of the full-length SSSI cDNA clone was obtained by combination of 3′- and 5′-RACE. 3′-RACE was performed with the primers P5 and P6 (GTTGATTGGTTTCATTGGAAG and GAC TCGAGTCGACATCG) by the method described by Frohman et al. (*20*). The template used in 5′-RACE was prepared with primer P4 by a SMART RACE cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA) following the manufacturer's procedure. Then it was subjected to PCR with primers P7, P8, and P9 (GAGACGAC-CATGACACGATGACCA, GGGCAAAGGCAAAGAACCACATAC, and AAGCAGTGGTAACAACGCAGAGTA, respectively). The fragments including the full-length of SSSI cDNA were amplified with primers P10 and P11 (GCAAACA TCACACCTCCACACAC and GTTCCTGCTGCCCCTTTGGACCA, respectively) by Platinum *Pfx* DNA polymerase (Invitrogen) following the manufacturer's protocol. All PCR products were sequenced after cloning into a pGEM-T-Easy vector (Promega, Madison, WI). And pGSSI850, pGSSIa, pGSSI3′, pGSSI5′, and pGSSIF were generated respectively in this cloning process. The sequence of taro SSSI has been deposited in GenBank under Accession No. AY142172.

Expression, Production, and Purification of Recombinant SSSI in *E. coli*. Two 1.9 and 1.8 kb fragments, which encode the entire coding region and transit the peptide truncated coding region, were PCR-amplified from pGSSIF using primer sets PA-PB (AATTCA-GGTACCATGGAGG TTCTACGGGTAGCA and AATTCGAAGCTT-TCTGACAT AAGGAGGATCTATGA, respectively) and PC (ATC-GAAGAATTCATGTGCGCTG CGGAGAAGAGGGA)-PB with the *Kpn*I/*Hind*III site introduced and *Eco*RI/*Hind*III site introduced, respectively. The PCR products were cloned into the pET29a vector (Novagen, San Diego, CA) to generate pESSI and pESSI′. The inserted genes were under the control of T7*lac* promoter, and in-frame fused at its 3′ end with a His-tag. The entire and transit peptide-truncated SSSI (SSSI-SP and SSSI-NSP, respectively) were produced by 2 mM isopropyl-β-D-thiogalactoside (IPTG) induction from *Esherichia coli* (*E. coli*) Rosetta(DE3) harbored with pESSI and pESSI′, respectively. SSSI-NSP was produced and purified by using Ni−NTA resins (Qiagen) under denaturing conditions described by the manufacturer. Then it was used as the antigen for anti-SSSI-NSP antiserum preparation.

Enzyme Assay. The transformants, induced with 2 mM IPTG at 20 °C for 20 h, were harvested and sonicated to prepare the crude enzyme solution. The primer-dependent and primer-independent starch synthase activities were measured using ADP[U-^{14}C] glucose as previously described (*21*, *22*). The reaction was performed at 30 °C, 90 min, and terminated by boiling for 2 min. One unit of activity is defined as 1 nmol ADP-glucose incorporated into α-glucan per minute at 30 °C. Protein concentration was determined by the Lowry method (*23*).

Southern Hybridization. Taro genomic DNAs (10 μg), digested completely with restriction endonucleases, were separated by 0.7% agarose DNA electrophoresis and blotted onto Hybond-N+ membranes (Amersham Biosciences). Hybridization and washing were carried out by the method described in "Zeta-Probe GT Blotting Membranes Instruction Manual" (BIO-RAY, Alfred Nobel Drive Hercules). The probe (0.4 kb), which had been excised from pGSSIa using *Eco*RI, was labeled with (α-^{32}P) dCTP using Rediprime II random prime labeling system (Amersham). After washing, the membranes were covered with polyethylene wrap and exposed while still wet to X-ray film (Amersham).

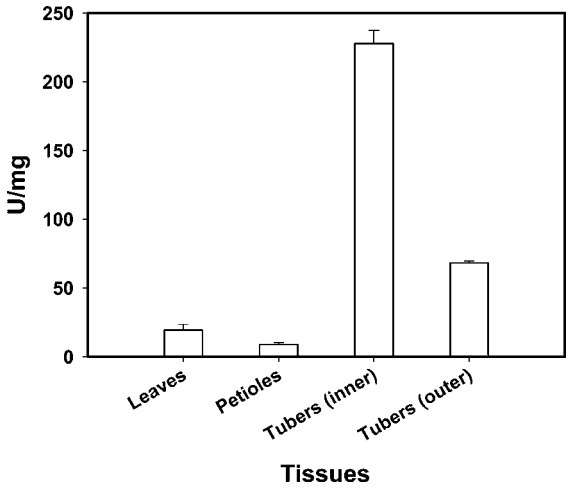

Figure 1. Comparison of starch synthase activities in crude extracts of different tissues from taro.

Western Blotting. Proteins were separated by 10% SDS−PAGE and electrophoretically transferred into Hybond-C Extra membrane (Amersham). The membranes were probed with anti-SSSI-NSP antiserum and then with peroxidase-conjugated AffiniPure goat anti-rat IgG (H + L) (Jackson ImmunoResearch, Cambridgeshire, U.K.). A positive SSSI signal was detected by incubation in TBS buffer (20 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 0.02% 4-chloronaphthol and 0.1% hydrogen peroxide.

RESULTS

Isolation of Taro SSSI cDNA Clones. Taro leaves and tubers which exhibited marked starch synthase activities (**Figure 1**) were selected as the primary sources for the preparation of RNA. A full-length SSSI cDNA obtained is shown in **Figure 2A**. RT-PCR with degenerate primers P1 and P2 generated a partial taro SSSI cDNA of 0.8 kb (**Figure 2B**). The product was cloned into pGEM-T Easy vector to generated pGSSI850 and was confirmed by sequencing and application of the "BlastX" program.

Another partial SSSI of 0.4 kb and the 3′-terminal end of SSSI of 0.9 kb were generated with the primers, designed according to the above clone, by RT-PCR and 3′-RACE (**Figure 2B**). And 5′-terminal cDNA of 0.7 kb was generated with the primers, designed according to the cDNA sequence of the 0.4 kb fragment, by SMART-RACE (**Figure 2B**). Finally, a full length of SSSI cDNA (2.1 kb) was generated by the primers, designed according to RACE products (**Figure 2B**). All products of RT-PCR and RACE were cloned into pGEM-T Easy vector to generate pGSSIa, pGSSI3′, pGSS5′, and pGSSIF. The sequences of all products were also confirmed by sequencing and application of the "BlastX" program.

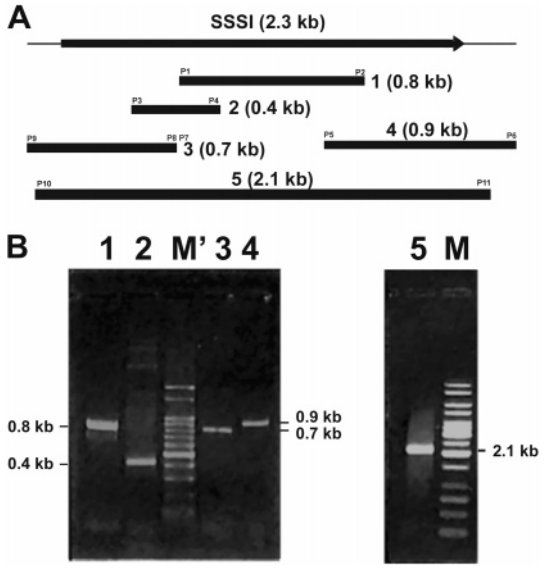

Figure 2. (**A**) Strategy for taro SSSI cDNA cloning. The topmost diagram shows the full-length cDNA. Boxes 1–5 represent cDNAs amplified by RT-PCR and RACE; (**B**) Agarose gel electrophoresis of RT-PCR and RACE products of SSSI. The partial SSSI cDNAs were amplified by RT-PCR using P1/P2 and P3/P4 primers, respectively (lanes 1 and 2); the product of the 5′ SMART-RACE was amplified using P5/P6 primers (lane 3); the product of the 3′-RACE product was amplified using P7/P8 primers (lane 4); the full-length of SSS I cDNA was generated by RT-PCR using the P10/P11 primers (lane 5): lane M′, 100 bp ladder (MBI); lane M, 1 kb ladder (MBI).

The techniques we used to identify novel SSSI from taro, i.e., RT-PCR and RACE, carry acknowledged risks. Polymerase-mediated cloning procedures might produce mutants. Furthermore, the products of both techniques may come from different isozymes. The use of *pfx* DNA polymerase which possesses a proofreading function in the final full-length cDNA cloning reduces the possibility of mutation in PCR. Additional primers (P10 and P11) were designed to generate a full-length cDNA with RT-PCR. All RT-PCR and RACE products were cloned and sequenced to confirm that they arose from the same transcript.

Characterization of Taro SSSI cDNA. The 2340 bp taro SSSI cDNA contained a coding region of 1932 bp, flanked by 5′- and 3′-untranslated regions of 154 and 254 bp, respectively (**Figure 3**). The open reading frame began with an ATG codon at position 155−157 and ended with the TGA codon at position 2084−2086. The open reading frame encoded a 643 residue protein with a theoretical molecular mass of 70 779 Da and a pI of 6.4, which both were estimated by "Compute pI/Mw Tool" (*24*). Use of the ChloroP 1.1 program (*25*) yielded predictions for SSSI of a putative 54 amino acid transit peptide with a cleavage site (CAAEK) located at amino acid 55 of the protein. A motif analysis of taro SSSI revealed a putative ADP-glucose binding site (KTGGLGDV) located at amino acids 148−155. In addition, general features of a glycosyl transferase group I located at amino acids 436−615 and a N−6 adenine-specific DNA methylase motif located at amino acids 635−641 were evident. It is noteworthy to point out that amino acids 545−550, RYGTVP, appeared very similar to the 14-3-3 protein phosphoserineythreonine-binding motif.

Alignment of protein sequences showed that the taro SSSI displayed an identity of 64, 61, 65, and 67% and a similarity of 70, 68, 70, and 73% with species obtained from maize, rice, wheat, and potato, respectively. Analysis with the GrowTree program of the GCG package showed that among the four classes of plant SSS groups, the cloned taro SSS belonged to the SSSI group and clustered more closely with that of dicot plants than monocot plants (**Figure 4**).

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   1 GATCGCAAAGGCAGACATCACACCTCCACACGCGCACGTTGGTCGGTTCCAGAAGGCGGA  60
  61 AGCAACTGCTCTCCGCTCCTGCCTCCCTTTTATCCCCGCTCCTCTGGATCTCCCCATCTT 120
 121 CCATCTCTCTCTCTCTCTCTCTCTCTCCGATCATGGAGGTTCTACGGGTAGCACGCCC 180
                                   M  E  V  L  R  V  A  R  P
 181 CGCATTGGCCGCCAGCAGCGCCCCCGGATCGCGGCCACTGCGTGGGGCTCCACGCCGCCC 240
      A  L  A  A  S  S  A  P  G  S  R  P  L  R  G  A  P  R  R  P
 241 GCCGCCTGGAGCTTGGCGGGCCGCCGGCTCCGCCCCGCGTCTGCGCCGGCGGCTCGGGGA 300
      P  P  G  A  W  R  A  A  G  S  A  P  R  L  R  R  R  L  G  D
 301 TCTCCCTCTGACGTTCTGCGCTGCGGAGAAGAGGGAGGAGGTGTTGCCTCCGGCTTTCGA 360
      L  P  L  T  F  C  A  A  E  K  R  E  E  V  L  P  P  A  F  E
 361 GGAGAAGGATGTGTCAGAGGTGTTGCTCCCCGCGGAGGCGCCTGGGAGTGGTCCTTGCTT 420
      E  K  D  V  S  E  V  L  L  P  A  E  A  P  G  S  G  P  C  F
 421 TGATGAGGCCGCCGGAGGTCCTCGGGGCGGAAGGAAGTAAAGGGAGAATGAGATCCAGTAC 480
      D  E  A  P  E  V  L  G  A  E  G  S  K  G  R  M  R  S  S  T
 481 TATTGCAGCTACTGTTAGTGGAGAAGAAGCATGAGAATGTTGGTGAGGAGAAGCAATCTCG 540
      I  A  A  T  V  S  G  E  K  H  E  N  V  G  E  E  K  Q  S  R
 541 ACCTGTGATAACAAATAACATTATTTTTGTCACTGCAGAAGCTGCTCCTTACTCCAAGAC 600
      P  V  I  R  N  N  I  I  F  V  T  A  E  A  A  P  Y  S  K  T
 601 TGGAGGTCTGGGAGATGTATGTGGTTCTTTGCCTATTGCCCTTGCTGCCCGTGGTCATCG 660
      G  G  L  G  D  V  C  G  S  L  P  I  A  L  A  A  R  G  H  R
 661 TGTCATGGTCGTCTCACCAAGGTATTTAGATGGTGTATCAGATGAGAAATTTGCAAATGC 720
      V  M  V  V  S  P  R  Y  L  D  G  V  S  D  E  K  F  A  N  A
 721 AGTTGATACTGGGCGCCGCATCAAGATTTCATGCTTTGGAGGACAGCAAGAAGTTGCTTT 780
      V  D  T  G  R  R  I  K  I  S  C  F  G  G  Q  Q  E  V  A  F
 781 TTTTCAAGAGTATAGGGCTGGTGTAGATTGGGTGTTTGTTGATCATCTCTCGTATCACAG 840
      F  Q  E  Y  R  A  G  V  D  W  V  F  V  D  H  L  S  Y  H  R
 841 ACCAGGGAACCCTTATGGTGATGTATATGGTGCGTATGGTGATAATCAGTTTCGGTTCAC 900
      P  G  N  P  Y  G  D  V  Y  G  A  Y  G  D  N  Q  F  R  F  T
 901 ACTGCTCTGCCATGCAGCATGTGAGGCTCCACTGGTGCTTCCACTGGGAGGATTTACCTA 960
      L  C  H  A  A  C  E  A  P  L  V  L  P  L  G  G  F  T  Y
 961 CGGAGATAAGTGCTTGTTCCTTGCCAATGATTGGCATGCAGCCCTGGTGCCAGTCCTTCT 1020
      G  D  K  C  L  F  L  A  N  D  W  H  A  A  L  V  P  V  L  L
1021 GAGTGCAAAATACCGCCCAAATGGTGTTTACAAAGATGGTCGCTGCATTCTTGTCATTCA 1080
      S  A  K  Y  R  P  N  G  V  Y  K  D  G  R  C  I  L  V  I  H
1081 TAACCTTGCACATCAGGGCGTGGAGCCAGAGACGACTTATCCGTTCCTAGGATTGCCATC 1140
      N  L  A  H  Q  G  V  E  P  E  T  T  Y  P  F  L  G  L  P  S
1141 CGAGTGGTATGGGTCTGTTGAGTGGGTCTTTCCTACTTGGGCAAGGACACATGCTCTTGA 1200
      E  W  Y  G  S  V  E  W  V  F  P  T  W  A  R  T  H  A  L  D
1201 TCCAGGCAAGTCTGTCAATCTTCTGAAAGGAGCAGTAGTCACAGTAGATCCGCATAGTCAC 1260
      P  G  K  S  V  N  L  L  K  G  A  V  V  T  V  D  R  I  V  T
1261 AGTTAGTCAGGGTTACTCTTGGGAGATAACAACCCCTGAGGGAGGATATGGTCTCCATGA 1320
      V  S  Q  G  Y  S  W  E  I  T  P  E  G  G  Y  G  L  H  E
1321 ACTCTTAAATAGCAGAAAGAGTGTACTCAATGGAATTACTAATGGCATTGATGTTGGTGA 1380
      L  L  N  S  R  K  S  V  L  N  G  I  T  N  G  I  D  V  G  D
1381 CTGGGATCCAGCCTCGGACCAGCACATACAATTTCATTATTCTGTCAATGACCTATCTGG 1440
      W  D  P  A  S  D  Q  H  I  Q  F  H  Y  S  V  N  D  L  S  G
1441 GAAGGTCAAATGCAAAATGGCTTTGCAGGAGGAATTTGGCCTTGATGTTCGTCCTGATTG 1500
      K  V  K  C  K  M  A  L  Q  E  E  F  G  L  D  V  R  P  D  C
1501 CCCATTGATTGGTTTCATTGGAAGGCTAGATATGCAGAAAGGGACTGATTTGATCATGAT 1560
      P  L  G  F  I  G  R  L  D  M  Q  K  G  T  D  L  M  M
1561 GGCAATGCCGGAGCTTATGAAAGAAGATATTCAATTTGTTATGCTTGGTTCTGGAGATGC 1620
      A  M  P  E  L  M  K  E  D  I  Q  F  V  M  L  G  S  G  D  A
1621 AGGTACAGAACAATGGATGAGAACTGCAGAATCTATTTATAGAGACAAGTTCCGTGGATG 1680
      G  T  E  Q  W  M  R  T  A  E  S  I  Y  R  D  K  F  R  G  W
1681 GGTTGGGTTTAATGTTGCTATTTCTCATCGGATAACTGCTGGGTGTGACATACTGTTAAT 1740
      V  G  F  N  V  A  I  S  H  R  I  T  A  G  C  D  I  L  L  M
1741 GCCATCAAGGTTGAACCTTGTGGTCTTAATCAATTGTATGCTATGAGATATGGAACTGT 1800
      P  S  R  F  E  P  C  G  L  N  Q  L  Y  A  M  R  Y  G  T  V
1801 TCCAGTTGTCCATGCCACTGGTGGCCTTAAGGATACGGTTGAGAATTTCAATCCATATGC 1860
      P  V  V  H  A  T  G  G  L  K  D  T  V  E  N  F  N  P  Y  A
1861 TAATGAAAGGAGTGGTAAGGGAACAGGGTGGGCTTTTCTCCTTTATCAAAGGATAGCAT 1920
      N  E  R  S  G  K  G  T  G  W  A  F  S  P  L  S  K  D  S  M
1921 GCTGAAGGCATTGAACTTGGCAATTCGGACATACAGGGATTTCAAGCCCTCATGGGAAGG 1980
      L  K  A  L  N  L  A  I  R  T  Y  R  D  F  K  P  S  W  E  G
1981 ACTCATGAAACGAGGGATGTCAAAAGATTTCACATGGGACAACGCCGCTGCTCAATATGA 2040
      L  M  K  R  G  M  S  K  D  F  T  W  D  N  A  A  A  Q  Y  E
2041 GCAAGTGTTCGGGTGGGCCTTCATAGATCCTCCTTATGTCAGATGATGCCCAGACAAACC 2100
      Q  V  F  G  W  A  F  I  D  P  P  Y  V  R  *
2101 CCTGTCGGCCAGATTCTTTCCCTGATTTCTTCCCCACGTTCCAGTTTTGCAGTAGCTAAC 2160
2161 ACATACTTTTCGTCTTCTCACTGTTCAAATGTCAAGGGTGGTCCAAAGGGCAGCAGGAAC 2220
2221 TTTGGTGAAATGTCTCTGTCACCTTCCTTCCGGACATTGGAAACCAATAAAGCCCCTGCA 2280
2281 TAAGCAATGACGTTGTACAGTGGACATTTCAATATTTAGTTCTATTCTTTGTGTAATGCC 2340
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Figure 3. Nucleotide sequence of taro SSSI cDNA. The transit peptide (located at amino acids 1–54), putative ADP-glucose binding motif (located at amino acids 148–155), glycosyl transferase group I motif (located at amino acids 436–615), and N-6 adenine-specific DNA methylase signature (located at amino acids 635–641) are underlined. The 14-3-3 protein binding consensus motif (located at amino acids 545–550) is shown in bold as well as italic. The potential polyadenylation sites are underlined and shown in italic.

Genomic DNA Blot Analysis and Expression Pattern of SSSI. Southern blot analysis of taro genomic DNA generated single signals of 1.4 and 3.9 kb, respectively, suggesting that taro SSSI has a high potential to be a single-copy gene (**Figure 5**).

RT-PCR analysis, using primers P3 and P4, revealed SSSI was expressed both in leaves and tubers, but not in the petiole (**Figure 6A**). With two other sets of primers, i.e., P1 and P2, and P10 and P11, a high SSSI expression in leaves and a comparatively low expression in tubers were detected (**Figure 6A**). Western blot analysis of total proteins also showed that the expression of SSSI was stronger in leaves than in tubers (**Figure 6B**). In addition, the 72 and 66 kDa SSSI species were found in leaves, but only the smaller one was evident in tubers.

A comparison of expression in different developmental stages of tubers, i.e., tubers of different fresh weight, indicated that

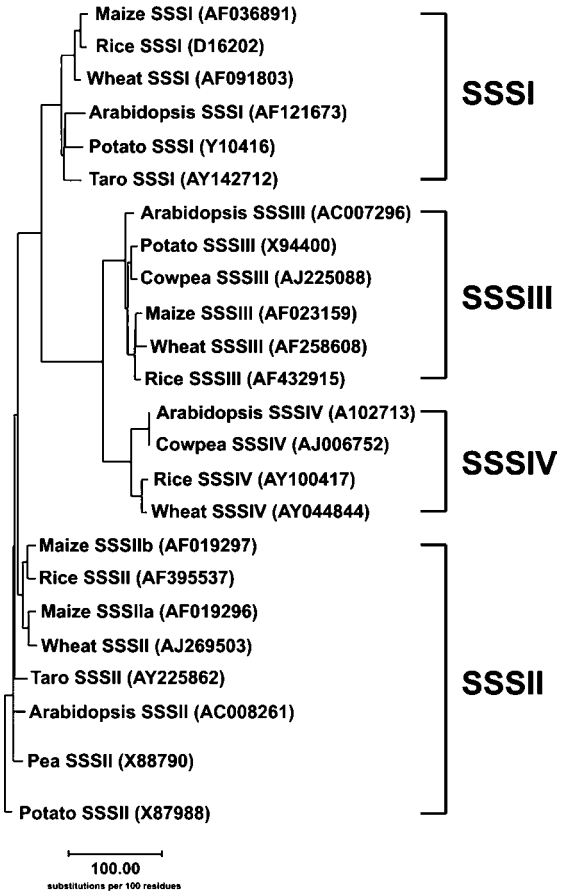

Figure 4. Relationships between the primary amino acid sequences of plant starch synthases. The dendrogram was generated by the program Growtree in the GCG package. All sequences of SSS were obtained from GeneBank/EMBLE/DDBJ.

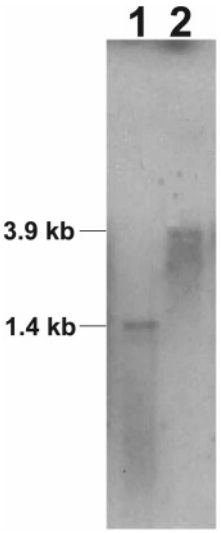

Figure 5. Southern blot analysis of taro genomic DNA. The 20 μg genomic DNA was digested with *Eco*RI (lane 1) and *Bam*HI (lane 2) and subjected to Southern blot analysis. Hybridization was carried out using the ^{32}P-labeled 0.4 kb partial SSSI cDNA.

the SSSI expression was evident only in tubers of 597 ± 37 g fresh weight (**Figure 7A**). In terms of the SSSI protein level, an increasing quantity was found in tubers of 106 ± 44 to 597 ± 37 g of fresh weight. Tubers of 1062 ± 72 g of fresh weight displayed a decreased amount of the protein (**Figure 7B**).

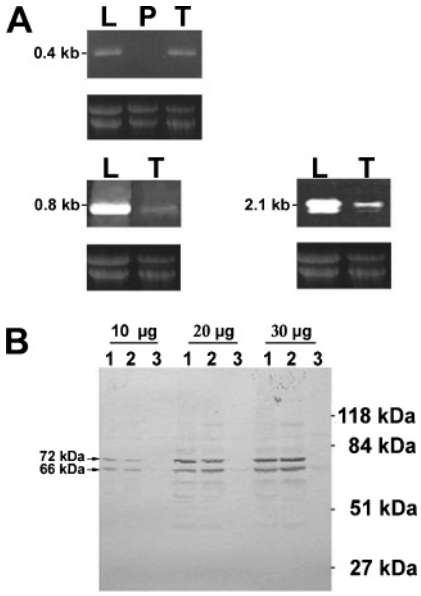

Figure 6. RT-PCR and Western blotting detection of SSSI expressed in different tissues of taro. (**A**) RT-PCR analysis of the tissue-specific expression of SSSI in leaves (lane L), petioles (lane P), and tubers (lane T) of taro. An aliquot of 10 μg RNA was used in RT-PCR. And the 0.4, 0.8, and 2.1 kb products were generated by the primers P3/P4, P1/P2, and P10/P11, respectively. (**B**) Total soluble extracts were fractionated by SDS–PAGE and probed by anti-SSSI-NSP. The amounts of samples were loaded in the lanes as indicated: lane 1, extract of young leaves; lane 2, extract of mature leaves; lane 3, extract of tubers.

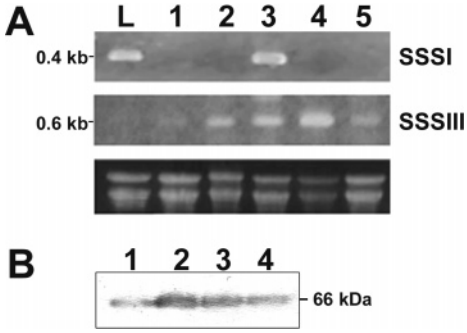

Figure 7. RT-PCR and Western blotting detection of SSSI expressed in different developmental stages of tubers (**A**) RT-PCR analysis of the temporal expression of SSSI in the up position of tubers of 1062 ± 72 g fresh weight (lane 1), bottom position of tubers of 1062 ± 72 g fresh weight (lane 2), the tubers of 597 ± 37 g fresh weight (lane 3), the tubers of 304 ± 56 g fresh weight (lane 4), and the tubers of 106 ± 44 g fresh weight (lane 5). An aliquot of 10 μg RNA was used in RT-PCR. The 0.4 and 0.6 kb products were generated to monitor the expression of SSSI and SSSIII, respectively. (**B**) Western blotting analysis of SSSI in the tubers of 1062 ± 72 g fresh weight (lane 1), the tubers of 597 ± 37 g fresh weight (lane 2), the tubers of 304 ± 56 g fresh weight (lane 3), and the tubers of 106 ± 44 g fresh weight (lane 4). An aliquot of 300 μg of protein was applied in SDS–PAGE and probed with anti-SSSI-NSP.

In addition, SSSI was primarily detected in the soluble extracts, but not in starch granules, from tubers of 597 ± 37 g fresh weight (**Figure 8**).

Enzymatic Activity of Recombinant SSSI. Direct evidence that the cloned SSSI encoded starch synthase was obtained by expressing the gene in *E. coli*. The primer-dependent specific activities of the recombinant SSSI and the truncated SSSI-NSP (without the transit peptide) were increased 2.7- and 1.5-fold, respectively, relative to the baseline level of glycogen synthase

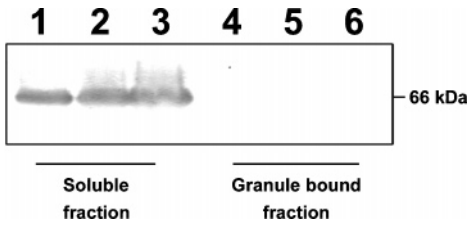

Figure 8. Immunological detection of SSSI in soluble and granule-bound fractions. The samples were separated by SDS–PAGE and probed with anti-SSSI-NSP. Lanes 1–3, 150, 200, and 300 μg of total protein in soluble fraction, respectively; lanes 4–6: 150, 200, and 300 μg of total protein separated from starch granule, respectively.

Table 2. Starch Synthase Activity in *E. coli* Soluble Extracts[a]

plasmid	insert	transit peptide	SS activity with primer (U[b]/mg)	SS activity without primer (U[b]/mg)
pET29a	none		154.8 ± 3.2	3.9 ± 0.2
pETSSI-SP	SSSI	with	421.0 ± 0.1	77.4 ± 0.2
pETSSI-NSP	truncated SSSI	without	231.8 ± 10.2	21.2 ± 1.3

[a] *E. coli* cell harbored with indicated plasmids were induced by 2 mM IPTG for 20 h at 20 °C. Total soluble extracts were assayed for starch synthase activity with or without amylopectin primer. [b] 1 U (unit) is defined as 1 nmol of ADP-glucose that was transferred per minute at 30 °C, pH 8.5.

activity (**Table 2**). Additionally, the primer-independent specific activities of the SSSI and SSSI-NSP were increased 20- and 5.5-fold, respectively. Detailed characterization of the expressed proteins will be described elsewhere.

DISCUSSION

Alignment of the SSSI protein sequence with that of four other SSSI species shows high identity and similarity, especially with potato SSSI reported by Kossmann et al. (*15*). The transit peptide cleavage site for taro SSSI identified by program "ChloroP" in this study is putative. The cleavage sites of maize SSSI and SSSIIa predicted by the same program (data not shown) were similar to the experimentally defined ones reported previously (*26*, *27*). Additionally, the signal identified by the anti-SSSI-NSP antibody in taro leaf and tuber extracts had a molecular mass of 66 kDa, which was similar to that of the putative transit peptide truncated SSSI. These results provide the indirect evidence for a cleavage site prior to residue 55. But it still needs to be defined experimentally.

The KXGGL motif in the amino-terminal region of taro SSSI is highly conserved in all starch synthase isozymes as well as in *E. coli* glycogen synthase. Since this conserved region in *E. coli* represents the substrate ADP-glucose binding site of glycogen synthase (*28*), it may well compose the substrate ADP-glucose binding site in taro SSSI.

The hexapeptide motif (RYGTVPVV) in taro SSSI, also conserved in all SSSIIIs, is identical to the14-3-3 protein binding site motif (*29*). *Arabidopsis* 14-3-3 proteins are found, by immunocapture using antibodies to maize SSSIII, to affect starch accumulation in leaves through a negative regulation of this synthase (*30*). On the basis of these results, we propose that taro SSSI may also be regulated by a 14-3-3 protein.

SSSI is predominantly expressed in taro leaves and to a lower extent in tubers, a pattern similar to that in potato (*15*), with the corresponding SSSI protein more abundant in leaf tissue. These results are consistent with the suggestion that SSSI contributes more to the deposition of transient starch in leaves than the synthesis of storage starch in tubers. However, the finding of high levels of SSSI transcript and SSSI protein in tubers of 597 ± 37 g fresh weight indicates that SSSI might also possess some specific functions in the synthesis of storage starch in the tuber stage. The observations of SSSI protein throughout all stages of tuber but no SSSI transcript in tubers larger or smaller than 597 ± 37 g fresh weight might be due to the fact that the copy number of SSSI transcripts was too low to be detected in our RT-PCR condition.

While the 66 and 72 kDa SSSIs are present in leaves, only the lower molecular mass species is present in tubers; and the same results have also been observed on 2-D PAGE (data not published). Maize and rice SSSI have been identified as a single-copy gene (*31*, *16*). Southern blot analysis revealed that taro SSSI may be a single-copy gene. These findings suggested that these two SSSIs have high potential to be the products sourced from the one gene. The similar result was also reported that three rice SSSIs sourced from the same gene in rice (*16*). Furthermore, the molecular weight of the two species conforms to that of a full-length SSSI and its transit peptide truncated form, both displaying primer-dependent and -independent starch synthase activities. It leads us to conclude that the SSSI gene in the taro genome encodes a starch synthase that is present in leaves in two forms and each one may possess its specific function in the biosynthesis of starch. But more experiments are required to define the details. In contrast to maize SSSI that exists in both soluble and granule-bound fractions (*17*), taro SSSI is found almost exclusively in the soluble fraction of tuber extracts, indicating that it is not stably associated with starch granules. Thus, the roles of taro SSSI, a novel member of starch synthase, may well differ from those of SSSI of other species.

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