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Production of amylose-free sweetpotato plants by RNAi

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Abstract. In sweetpotato roots (*Ipomoea batatas* (L.) Lam., cv. Kokei 14) 18 to 20 % of the starch is amylose and the other major component is amylopectin. Granule-bound starch synthase I (GBSSI) is one of the key enzymes which catalyzes the formation of amylose in starch granules, a linear á-(1,4)D-glucan polymer, from ADP-glucose. In this study, sweetpotato plants were transformed to produce amylose-free starch by RNA interference (RNAi). The gene construct that encoded double stranded RNA (dsRNA) of *GBSSI* first exon was introduced into the sweetpotato genome by *Agrobacterium tumefaciens*-mediated transformation to inactivate the endogenous *GBSSI* gene. Starches from 80% of transgenic lines showed red-brown staining pattern by iodine staining, indicating a reduction of amylose content. Starches from these transgenic plants, which showed red-brown staining pattern were confirmed to be amylose-free by calculations from the blue value at 680 nm.

Introduction

Sweetpotato is one of the most important crops in the world and provides not only staple food but is also important as an industrial raw material. Genetic engineering of starch has great potential for the quality improvement of sweetpotato starch for the development of new food and industrial products. Amylose and amylopectin are the two main polysaccharide components of most natural starches. In sweetpotato plants, a relatively narrow range in amylase content (10 – 20 %) has been observed in comparison with other crops (Noda *et al.*, 1998). Since an amylose-amylopectin ratio is an important factor influencing the textual properties of starch, a new sweetpotato variety that contains starch, which is amylose free or has low amylose content, would help develop new industrial applications.

The granule-bound starch synthase I (GBSSI) is one of the key enzymes in the biosynthesis of starch, which catalyzes the formation of amylose in starch granules, a linear á-(1,4)D-glucan polymer, from ADP-glucose. Amylose-free or low amylose starch has been produced by the antisense *GBSSI* RNA technique in potato (Visser *et al.*, 1991, Kuipers *et al.*, 1994) and rice (Terada *et al.*, 2000). Recently, Kimura *et al*. (2001) introduced the sense *GBSSI* cDNA into the sweetpotato genome and obtained one transgenic plant with amylose-free starch form a total 26 transgenic plants. They revealed that co-suppression of endogenous *GBSSI* resulted in a reduction in the amount of amylose by sense suppression of the *GBSSI* gene in sweetpotato.

Recently, double stranded RNA-mediated gene silencing has been found to be an effective technology in the genetic improvement of crops as well as in functional genomic studies (Levin *et al.*, 2000). In this study, we introduced the construct that contains both sense and antisense of *GBSSI* fragments into sweetpotato plants to inactivate the endogenous *GBSSI* gene. Amylose-free transgenic sweetpotato were obtained efficiently by the double stranded RNA mediated technique in a higher frequency than by the co-suppression

technique in sweetpotato (Kimura *et al.,* 2001) or the antisense technique in potato (Visser *et al.,* 1991).

Materials and Methods

Plasmid construct. The gene-silencing constructs were cloned into the binary vector pCAMBIA1300 (Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, England) as HindIII-EcoRI fragments, replacing the â-gluclonidase-containing HindIII-EcoRI region of pCAMBIA. The pGBSSI-SIAS contains the sense orientation of GBSSI first exon, first intron and the antisense orientation of the first exon (Fig. 1).

Transformation. Embryogenic calli were induced from shoot meristems of sweetpotato, cultivar Kokei 14, on 4F1 medium, which is LS medium (Linsmaier and Skoog, 1965) containing 1 mg/l 4-fluorophenoxyacetic acid (4FA), 3% (w/v) sucrose and 0.32% (w/v) gellan gum (Otani and Shimada 1996). Transformation was performed according to the method of Otani *et al*. (1998).

Iodine staining of roots and measurement of amylose content. The sweetpotato roots, which grew for four months in the biohazard greenhouse, were washed, cut into pieces and stained at the surface of root slices with iodine solution (0.1 % I_2, 1 % KI). The starches were extracted from the roots and the amylose content calculated from the blue value at 680 nm according to the method by Noda *et al*. (1998). Via *Agrobacterium tumefaciens*-mediated transformation the double stranded constructs pGBSSI-SIAS were integrated into the genome of the sweetpotato cultivar Kokei 14. Eighty-nine individual hygromycin-resistant plants were regenerated and grown in Wagner pots (1/5000 a) in the biohazard green house. After four months growth, the number of veins over 10 cm, total length of the veins, the number of root roots over 10 g and total length of the root roots were determined.

Results and Discussion

No morphological differences were observed between non-transgenic plant and the hygromycin resistant plants. Table 1 shows the results of a non-transgenic plant and 15 transgenic lines. Number and total length of veins and number and total of roots showed no significant differences between a non-transgenic plant and the transgenic plants, although individual differences were large.

The hygromycin resistant lines were investigated for starch character by iodine staining. Twelve transgenic lines of the 15 lines contained red-brown staining starch while the other three lines contained blue staining starch similar to the non-transgenic plant (Table 1). This shows that 80% of the transgenic plants have starches with no or low amylose and had been modified. Starches were isolated from roots of the 15 transgenic lines and the non-transgenic plant to examine their amylose contents.0The twelve transgenic lines which were stained red-brown by iodine were under 0.166 of blue value, which is the blue value of amylopectin,

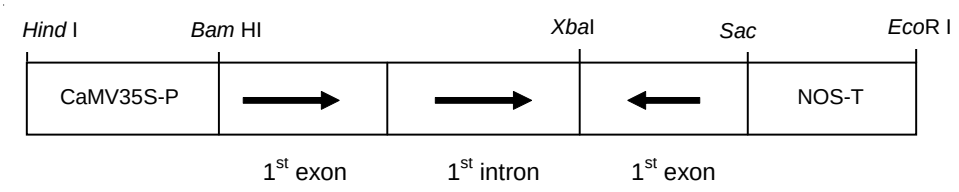

Figure 1: Composition of the double strand GBSSI construct containing the first intron between the first exon *GBSSI* in sense orientation and the first exon in antisense orientation.

Table 1: Characteristics of a non-transgenic Kokei 14 and the 15 lines of hygromycin resistant plants.

Strain	No. of vines per 10 cm	Total length of vines (cm)	No. of roots per 10 g	Total weight of roots (g)	Iodine staining pattern
Kokei 14	7	385.6	5	305.8	B[3]
GR-01	5	406.4	7	237.4	R
-02	5	440.8	3	192.6	B
-03	4	347.9	2	179.7	R
-04	4	259.7	6	338.0	R
-05	6	336.9	9	299.3	R
-06	5	295.7	4	253.7	R
-07	4	244.8	4	252.1	B
-08	8	156.6	7	303.8	R
-09	7	378.0	6	324.9	B
-10	6	321.9	7	212.5	R
-11	4	410.1	6	222.4	R
-12	5	264.0	5	221.3	R
-13	5	209.0	2	328.9	R
-14	4	242.5	4	212.5	R
-15	5	323.2	4	248.0	R
Average T lines with amylose	5.3±1.5[1]	354.5±100.1	4.3±1.5	254.8±50.9	B
T lines without amylose	5.1±1.2ns[2]	297.8±75.8ns	5.3±2.1ns	256.5±66.3ns	R

[1]Mean ± sd; [2]T test; [3]B: blue-black staining pattern, R: red-brown staining pattern.

indicating amylose free starch. The other three lines and non-transgenic Kokei-14 showed blue values of 0.350 to 0.362, and they, therefore, contained around 17 % amylose. The yield of the amylose-free roots showed no differences compared to the yield of roots having normal amylose content (Table 1).

The transgene was confirmed in all lines of the transgenic plants by Southern hybridization (data not shown), and the amylose-free lines did not express the *GBSSI* mRNA revealed by Northern analysis nor did they produce the GBSSI protein revealed by SDS-PAGE (data not shown). These results suggest that double stranded RNA inhibited the expression of *GBSSI* gene completely in 73.7 % of the transgenic plant lines. Kimura *et al*. (2000) obtained amylose-free sweetpotato by sense silencing at the frequency of 0.4 % (1/26). RNA interference is an effective technology which may be used in the genetic improvement of crops.

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