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cDNA Cloning, Expression, and Characterization of Taro SSII: A Novel Member of Starch Synthase II Family

DA-GIN LIN AND CHII-LING JEANG*

Department of Food Science, National Chung Hsing University, Taichung, Taiwan, Republic of China

A novel soluble starch synthase II (SSII) gene was isolated from taro (*Colocasia esculenta* var. *esculenta*) tubers. This 2939 bp SSII transcript encodes 804 amino acids with a putative transit peptide of 52 residues. It displays 58−63% identity and 63−69% similarity with SSIIs from other sources. Alignment and phylogenetic analyses showed that taro SSII is more closely related with dicot SSIIs than with the monocot ones, though taro is a monocot. The identification of taro SSII clone as starch synthase was confirmed by the expression of its enzymatic activity in *Escherichia coli*. Genomic DNA blot analysis revealed a single copy or low number copies of SSII in taro. Expression profile showed that more transcript and protein were accumulated in tubers of 597 ± 37 g fresh weight, that is, a stage of rapid starch synthesis, than tubers of other stages. By Western blot analysis, SSII was found in both soluble and granule bound portions of tuber extracts, and more SSII protein was found in aged leaves than in leaves of other stages. These results suggest that taro SSII is a novel starch synthase for the synthesis of both transit and storage starch.

KEYWORDS: Cloning; expression; characterization; starch synthase; taro (*Colocasia esculenta* var. *esculenta*)

INTRODUCTION

Starch, the major storage compound accumulated in leaf chloroplasts or amyloplasts of storage organs, is a source of energy for plants during periods of growth and dormancy. It remains the most important source of calories in the diet of both humans and animals, and it is widely utilized in paper, textile, plastics, food, and pharmaceutical industries. The structure and proportion of its two components, that is, amylose and amylopectin, determine the physicochemical properties of starch, such as swelling, solubility, plasting, viscosity, and retrogradation (*1*, *2*).

In recent years, an increasing need for starch with novel properties has prompted the research community to concentrate its efforts on unraveling starch biosynthesis pathways. A clarification of the mechanism of starch synthesis has enabled the genetic modification of crops in a rational manner to produce novel starch with improved functionality (*3*).

Starch synthase (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 of this enzyme have been described, that is, soluble starch synthase (SS) and starch granule bound starch synthase (GBSS) (*4*, *5*). The *waxy* mutants of some plant species contain little or no amylose and exhibit less GBSS activity than normal plants (*6*, *7*), consistent with the findings that the *waxy* locus codes for the GBSS (*8*−*10*). Therefore, the lack of amylose in waxy mutants is related to GBSS deficiency, implicating that this enzyme is critical for amylose synthesis.

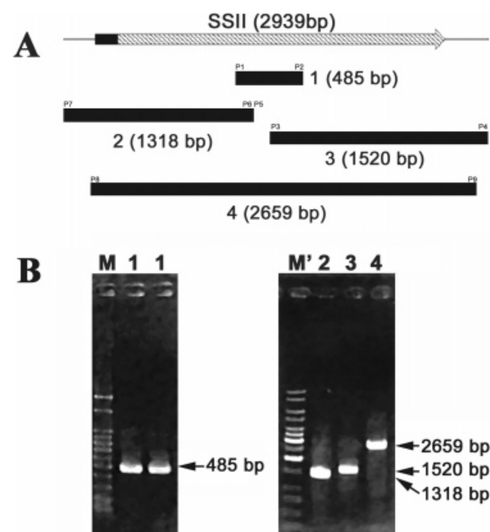

Figure 1. (**A**) Strategy for SSII cDNA cloning. The topmost diagram shows the full-length SSII cDNA. Boxes 1–4 represent cDNA fragments generated by RT-PCR and RACE. (**B**) Agarose gel electrophoresis for the products of RT-PCR and RACE. Lane 1: partial SSII cDNA generated by P1 and P2 primers; lane 2: the product of 5′-RACE amplified by P5, P6, and P7 primers; lane 3: 3′-RACE product generated by P3 and P4; and lane 4: the SSII cDNA encoding entire open reading frame generated by P8 and P9 primers. Lane M: 100 bp ladder and lane M′: 1 kb ladder (MBI).

Multiple forms of SS are found in plant leaves and storage tissues (*11*); to date, four classes, that is, SSI, SSII, SSIII, and SSIV, based on primary sequences, have been reported (*12*). How these isozymes affect the amount and composition of starch

* Author to whom correspondence should be addressed. Tel: 886−4−22862797; fax: 886−4−22876211; e-mail: cljeang@nchu.edu.tw.

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synthesized in plants remains to be elucidated. Elimination of SSII in pea embryo (*rug5* mutant) drastically altered the morphology of starch granules; in addition, chain-length distribution of amylopectin was also affected significantly, with a decrease in the number of medium-length chains and an increase of both short (DP < 10) and long (DP > 25) chains (*13*). The altered chain-length distribution and subtle effects on starch structure and total starch synthase activity were also observed in transgenetic potato tubers with low level of SSII (*14*). In monocots, two classes of SSII (SSIIa and SSIIb) have been found, but the role of SSIIb in endosperm starch biosynthesis is still unknown. Elimination of SSIIa in cereals caused a reduction of starch content, an alteration of starch structure, and a distinct chain distribution (*15*, *16*). These findings suggest that SSII of dicots and SSIIa of monocots may play similar roles in amylopectin biosynthesis. Since plant organs vary greatly in SSIIs that they possess, the relative contribution of these SSIIs to starch synthase activity may vary accordingly. For example, SSII contributes more than 60% of the soluble activity in pea embryo, in contrast to a maximum of 15% of the soluble activity in potato tubers (*13*, *14*). Nevertheless, details about functions of SSII in monocots remain unclear. To facilitate further studies of SSII in starch biosynthesis, it is essential to characterize this enzyme and to establish its primary structure.

We herein report the isolation, expression in *Escherichia coli*, and characterization of a novel SSII cDNA from *Colocasia esculenta* var. *esculenta* (taro, a monocot plant), an important food crop in Africa, Asia, and South America with its starch holding a high value in food industry. Alignment and phylogenetic analyses of SSII cDNA were performed, and transcription and protein production in tuber and leaf tissues were also investigated.

MATERIALS AND METHODS

Plant Material. Taro (*Colocasia esculenta* var. *esculenta*) tubers were harvested at different developmental stages on the basis of their fresh weight, that is, 106 ± 44 g, 304 ± 56 g, 597 ± 37 g, and 1062 ± 72 g, and leaves in different developmental stages, that is, bud, young, mature, and aged stages, were sampled. The collected leaves, petioles, and tubers were frozen immediately in liquid nitrogen, were lyophilized, and then were stored at −20 °C until required.

RNA Isolation. The method described in *Current Protocols in Molecular Biology* (*17*) was modified to overcome the problem caused by the large amounts of polysaccharides, for example, starch and water-soluble mucilage, in taro tissues. To selectively remove these polysaccharide contaminants, 20% ethanol and 0.5 M potassium acetate were added to the RNA solution after extraction and centrifugation; then, chloroform was used to replace phenol/chloroform to remove protein contaminants. Finally, RNA was stored at −70 °C until required. Also, poly(A) RNA was purified with Oligotex (Qiagen, Valencia, CA) following the manufacturer's protocol.

Taro SSII cDNA Cloning. A schematic representation of cloning strategy is shown in **Figure 1A**. The first strand cDNA mixture was prepared by SuperscriptII RNaseH$^-$ reverse transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol and then was subjected to PCR with two degenerated primers, P1(YAAAACAGGTGGNCTBGGAGATGT) and P2 (GRCCCTGRTGVGCKATGTTATGWAT). On the basis of the results of RT-PCR, information of full-length SSII cDNA clone was obtained by a combination of 3′ and 5′-rapid amplification of cDNA ends (RACE). 3′-RACE was performed with primers P3 and P4 (GCCATCGTGAAAGCAACAT and GACTCGAGTCGACATCG) by the method described by Frohman et al. (*18*). The template used in 5′-RACE was prepared with primer P5 (GAAAACGAAATCCA CACCATCAAT) 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 P6 and P7 (GAAAACGAAATCCACACCATCAAT and GTTG AGGTCCAGAATATTGCCAT). The fragments including SSII coding region were amplified with primers P8 and P9 (GTAAGGAGGACAGA AAGATGGCATCTTTGGGACGA and GAGCCATACTACACGGCTGCAAT), designed according to sequences of RACE products, by Platinum *Pfx* DNA polymerase (Invitrogen) following the manufacturer's procedure. The above four cDNA fragments, obtained from RT-PCR and RACE (boxes 1−4, **Figure 1A**), were cloned into pGEM-T-Easy vector (Promega, Madison, WI) to generate pGSSIIa, pGSSII3′, pGSSII5′, and pGSSIIF, respectively, and then they were confirmed by sequencing. The sequence of taro SSII has been deposited in GenBank under Accession No. AY225862.

Expression, Production, and Purification of Recombinant SSII in *E. coli*. Two fragments (2.4 kb and 2.3 kb), encoding the entire coding region and transit peptide truncated coding region, were PCR amplified from pGSSIIF using primer sets PA-PB (ATCGAAGGATCCATGGCATCTTTGGGACGA and GATCATCACTCGAGCCAC TGGTACTTGGCAGCAAC) and PC (ATCGAAGGATCCGCGGGCAATGGCGAA AGGCAC)-PB both with *Bam*HI/*Xho*I introduced. pGEXSSII and pGEXSSII′, constructed by cloning the above products in the *Bam*HI and *Xho*I sites of pGEX-4T-2 vector (Amersham Biosciences, Buckinghamshire, U.K.), were transformed into *E. coli* Rosetta(DE3) (Novagen, Darmstadt, Germany) for producing the entire and transit peptide truncated SSII fusion proteins. The cultured cells, harboring the indicated plasmids, were induced by 2 mM isopropyl-beta-D-thiogalactoside (IPTG) at 30 °C for 5 h. Then, the harvested cells were sonicated, and after removing cell debris it was used as a crude enzyme solution. Furthermore, GST-SSII was purified by GSTrap FF column (Amersham Biosciences) by the method described by the manufacturer and then was used as an antigen for antiserum preparation.

Enzyme Assay. The starch synthase activity of the abovementioned crude enzyme solution was examined using ADP(^{14}C) glucose as previously described (*19*). The reaction was performed at 30 °C for 90 min and was terminated by boiling for 2 min. One unit of activity is defined as 1 nmol ADP-glucose incorporated into α-glucan per min at 30 °C. Protein concentration was determined as described elsewhere (*20*).

Southern Hybridization. Taro genomic DNAs, digested completely with restriction endonucleases, were separated by 1.0% agarose DNA electrophoresis and were blotted onto Hybond-N+ membranes (Amersham Biosciences). Hybridization and washing were carried out by the method described in *Zeta-Probe GT (Genomic Tested) Blotting Membranes Instruction Manual* (Bio-Rad, Hercules, CA), and ^{32}P labeled DNA probe of 0.5 kb was excised from pGSSIIa and was labeled with (α-^{32}P) dCTP using Rediprime II random prime labeling system (Amersham Biosciences). After washing, the membranes were covered with polyethylene wrap and were exposed while still wet to X-ray film (Amersham Biosciences).

Preparation of Total Protein from Different Tissues and Fractionation of Taro Tuber Extracts. The ground tissues were reconstituted in extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, pH 10, 50 mM EDTA, 0.1 M KCl, and 2% β-mercaptoethanol). The total protein was transferred into water-saturated phenol phase and then was precipitated, washed, and dissolved by the methods described in *2-D Electrophoresis Using Immobilized pH Gradients: Principles and Method* (Amersham Biosciences). Besides, fractionation of taro tuber extracts was preformed by the method described Cao et al. (*21*). The amount of protein was determined, and then the sample was stored at −70 °C until required.

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

RESULTS AND DISCUSSION

Isolation of Taro SSII cDNA Clones. In this work, a partial SSII cDNA of 0.5 kb was generated initially by RT-PCR with

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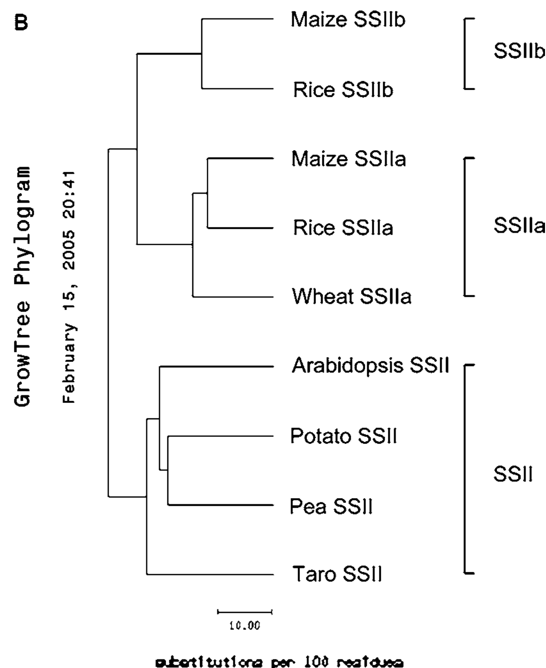

Figure 2. Alignment of the primary sequences of several plant SSII members (**A**) and phylogenetic tree of SSIIs (**B**). Sequences of all members were obtained from the Genbank database: taro SSII (Accession No. AY225862), pea SSII (Accession No. ×88790), potato SSII (Accession No. X87988), wheat SSIIa (Accession No. AF155217), rice SSIIa (Accession No. AF419099), rice SSIIb (Accession No. AF395537), maize SSIIa (Accession No. AF019296), maize SSIIb (Accession No. AF019297), and arabidopsis SSII (Accession No. AY054467). This alignment was performed by Clustal W program. The arrow indicates the transit peptide processing site. The asterisks indicate residues involved in the putative ADP-glucose binding site, HPr serine phosphorylation site, and glycosyl transferase group I motif, respectively, and glycogen/starch synthase, ADP-glucose type, signature is underlined. The conserved regions in dicot or monocot SSIIs are shown in boxes. On the basis of the sequence alignment, a phylogenetic tree was constructed.

degenerated primers (**Figure 1B**). Then, the 5′ end of SSII of 1.3 kb was generated by 5′-RACE and the 3′ end of 1.5 kb was generated by 3′-RACE (**Figure 1B**). Finally, a near full-length fragment (about 2.6 kb) encoding the entire open reading frame was obtained by *Pfx* DNA polymerase with proofreading function (**Figure 1B**) and then was cloned for the confirmation of the above products. With the generated recombinant plasmid, pGSSIIF, as the template for constructing expression system, all RT-PCR and RACE products were found to have identical overlapping regions. It is thus confirmed that all partial cDNA fragments did come from the same transcript.

The taro SSII cDNA is 2939 bp in length and contains a coding region of 2415 bp flanked by 5′- and 3′-untranslated regions of 219 and 305 bp, respectively. The open reading frame, beginning with an ATG codon at position 220−222 and ending with the TGA codon at position 2632−2634, encodes 804 residues protein with a theoretical molecular mass of 89 014 Da and pI of 5.8. It is predicted that this SSII contains a putative 52 amino acid transit peptide according to the ChloroP 1.1 program (*22*) (**Figure 2A**).

Alignment of taro SSII protein with other SSIIs showed that it displays 58, 58, 58, 60, 61, 62, 63, and 63% identity and 64, 63, 64, 67, 67, 69, 68, and 68% similarity with wheat SSIIa, rice SSIIa, maize SSIIa, maize SSIIb, rice SSIIb, pea SSII, arabidopsis SSII, and potato SSII, respectively. The alignment results also showed that while all dicot SSIIs shared two conserved regions, that is, boxes 1 and 2, all monocot SSIIs shared another four conserved regions, that is, boxes A− D (**Figure 2A**). Yet, SSII of taro, a monocot plant, contains the two conserved regions that appear in SSIIs of dicot plants. Also, among the three major classes of SSIIs (**Figure 2B**), only two, SSIIa and SSIIb, have been found in monocots; the third class includes SSIIs found only in dicot plants and taro. These results clearly suggest that taro SSII is distinct from the monocot SSIIs and yet is closely related to dicot SSIIs.

Motif scanning, analyzed by InterPro Scan and ScanProsite (in ExPASY Web site), revealed several unique features in taro SSII protein: the putative ADP−glucose binding motif KTG-GLGDV at amino acids 327−334; a general feature of the glycosyl transferase group I (Pfams Accession No. PF00534) located at amino acids 605−768; glycogen/starch synthase, ADP-glucose type signature (TIGRFAMs Accession No. TIGR02095) located at amino acids 313−800; and HPr serine phosphorylation site signature (Pfams Accession No. PS00589) at amino acids 469−484 (**Figure 2A**).

Genomic DNA Blot Analysis. Single signals of 6.4, 11.8, and 0.7 kb were observed when genomic DNA was digested with *Eco*RI, *Bam*HI, and *Hin*dIII, respectively, while two signals, 3.9 and 0.7 kb, were observed in the products of *Dra*I digestion (**Figure 3**). These results indicate the presence of only one copy or low number copies of SSII gene in the genome of taro.

Expression and Purification of Recombinant SSII in *E. coli*. Direct evidence that cloned SSII encodes starch synthase was obtained by expressing the gene in *E. coli* Rosetta. The two recombinant types constructed in this work are (1) the entire SSII (GST-SSIISP from cells harboring pGEXSSII) and (2) a transit peptide truncated SSII (GST-SSII from cells harboring pGEXSSII′). The starch synthase activities of GST-SSIISP and GST-SSII were increased 1.9- and 2.3-fold, respectively, relative to the baseline level of glycogen synthase activity (**Table 1**). Under the same induction condition, more target protein was produced from cells harboring pGEXSSII′ than from cells harboring pGEXSSII (**Figure 4A**), suggesting that the transit peptide of GST-SSIISP reduced the production of recombinant protein. Therefore, GST-SSII was produced and purified to a

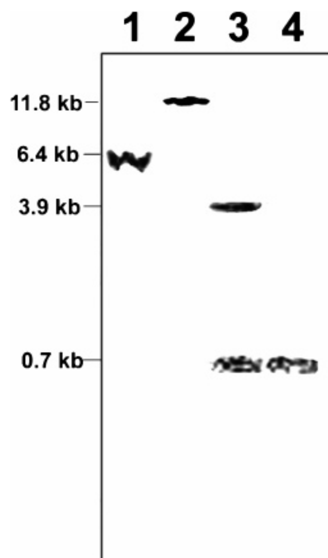

Figure 3. Southern blot analysis of taro genomic DNA. Twenty micrograms genomic DNA was digested with different restriction endonucleases and was subjected to Southern blot analysis. Lane 1: with *Eco*RI; lane 2: with *Bam*HI; lane 3: with *Dra*I; and lane 4: with *Hin*dIII. Hybridization was carried out using the ^{32}P-labeled 0.5 kb partial SSII cDNA in pGSSIIa.

Table 1. Starch Synthase Activity of *E. coli* Soluble Extracts. *E. coli* Cell Harboring the Indicated Plasmids Were Induced by 2 mM IPTG at 30 °C for 5 h

	pGEX-4T-2/Rosetta	pGEXSSII/Rosetta	pGEXSSII′/Rosetta
specific activity (U/mg)[a]	7114 ± 136	13349 ± 566	14373 ± 2795

[a] 1 U (unit) is defined as 1 nmol ADP-glucose transferred to potato amylopectin per minute at 30 °C.

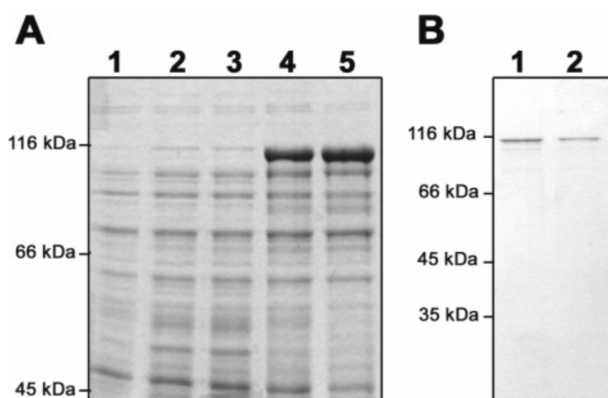

Figure 4. Production and purification of recombinant SSIIs in *E. coli.* (**A**) The total protein samples, from cells harboring indicated plasmids after induction of 2 mM IPTG, were separated by SDS–PAGE and were stained by Coomassie Blue. Lane 1: *E. coli* harboring pGEX-4T-2; lane 2, 3: *E. coli* harboring pGEXSSII; and lane 4, 5: *E. coli* harboring pGEXSSII′. All samples were adjusted at the same A600 value prior to the preparation of SDS–PAGE sample. (**B**) The fusion protein, GST-SSII, purified by GSTrap FF chromatography, was separated by SDS–PAGE and was stained by Coomassie Blue. Lane 1, 2: purified GST-SSII.

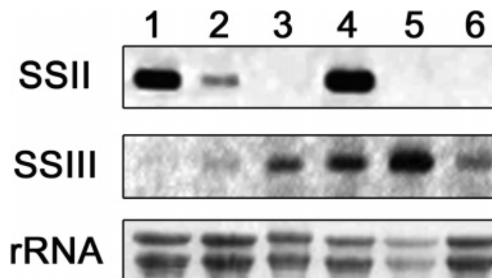

Figure 5. RT-PCR analysis of the expression of SSII in leaves and tubers. Lane 1: young leaves; lane 2: upper portion of tubers of 1062 ± 72 g fresh weight; lane 3: bottom portion of tubers of 1062 ± 72 g fresh weight; lane 4: tuber of 597 ± 37 g fresh weight; lane 5: tuber of 304 ± 56 g fresh weight; and lane 6: tubers of 106 ± 44 g fresh weight. An aliquot of 10 μg total RNA was used in RT-PCR. The 0.5 kb and 0.6 kb products were generated to monitor the expression of SSII and SSIII, respectively.

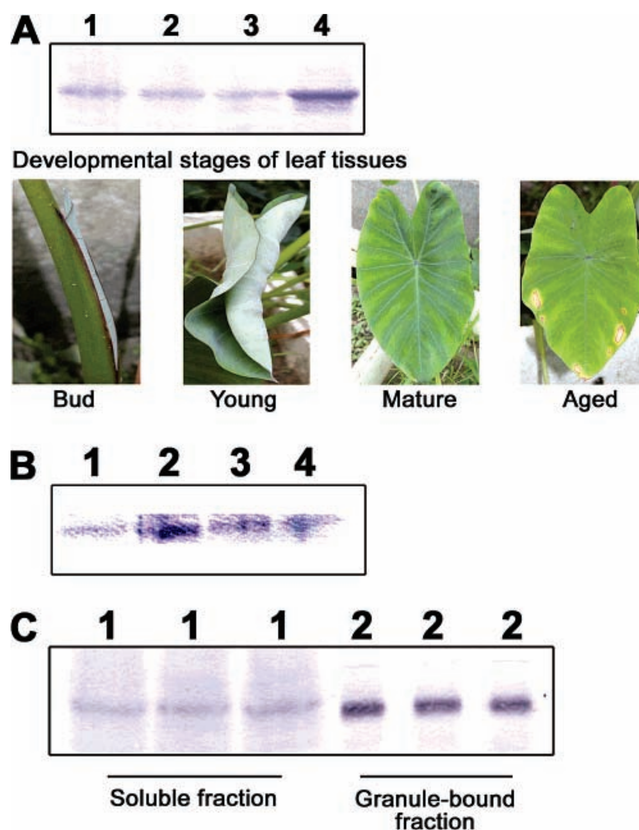

Figure 6. Immunological detection of SSII in taro extracts. (**A**) Temporal expression of SSII in leaves. Lane 1: bud leaves; lane 2: young leaves; lane 3: mature leaves; and lane 4: aged leaves. (**B**) Temporal expression of SSII in tubers. Lane 1: tubers of 1062 ± 72 g fresh weight; lane 2: tubers of 597 ± 37 g fresh weight; lane 3: tubers of 304 ± 56 g fresh weight; and lane 4: tubers of 106 ± 44 g fresh weight. (**C**) Fractionation of tuber extracts. Lane 1: soluble fraction and lane 2: granule bound fraction. A defined amount of each sample (50 μg protein) was used in analysis A; 100 μg protein was used in analysis B; and C was separated by SDS–PAGE and then was immunostained by the antibodies, raised against GST- SSII.

homogeneous state by GSTrap FF chromatography (**Figure 4B**). It was used as antigen for the preparation of antibody.

Transcriptional and Translational Profiles of SSII in Taro. The expression of SSII was high in taro leaves and tubers of 597 ± 37 g fresh weight and was comparatively low in tubers of 1062 ± 72 g fresh weight (**Figure 5**). Also, the transcriptional profile of SSII was different from that of SSIII, suggesting that these two starch synthases have different functions in the starch biosynthesis of taro.

A protein with an estimated size of 96 kDa on SDS–PAGE was recognized (**Figure 6A**) by the antibody raised against the recombinant GST-SSII. It was larger than the theoretical molecular mass of taro SSII, 89 014 Da. Similar observations were reported in other plants, for example, maize SSI and potato

SSII (*14*, *24*, *25*), and ScanProsite (in ExPASy web site) analysis showed that these SS might be glycosylated, phosphorylated, or amidated. Among these posttranslational modifications, only phosphorylation has been proved for wheat SSIIa in amylopast (*26*) and is speculated to be involved in the regulation of starch synthase activity. In this study, a HPr serine phosphorylation site signature highly conserved in all SSIIs at 469−484 residues implies that, in starch synthesis, SSII might be regulated by phosphorylation with HPr or HPr-like protein. The latter is involved in the regulation of certain important metabolisms in Gram-positive bacterium (*27*).

A large amount of SSII was detected in aged leaves, a somewhat unusual phenomenon. Increasing quantities of SSII protein were found in tubers of 106 ± 44 g up to 597 ± 37 g fresh weight, yet tubers of 1062 ± 72 g fresh weight displayed a decrease of this protein (**Figure 6B**). In addition, SSII in tubers of 597 ± 37 g fresh weight was found primarily in the starch granule portion of tuber extracts, with a comparatively low content in the soluble portion (**Figure 6C**). Large amounts of SSII transcript and protein observed in tubers of 597 ± 37 g fresh weight, representing a stage of rapid growth and starch synthesis (*28*), indicates that the accumulation of starch in taro tubers requires the involvement of this enzyme. Finding SSII transcript in both leaves and tubers implies that this enzyme is involved in both transient and storage starch synthesis in taro.

The unique features of taro starch granules, which are 1.2−6 μm in diameter and smaller than starch granules from other corps, make this plant an ideal material for the study of starch synthesis (*29*, *30*). While multiple SS isoforms have been identified in taro (unpublished results), the biochemical and physiological function of each individual soluble starch synthase remain to be investigated. Identification in this study of SSII gene, which encodes a novel starch synthase for the synthesis of both transit and storage starch, provides an opportunity to fill the gaps and to define its precise functional role in amylopectin synthesis in taro.

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