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Original Paper

Increase of amylose content of sweetpotato starch by RNA interference of the starch branching enzyme II gene (*IbSBEII*)

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Abstract In the storage roots of sweetpotato (*Ipomoea batatas* (L.) Lam. cv. Kokei 14), 10 to 20% of the starch is essentially unbranched linear amylose and the other major component is branched amylopectin. The starch branching enzymes, which are responsible for production of amylopectin to form α-1,6-linkages in the glucan can be divided into two classes, class A (e.g. potato and maize SBEII, pea SBEI) and class B (e.g. potato and maize SBEI, pea SBEII). On the bases of the registered cDNA of sweetpotato SBEII (*IbSBEII*) encoding class A branching enzyme, we constructed double-stranded RNA (dsRNA) interference vectors and introduced them into sweetpotato genome via *Agrobacterium*-mediated gene transformation. We obtained eight independent transgenic plants by using two kinds of RNA interference (RNAi) constructs, encoding *GBSSI* 1st intron-spliced RNA or a GUS fragment-spliced RNA, respectively. All transgenic plants were confirmed not to express *IbSBEII* by RT-PCR and to have the starch with a higher amylose content than the non-transgenic control (up to 25% compared to 10% in the control). Both constructs induced the same level of silencing of *IbSBEII* in all transgenic plants. The morphological characters showed no significant differences between the transgenic and control plants. Starch yield of transgenic tubers was slightly lower than that of non-transgenic tubers. The starch granules of the transgenic plants were similar to those of typical sweetpotato starchs in shape and the distribution in granule size, but slightly different in grain structure.

Key words: Amylose, *IbSBEII*, RNA interference, starch branching enzyme II, transgenic sweetpotato.

Sweetpotato is one of the most important crops in the world and provides not only staple food but also important industrial raw materials. Starch is the major storage carbohydrate, which is composed essentially of linear amylose and branched amylopectin. In sweetpotato starch the range in amylose contents is relatively narrow (10 to 25%) compared with other crops (Noda et al. 1998). The ratio of amylose to amylopectin has a great influence on the physicochemical properties of starch. Genetic engineering of starch has a high potential for the quality improvement of sweetpotato starch and helps the development of new dietary and industrial applications.

Biosynthesis of starch from adenosine diphosphoglucose (ADP-glucose) is catalyzed by multiple enzymes. The enzyme GBSSI (granule bound starch synthase I) is required to produce the linear polysaccharide amylose, and the soluble starch synthases and starch branching enzymes act in concert to produce amylopectin. The starch branching enzymes, responsible for forming α-1,6-linkages in the glucan can be divided into two classes, class A (e.g. potato and maize SBEII, pea SBEI) and class B (e.g. potato and maize SBEI, pea SBEII). Although SBEI (class B) and SBEII (class A) of sweetpotato have not been characterized yet, the *SBEII* cDNA have been registered.

In potato, antisense inhibition of *SBEII* resulted in a moderate increase in the apparent amylose level up to 38% (Jobling et al. 1999), although antisense inhibition of *SBEI* did not result in an increased amylose level (Jobling et al. 1999). However, recently, they succeeded in generating potato starch with a very high amylose content by simultaneous inhibition of *SBEI* and *SBEII* by antisense RNAs (Schwall et al. 2000).

Recently, double-stranded RNA (dsRNA)-mediated gene silencing has been found to be effective technology in the genetic improvement of crops as well as in functional genomic studies (Waterhouse et al. 1998). We transformed sweetpotato with the intron-spliced hairpin RNA of the first exon of sweetpotato *GBSSI*, giving the high degree of silencing of the endogenous *GBSSI* gene and the transfomants had amylose-free starch (Shimada et al. 2005).

Abbreviations: CaMV, cauliflower mosaic virus; dsRNA, double-stranded RNA; GBSSI, granule bound starch synthase I; RT-PCR, reverse transcriptase polymerase chain reaction; RNAi, RNA interference; SBEI, starch branching enzyme I; SBEII, starch branching enzyme II.

This article can be found at http://www.jspcmb.jp/

In this paper, we introduced the construct encoding dsRNA of sweetpotato *SBEII* (*IbSBEII*) into sweetpotato genome to inactivate the endogenous *IbSBEII*, resulting in increase in apparent amylose content in the starch.

Materials and methods

Plant material

Sweetpotato (*Ipomoea batatas* (L.) Lam.) cultivar Kokei 14, which is the leading variety in Japan, was used.

Plasmid construction

The nucleotide sequence of the *IbSBEII* was referred to the GenBank/EMBL/DDBJ nucleotide sequence database with the accession number AB042937. A part of the *IbSBEII* cDNA was prepared by PCR with primers IbSBEII.1123.L.*Bam*HI (5′-cgggatccacggccaaaaagacca-aagtcac-3′; *Bam*HI site underlined), IbSBEII.1594. R.*Xba*I (5′-gctctagaccaccatcttgcgtttgagagcag-3′; *Xba*I site underlined) and 1st strand cDNA of *Ipomoea batatas* cv. Kokei 14 as a template. The resultant fragment (about 500 bp) was subcloned into the *Bam*HI and *Xba*I unique site of pBluescriptIIKS$^-$. The product was named pIbSBEII-No.14. A 1.0 kbp DNA fragment of β-glucuronidase gene was prepared by PCR using primers GUS.783.L.*Eco*RI (5′-ggaattcgtgtgatatctacccgcttcg-3′; *Eco*RI site underlined) and GUS.1789.R.*Bam*HI (5′-cg*ggatcc*gtttttcaccgaagttcatgc-3′; *Bam*HI site underlined), and plasmid pBI121 (Clontech) as a template. The resultant fragment was subcloned into *Eco*RI and *Bam*HI site of pBluescriptIISK$^+$. The product was named pGUS1.0. A 0.3 Kbp of DNA fragment of the 1st intron of the *IbGBSSI* gene was prepared by PCR with primers IbGBSSI.Int1.L.*Eco*RI (5′-ggaattcc*ttggcagtaagtctcaca-3′; *Eco*RI site underlined) and IbGBSSI.Int1.R.*Bam*HI (5′- cgggatccgcgcgcctggaa-aatggat-3′; *Bam*HI site underlined), and plasmid pGBSSI-S No.54 as a template. The *Xba*I - *Bam*HI and *Eco*RI - *Sac*I fragments of the pIbSBEII-No.14, and the *Bam*HI - *Eco*RI fragment of pGUS1.0 or the 1st intron of the *IbGBSSI* were cloned into the *Bam*HI and *Sac*I unique site of the binary plasmid pCAM35SBar (Otani et al. 2003) so as to replace the *bar* gene. The plasmids, pTIbSBEII-ASGS and pTIbSBEII-ASIS, are harboring the partial *IbSBEII* genes in antisense and sense orientation inserted by a GUS fragment and *IbGBSSI* Ist Inton between them, respectively (Figure 1).

Transformation

The plasmids were introduced into *Agrobacterium tumefaciens* strain EHA101, and used to transform embryogenic calli induced from shoot meristems as described by Otani et al. (1998) with some modification: *Agrobacterium* was co-cultured at 23 C and on the medium containing 3% (w/v) maltose and 1% (w/v) glucose instead of 3% (w/v) sucrose.

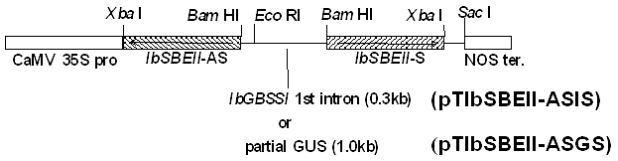

Figure 1. Constructs for RNA interference of *IbSBEII*. pTIbSBEII-ASIS and pTIbSBEII-ASGA contain partial GUS gene (1.0 kb) and *IbGBSSI* 1st intron as a spacer, respectively.

In vitro rooted transgenic plantlets were transferred to pots with vermiculite and grown in the growth chamber for 2 weeks. Then plantlets were transferred to the Wagner pots (1/5000a) containing Kureha Engei-baido Compost (Kureha Chemical Industry, JAPAN), in a biohazard green house.

Starch extraction and determination of amylose content

The tuberous roots were washed, cut into pieces and homogenized in a mixer with water. The homogenate was successively filtered twice through 250-μm metallic sieves and allowed to settle. The resuspension in water and settlement step was repeated four times. The starch was dried in air. The amylose content of the crude starch was determined from the blue value (absorbance at 680 nm) by spectrophotometry according to the method described by Noda et al. (1998) and calculated by the method of Takeda et al. (1983).

Southern hybridization

The CTAB method (Kim et al. 2002) was revised for extraction of genomic DNA from Kokei 14 and transgenic hygromycin resistant plants. We digested genomic DNA (about 10 μg) from Kokei 14 with restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII and hybridized with 400 bp *IbSBEII* fragment. Genomic DNAs from transgenic plants were digested with *Hin*dIII, and hybridized with a hygromycin-specific probe (Figure 4B). We used the DIG-direct-labeling reagent (Amersham Pharmacia, USA) and detection system with CDP star (Amersham Pharmacia, USA).

RNA extraction and Reverse-Transcriptase (RT)-PCR

Transcript levels were measured by RT-PCR. Total RNA was isolated from root tubers as described by Kim et al. (2002), and treated extensively with *RNase*-free *DNase* I to remove any contaminating genomic DNA. The first-strand cDNA was synthesized using *Pfu* Turbo *polymerase* (Stratagene, USA) from 2 μg of total RNA in a 20 μl reaction volume, and 2 μl of the reaction mixture was subjected to subsequent PCR in a 50 μl reaction volume. *IbSBEII* (5′ccacggccaaaaagaccaaagtcac 3′ and 5′accaccatcttgcgtttgagagcag 3′), and *Tublin* (5′

caactaccagccaccaactgt 3′ and 5′ caagatcctcacgagcttcac 3′) were amplified using the indicated primers according to the following cycling conditions: RT-PCR for 25 cycles, depending on the linear range of PCR amplification for each gene, with each cycle at 94°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1 min, with a final cycle at 72°C for 5 min to allow the completion of the polymerization.

Results

Sweetpotato SBEII and its expression

Southern blot analysis with a *IbSBEII* specific probe under stringent conditions against sweetpotato genomic DNA revealed more than two bands when digested with *Eco*RI, *Hin*dIII and *Bam*HI (Figure 2). The number of hybridizing bands indicates the presence of at least two copies of *IbSBEII* in the sweetpotato genome, suggesting that there are multi-copies of *IbSBEII* in the sweetpotato genome which is a hexaploid plant. It is unclear at present whether this result is due to the hexaploid and heterozygous nature of sweetpotato.

We examined the expression of the *IbSBEII* in RNA isolated from flowers, leaves, petioles, stems, white fibrous roots, thick pigmented roots and tuberous roots by RT-PCR. Figure 3 shows the result of RT-PCR with the *IbSBEII* specific primers. *IbSBEII* was expressed in both storage starch- and transitory starch-synthesizing tissues, i.e. flowers, leaves, petioles, stems and tuberous roots.

Transgenic sweetpotato with RNAi SBEII construct

Plasmids pTIbSBEII-ASGS and pTIbSBEII-ASIS (Figure 1), having constructs for double stranded RNA of partial *IbSBEII*, were driven by the CaMV35S promoter and had the hygromycin-resistant gene under CaMV35S promoter as a selectable marker gene (Figure 4B). We obtained four hygromycin resistant lines (ASGS-1, -2, -3, -4) which were transformed with the pTIbSBEII-ASGS plasmid, and four hygromycin resistant lines (ASIS-1, -2, -3, -5) which were transformed with the pTIbSBEII-ASIS plasmid. The transgenic plants and the control non-transgenic plants were grown in a biohazard green house.

Southern blot analysis with a hygromycin-specific probe revealed one to four bands when the genomic DNA from eight hygromycin-resistant plants were digested with *Hin*dIII, while non-transgenic Kokei 14 showed no hybridization band (Figure 4A). This indicates that the transfomants had a single or two copy insertion of T-DNA in different patterns, and they were independent transgenic lines.

The expression of *IbSBEII* in the root tubers of transgenic lines was examined by RT-RCR with the

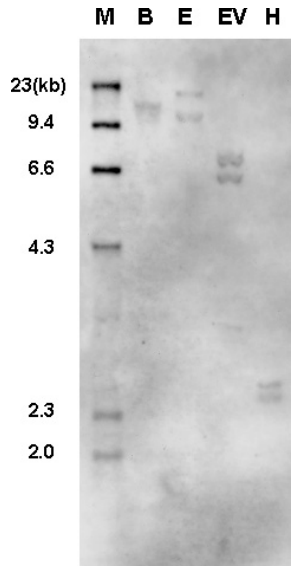

Figure 2. Southern-blot analysis of *IbSBEII* of sweetpotato Kokei 14. Ten micrograms of genomic DNA was digested with *Bam*HI (B), *Eco*RI (E), *Eco*RV (EV), and *Hin*dIII (H). Blots were probed with 400 bp of specific fragment of *SBEII* at high stringency condition. Lane M: molecular size marker (*λ*/*Hin*dIII).

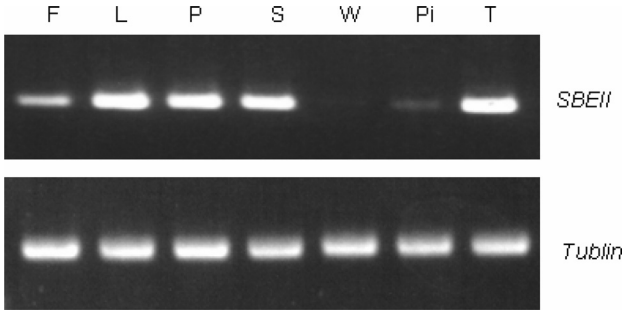

Figure 3. RT-PCR analysis of *IbSBEII* (A) in different organs of the sweet potato, F, flowers; L, leaves; P, petioles; S, stems; W, white fibrous roots; Pi, pigmented roots; T, tuberous roots. tublin (B) was used as a control.

IbSBEII specific primers. Figure 5 showed the results of the RT-PCR and indicated that the expression of *IbSBEII* decreased drastically in the all transgenic lines.

Characteristics of transgenic plants

After grown for four months in a biohazard greenhouse, the morphological characters of the transgenic lines were compared with those of non-transgenic control plants. The number and total length of veins and the number and total weight of storage roots showed no significant differences between them. Starch yield of transgenic tubers were slightly lower than that of non-transgenic tubers (Table 1).

Amylose content

Each starch solution was stained with iodine solution. Figure 6 shows the stained solution of starch from three transgenic lines compared with that from the non-

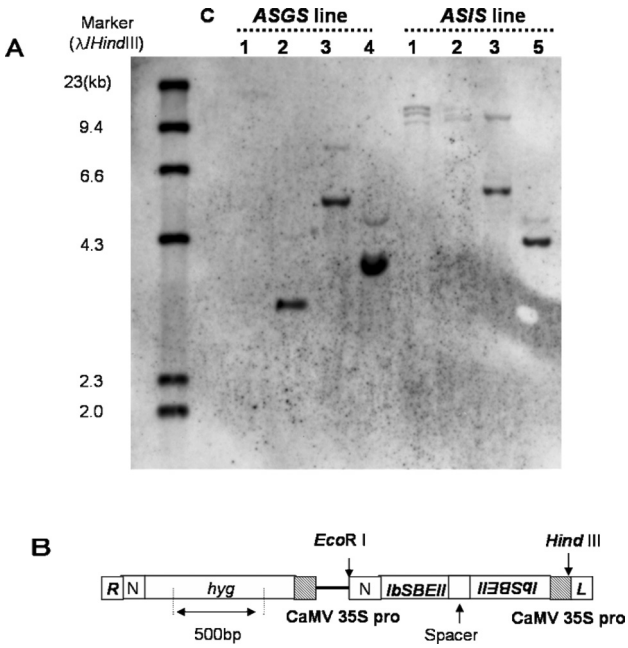

Figure 4. Southern hybridization analysis of DNA isolated from leaves of independent transgenic plants. A. Southern-blot analysis of *IbSBEII* RNAi lines. Ten micrograms of genomic DNA was digested with *Hind* III. Blots were probed with 500 bp of hygromycin gene at high stringency. Lane C: DNA from un-transformed plant DNA. B. Constructs used for plant transformation and the region of the probe (500 bp). R, Right T-DNA border; L, left T-DNA border; 35S, *hyg*: Hygromycin; N, NOS terminator.

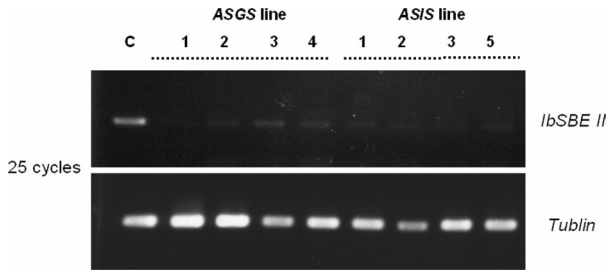

Figure 5. RT-PCR analysis (25 cycles) of *IbSBEII* (A) in transgenic lines of the sweetpotato. α-tublin (B) was used as a control.

transgenic line and amylose-free starch. Starch solutions from the transgenic lines were stained to a dark blue color than that from non-transgenic line, suggesting that the amylose contents in transgenic starch were high. When the amylose content was determined by the blue value absorbance at 680 nm, starches from the transgenic tubers contained 15.4% to 24.3% of amylose, while non-transgenic starch contained only 10% (Table 1). Starches of transgenic lines contained amylose two-fold as much as the control.

Starch granule

Preliminary observation of starch granules by light microscopy indicated that all starches had spherical shapes as typical sweetpotato starch (Figure 7). However, some starch granules from transgenic plants had unclear fissures on the surface of granules different from those from non-transgenic plants (Figure 7B). The particular observation is required to clarify the constitution of the transgenic starch granules. On the other hand, the distribution pattern of granule size was not different between the starch grains from transgenic and non-transgenic lines (data not shown).

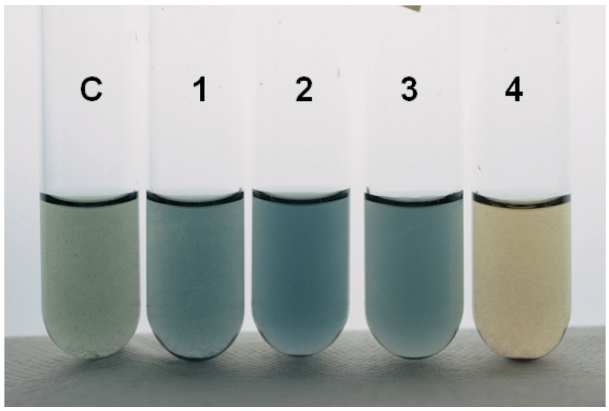

Figure 6. Iodine staining pattern of starch solution from non-transgenic Kokei 14 (c), transgenic lines with dsRNA of *IbSBEII* (1, ASIS-1; 2, ASGS-1; 3, GS-2) and a transgenic line with dsRNA of *GBSSI* (4).

Table 1. The yield and amylose content of starches from transgenic sweetpotato roots.

Sample[1)]	Total fresh weight (g)	Starch yield (g)[2)]	Amylose content (%)[3)]	Control/transgenic
Control	281.7	5.8	10.25±0.4	100
ASGS-1	259.6	5.2	17.90±0.7	175
ASGS-2	181.8	4.7	23.33±0.3	228
ASGS-3	241.3	4.1	15.67±0.5	153
ASGS-4	144.5	5.9	24.23±0.3	236
ASIS-1	277.0	4.5	20.00±0.1	195
ASIS-2	244.4	4.9	23.35±0.8	228
ASIS-3	173.5	4.4	24.85±0.6	242
ASIS-5	205.2	4.2	23.85±0.1	233

1) Control, non-transgenic plants; ASGS-1～-4, transgenic plants with pIbSBEII-SAGA; ASIS-1～-5, transgenic plants with pIbSBEII-ASIA.
2) Dry weight of starch in 30 g fresh weight tuber
3) Average±S.D. (n=3～5)

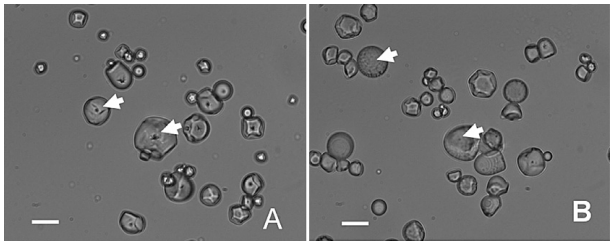

Figure 7. Light microscopy of starch granules. Wild-type granules (A) and granules from line ASIS-1 (B). Bar=20 μm.

Discussion

The starch branching enzymes, SBEI and SBEII, are characterized in potato as well as in maize and pea (Rydberg et al. 2001). However, the branching enzymes in sweetpotato have not been characterized yet, although only the cDNA of *SBEII* was registered in DDBJ/EMBL/GeneBank. We confirmed the *SBEII* genes in sweetpotato (*IbSBEII*) by Southern analysis and the expression of *IbSBEII* in both storage starch- and transitory starch-synthesizing tissues by RT-PCR. Based on the registered cDNA of sweetpotato *SBEII*, we constructed dsRNA interference vectors of *IbSBEII* and introduced them into sweetpotato genome to produce the transgenic plants having starch with a moderately high amylose content. This is the first report of sweetpotato with a higher amylose than Kokei 14.

Maize with amylose extension *ae* mutant produces starch with a higher amylose content, and the starches with approximately 50 or 70% amylose are commercially available. It is reported that high-amylose and low-amylopectin maize is produced by the reduction of total starch branching enzyme activity and the almost complete absence of SBEII isoform (Sidebottom et al. 1998). However, antisense inhibition of *SBEII* in transgenic potato resulted in only about a 1.3-fold amylose content compared with the control (Jobling et al. 1999). In this experiment of sweetpotato, the amylose content of transgenic sweetpotato with *SBEII* inhibited by RNAi was not as high as that in maize *ae* mutant. On the other hand, in transgenic potato plants with two isoforms, SBEI and SBEII, simultaneously inhibited, the amylose content was as high as the highest commercially available maize starches (Schwall et al. 2000). Therefore, we speculated that starch of sweetpotato with a high amylose content would be produced when the expression of not only *IbSBEII* but also sweatpotato *SBEI* (*IbSBEI*) could be inhibited. We cloned the sweetpotato *IbSBEI* gene and transformed the dsRNA gene silencing constructs of both *IbSBEI* and *IbSBEII* into sweetpotato genome.

Starch branching enzymes have a strong influence on the molecular structure of starch grains, especially on amylopectin chain length (Jobling et al. 1999). We are investigating the physicochemical characteristics of the starch from transgenic plants with RNAi of *IbSBEII*.

RNA interference is very effective technology in the crop improvement, for example, Ogita et al. (2004) succeeded in production of transgenic coffee plants with a low caffeine content by suppressing the expression of the enzymes for the caffeine biosynthesis using the double strand RNA-mediated interference. Recently, we successfully obtained transgenic amylose-free sweetpotato by RNA interference of *GBSSI* (Shimada et al. 2005) with about 75% efficiency, though we produced only one amylose-free sweetpotato plant in 26 transgenic plants by co-suppression method (Kimura et al. 2001). In this experiment, none of the eight independent transgenic hygromycin-resistant plants we obtained produced *IbSBEII* mRNA and had a higher amylose content than non-transgenic control. We used two kinds of RNAi constructs, encoding *GBSSI* 1st intron-spliced RNA and a GUS fragment-spliced RNA. Both constructs induced the same level of gene silencing of *IbSBEII*, although it was reported in *Arabidopsis* that the construct having intron as a spacer was more effective for the induction of gene silencing than the construct having GUS as a spacer (Smith et al. 2000).

High-amylose corn starch has several differentiated properties that can be applied in many foods (Richardson et al. 2000). In Japan, most sweetpotato starch is used for alcohol fermentation and the production of glucose and high-fructose syrup and has relatively narrow ranges of amylose content (10–25%) (Noda et al. 1998). Here, we demonstrated the possibility of producing high-amylose sweetpotato starch by the genetic modification of starch branching enzymes sweetpotato.

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