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Absence of amylose in sweet potato [*Ipomoea batatas* (L.) Lam.] following the introduction of granule-bound starch synthase I cDNA

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Abstract The full-length sense cDNA for sweet potato granule-bound starch synthase I (GBSSI) driven by the CaMV 35S promoter was introduced into the sweet potato by *Agrobacterium tumefaciens*-mediated transformation. Out of the 26 transgenic plants obtained, one plant showed the absence of amylose in the tuberous root as determined by the iodine colorimetric method. Electrophoresis analysis failed to detect the GBSSI protein, suggesting that gene silencing of the GBSSI gene occurred in the transgenic sweet potato plant. These results demonstrate that starch composition in the tuberous root of sweet potato can be altered by genetic transformation.

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Abbreviations *CaMV*: Cauliflower mosaic virus · *SDS-PAGE*: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

In the biosynthetic pathway of starch, starch synthase uses ADP-glucose to form a linear chain of α(1,4)-linked glucose units. Several isoforms of starch synthase have been shown to exist in diverse plant species. In potato, four isoforms of starch synthase have been reported – granule-bound starch synthase I (GBSSI) and three soluble starch synthases (SSs) (Vos-Scheperkeuter et al. 1986; Edwards et al. 1995; Abel et al. 1996; Marshall et al. 1996; Kossman et al. 1999). GBSSI is bound to the starch granule, while the SSs are predominantly located in the stroma of the plastid. Analyses of mutants of various plants have shown that GBSSI is the sole starch synthase that produces the amylose of storage starch. For example, the *amf* mutant of potato lacks GBSSI activity and contains amylose-free starch (Hovenkamp-Hermelink et al. 1987).

Sweet potato [*Ipomoea batatas* (L.) Lam.] is an important crop for food and a major material for industries. The development of new cultivars that have a novel physico-chemical property of starch is one objective of sweet potato breeding. It is thought that amylose-free starch or starch with a very low amylose content will have a major role in new industrial developments. However, the genotypes that have such a starch have not been found yet in sweet potato. In potato and rice, inhibition of GBSSI expression by antisense technology resulted in a large reduction in amylose content (Kuipers et al. 1994; Terada et al. 2000). In addition to antisense suppression, sense suppression of the GBSSI gene has been reported in potato (Flipse et al. 1996b). In this case also,

the storage starch had a very low amylose content. These studies demonstrate that, irrespective of plant species, it is possible to inhibit the expression of GBSSI and modify the amylose content in starch using genetic transformation.

The full-length cDNA and the gene for sweet potato GBSSI have been isolated and characterized (Wang et al. 1999; Kimura et al. 2000). We report here the production of an amylose-free phenotype transgenic sweet potato that was obtained by introducing the full-length GBSSI cDNA in the sense orientation.

Materials and methods

Construction of vector

A *Sph*I-*Sma*I fragment containing the 35S promoter derived from pBI221 and a *Eco*RI (filled-in)-*Kpn*I fragment containing the full-length GBSSI cDNA (Kimura et al. 2000) were ligated into the *Sph*I-*Kpn*I site of pHSG299 (Takara Shuzo, Tokyo). The 35S promoter-GBSSI construct was cut off with *Hin*dIII and *Kpn*I. A *Sac*I-*Eco*RI fragment containing the nopaline synthase (Nos) gene terminator derived from pBI221 was inserted into pHSG299 and cut off with *Kpn*I and *Eco*RI. The 35S promoter-GBSSI construct and the Nos terminator were then ligated into the *Hin*dIII-*Eco*RI site of pBluescript SK-. The GBSSI expression cassette was cut off with *Hin*dIII and *Eco*RI and inserted into the same site of pBI121 to yield pBIGBSSI.

A *Sph*I-*Xba*I fragment containing the 35S promoter derived from pBI221 and a *Xba*I-*Sac*I fragment containing the hygromycin phosphotransferase gene (HPT) were ligated into the *Sph*I-*Sac*I site of pUC18. The 35S promoter-HPT construct was cut off with *Sac*I and *Hin*dIII. A *Sac*I-*Eco*RI fragment containing the Nos terminator derived from pBI221 was inserted into pBluescript SK- and cut off with *Sac*I and *Hin*dIII. The 35S promoter-HPT construct and the Nos terminator were then ligated into the dephosphorylated *Hin*dIII site of pHSG398 (Takara Shuzo). The HPT expression cassette was cut off with *Hin*dIII and blunt-ended and then inserted into the filled-in and dephosphorylated *Eco*RI site of pBIGBSSI. The sense expression vector, pBIGBSSI-Hm (Fig. 1), was introduced into the *Agrobacterium tumefaciens* EHA 101 competent cell by a chemically based direct transformation (Walkerpeach and Velten 1994).

Plant transformation

The embryogenic callus from the apical meristem of sweet potato [*Ipomoea batatas* (L.) Lam.] cv. Kokei 14 was infected with EHA101/pBIGBSSI-Hm. The transformants were selected on a solidified LS medium (Linsmaier and Skoog l965) containing hygromycin (25 mg/l), and plantlets were regenerated as described by Otani et al. (1998).

Southern blot analysis

Genomic DNAs were extracted from the leaves of the regenerated plants using the modified CTAB method. Five-microgram aliquots of genomic DNA were digested with either *Xba*I or *Hin*dIII. After electrophoresis on a 0.8% agarose gel, the DNA was transferred onto a positively charged nylon membrane (Hybond-N^+, Amersham, UK). The full-length sweet potato GBSSI cDNA or 35S promoter, labeled by the random primer method with fluorescein-11-dUTP using Gene Images random prime labeling module (Amersham), was used as a probe. Filters were hybridized and washed according to the manufacturer's protocols.

Phenotypic assay of tuberous roots of transgenic sweet potatoes

The sweet potato tuberous roots were sliced, and the surface of the tuber section was stained with an iodine solution [0.18% (w/v) iodine, 1% (w/v) potassium iodine].

Determination of the amylose content of starch

The sweet potato tuberous roots were washed, cut into pieces and homogenized in a mixer with water. The homogenate was successively filtered twice through 250-µm and 63-µm metallic sieves and allowed to settle. After the supernatant had been discarded, the starch granules were resuspended in water and allowed to settle. The resuspension and settlement step was then repeated. The starch granules were dried with ethanol and acetone after washing, and the amylose content was calculated from the blue value at 680 nm according to the method described by Noda et al.(1998). The measurement was carried out three times.

Protein electrophoresis

Starch granule-bound proteins were extracted by boiling 60 mg of purified starch granules for 2 min in 460 µl of sample buffer [55 m*M* Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol]. After boiling, the solution was cooled on ice and then centrifuged at 15,000 *g* for 10 min. All of the supernatant (about 50 µl) was electrophoresed on a 10% SDS polyacrylamide gel and visualized by staining with Coomassie brilliant blue R-250.

Results and discussion

Transformation

To introduce the GBSSI cDNA into the sweet potato we designed a sense construct that was under the control of the CaMV 35S promoter (Fig. 1). Following selection by hygromycin, we obtained 26 sweet potato plants. On the basis of Southern blot analysis, these were all transgenic plants (data not shown except for six samples in Fig. 4). These plants all grew normally and formed tuberous roots.

Iodine staining of tuberous roots and analysis of amylose content

To select a co-suppressor that exhibited a reduction in the amylose content, we carried out iodine staining of

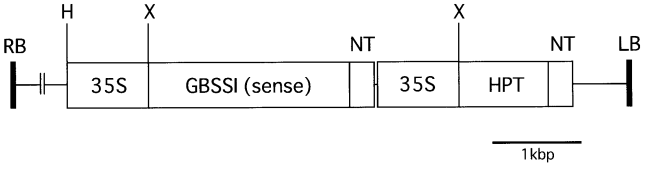

Fig. 1 Schematic diagram of the T-DNA region of transformation vector pBIGBSSI-Hm. *RB* Right border, *LB* left border, *35S* 35S promoter of cauliflower mosaic virus, *GBSSI* granule-bound starch synthase I cDNA, *NT* nopaline synthase gene terminator, *HPT* hygromycin phosphotransferase gene, *H Hin*dIII restriction enzyme recognition site, *X Xba*I restriction enzyme recognition site

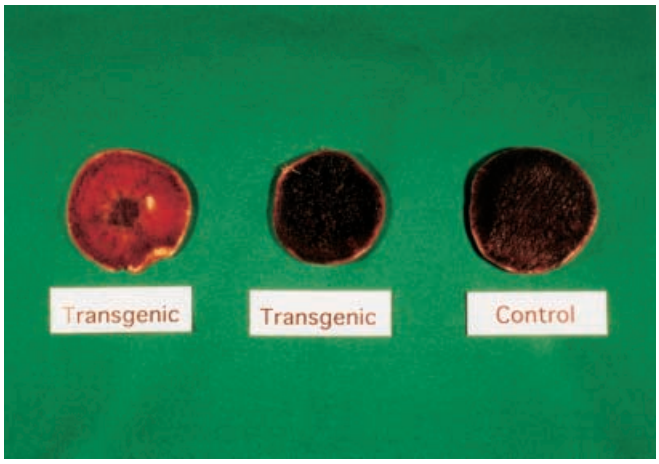

Fig. 2 Iodine staining of transgenic sweet potato tuberous roots. *Left* Transformant no. 1, *center* transformant no. 23, *right* untransformed control plant

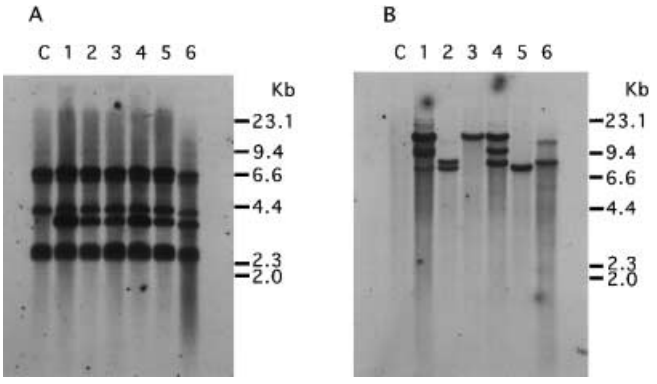

Fig. 4A,B Southern blot analysis of transgenic plants. **A** DNA was digested with *Xba*I and hybridized to the GBSSI probe. **B** DNA was digested with *Hin*dIII and hybridized to the 35S promoter probe. *Lanes*: *1* Transformant no. 1, *2* transformant no. 2, *3* transformant no. 7, *4* transformant no. 13, *5* transformant no. 15, *6* transformant no. 23, *C* untransformed control plant

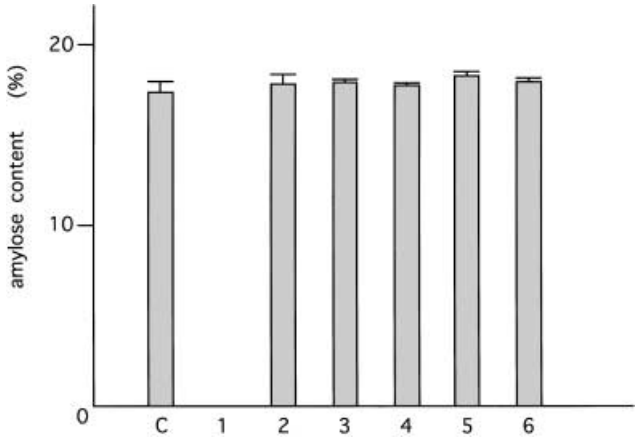

Fig. 3 Amylose content of starch from transgenic plants. *Error bars* indicate standard deviations. *1* Transformant no. 1, *2* transformant no. 2, *3* transformant no. 7, *4* transformant no. 13, *5* transformant no. 15, *6* transformant no. 23, *C* untransformed control plant

the transgenic sweet potato tuberous roots after cutting. Of the 26 plants, one transgenic plant, clone no. 1, had a red-brown staining pattern (Fig. 2, left), while the others and the nontransgenic plant had dark-purple staining patterns. This exceptional staining pattern indicates a reduction in the amylose content in the transgenic plant. The amylose content of starch was determined by the colorization method. Amylose was not detected in the starch from the tuberous root of transformant no. 1 (Fig. 3, lane 1), while the nontransgenic plant and the remaining transformants had 17.4–18.3% amylose in their starch.

Southern blot analysis

The presence of the introduced transgene was monitored by Southern blot analysis. Genomic DNA prepared from each plant grown in the greenhouse was digested by *Xba*I or *Hin*dIII. The full-length sweet potato GBSSI cDNA was used as a probe for the analysis of the *Xba*I-digested DNA, and the CaMV 35S promoter fragment was used for the analysis of the *Hin*dIII-digested DNA. When the *Xba*I-digested DNAs were analyzed, the expected 3.5-kb band corresponding to the fragment composed of the GBSSI cDNA, Nos terminator and CaMV 35S promoter (Fig. 1) was detected in the transformants but not in the control plant (Fig. 4A). The amylose-free transformant no. 1 showed a relatively intense signal of the 3.5 kb band, indicating that more copies had been integrated (Fig. 4A, lane 1). The *Hin*dIII-digested DNA analysis was performed to investigate the integration pattern. As the binary vector pBIGBSSI-Hm has only one restriction site for *Hin*dIII (Fig. 1), the presence of DNA bands of different lengths indicates integration at different sites. The amylose-free transformant no. 1 was found to have multiple integration sites in the nuclear genome (Fig. 4B, lane 1), corroborating the result of the *Xba*I-digested DNA analysis.

Analysis of the GBSSI protein in the tuberous roots of transgenic plants

Starch granules from the sweet potato tuberous root contain one major 60-kDa protein, which on the basis of sequencing analysis (data not shown) has been identified as the GBSSI protein. To monitor the amount of GBSSI protein, we carried out protein electrophoresis. The same starch sample analyzed in Fig. 3 was used in each plant to obtain the granule-bound proteins. The GBSSI protein was barely detected in transformant no. 1 but was detected in the other five transgenic plants (Fig. 5).

These results suggest that homology-dependent gene silencing of the GBSSI gene only occurred in the tuberous roots of transformant no. 1. In the case of potato, 2 transformants out of 45 showed complete inhibition

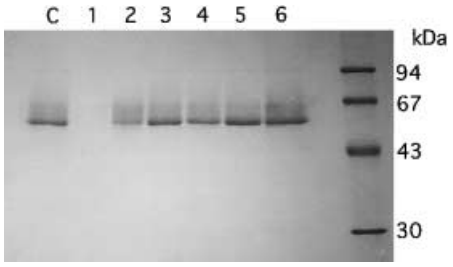

Fig. 5 SDS-PAGE patterns of proteins extracted from starch granules. *Lanes*: *1* Transformant no. 1, *2* transformant no. 2, *3* transformant no. 7, *4* transformant no. 13, *5* transformant no. 15, *6* transformant no. 23, *C* untransformed control plant

when the GBSSI cDNA driven by the 35S promoter was introduced (Flipse et al. 1996b). The frequency that we obtained in our sweet potato transformants (1 out of 26) was similar to that of potato. Because in our investigation there was no sequence homology in the promoter – which is associated with transcriptional gene silencing – between the endogenous GBSSI gene(s) and foreign GBSSI cDNA(s) driven by the 35S promoter, this gene silencing seems to be post-transcriptional. The relatively high copy numbers of the transgenes introduced into this plant (Fig. 4) may be related to gene silencing; for example, excessive production of the GBSSI mRNA to more than the threshold level (Smith et al. 1994) might occur. Further study is needed to confirm that the expression of both the endogenous GBSSI genes and the GBSSI transgenes is actually suppressed at the post-transcriptional level.

Among the five transgenic plants that showed a normal phenotype, differences in the amount of the GBSSI protein were observed (Fig. 5); however, no significant difference in amylose content was observed (Fig. 3), and the level was almost similar to that of the untransformed control. Transgenic potato also showed no significant increase in amylose content following introduction of the GBSSI gene when compared to that of wild-type potato (Flipse et al. 1996b). In studying the GBSSI gene dosage effect in potato, Flipse et al. (1996a) observed that the amylose content reached a maximum and did not increase further when GBSSI activity exceeded a certain level [One explanation for this – a limitation of physical space available within the amylopectin matrix (Flipse et al. 1996b; Smith et al. 1997)]. Although GBSSI activity has yet to be studied, the stability of the amylose content described above is likely due to this limitation.

This is the first report of sweet potato that lacks amylose in the tuberous root. Our investigation has demonstrated that it is possible to alter the starch composition of sweet potato by genetic transformation just like in other plants such as potato. Analysis of the physicochemical properties of the amylose-free starch is now in progress.

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