academicJournals

Vol. 12(41), pp. 5994-5999, 9 October, 2013
DOI: 10.5897/AJB11.3321
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Expression of an engineered tandem-repeat starch-binding domain in sweet potato plants

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Accepted 17 August, 2012

In this study, the transgenic sweet potato Xu55-2 modified with an engineered tandem repeat of a family 20 starch binding domain (SBD2) was analyzed by Western dot blot to investigate whether SBD2 proteins are capable of granule-targeting during starch biosynthesis. Furthermore, the impact of SBD2 accumulation in granules on the physicochemical properties of the transgenic starches was also investigated. Our results demonstrate that the high levels of SBD2 protein could be accumulated in granules. The SBD2 expression affect granule morphology without altering the primary structure of the constituent starch molecules, suggesting that SBD2 could be used as an anchor for effector proteins to sweet potato starch granules during biosynthesis.

Key words: Sweet potato (*Ipomoea batatas*), tandem starch-binding domain, transgenic starch, granule morphology.

INTRODUCTION

Sweet potato (*Ipomoea batatas*) is one of the important starchy resources in Asia. It accounts for over 80% of world sweet potato starch production, most of which are in China. Every year, over 10% of the production of 100 million tons of sweet potato in China is processed into starch. Sweet potato starch is used in the manufacture of sweeteners, sizing of paper and textile, production of bio-ethanol (Chen et al., 2003; Tian et al., 2009; Yang et al., 2011) and it is particularly valued as a food starch used in starch noodles, bakery foods and snack foods production (Chen et al., 2003; Kitahara et al., 2007). The use of sweet potato starch is primarily determined by its physic-chemical properties. However, there are almost no natural starches with essential properties for a particular application. Thus, different modifications must be made before applying natural starch.

Improvement of natural starch properties for industrial purposes can be achieved by chemical or physical modification after isolation, but also through the *in planta* modification. Over the years, a number of starches with new or improved properties such as amylose-free starches (Kuipers et al., 1994; Noda et al., 2002; Kitahara et al., 2007), high-amylose starches (Schwall et al., 2000; Kitahara et al., 2007) and a heavily-branched amylo-

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Abbreviations: SBD2, Tandem starch-binding domain; **CGTase,** cyclodextrin glycosyl-transferase; **GBSS I,** granule bound starch synthase I; **PVDF,** polyvinylidene-fluoride; **AEC,** 3-amino-9-ethylcarbazole; **AM,** apparent amylose; **LM,** light microscopy; **SEM,** scanning electron microscopy; **DSC,** differential scanning calorimetry; **XRD,** x-ray diffractometer.

pectin starch (Kortstee et al., 1996) have been obtained by either inhibition of native genes or expression of foreign genes. The examples mentioned above show that genetic engineering may provide a promising approach to produce novel starches with the desired properties *in planta*. In previous study, we developed a starch-binding domain (SBD)-technology for modification starches with new or tailor-made properties, in which microbial SBD-encoding region of cyclodextrin glycosyltransferase (CGTase) from *Bacillus circulans* is used as an anchor to target recombinant proteins into granules during the starch biosynthesis (Ji et al., 2003). Moreover, an engineered tandem repeat of SBD (SBD2) was expressed in potato, and the SBD2 shows much higher affinity for starch granules than single SBD, indicating that the SBD2 can be used as an alternative for making SBD fusion proteins (Ji et al., 2004). Sequentially, the SBD2 construct has been introduced into a sweet potato cultivar Xu55-2 to investigate whether the SBD2 can also be used as an anchor for its starch modification. The presence of the *SBD2* gene in the genomic DNA of transgenic sweet potatoes has been verified by polymerase chain reaction (PCR) amplification and confirmed by Southern blot analysis (Xing et al., 2008).

In this study, the transgenic sweet potatoes modified with the *SBD2* gene was investigated to determine whether SBD2 protein can accumulate in granules with a high level during starch biosynthesis, and whether accumulation of the SBD2 into granules has effects on the physicochemical properties of the transgenic starches.

MATERIALS AND METHODS

Preparation of transgenic plants

Agrobacterium tumefaciens strain EHA105 carrying the plasmid pBIN19/SBD2 was transformed into sweet potato cultivar Xu55-2 (Xing et al., 2008). The *SBD2* gene, in which two copies of SBD is linked through an artificial proline-threonine (PT)-rich linker, is expressed in sweet potato plants under the control of the tuber-specific potato granule bound starch synthase I (GBSS I) promoter. Amyloplast entry of SBD2 is mediated by the potato GBSS I transit peptide (Ji et al., 2004). Transgenic sweet potatoes Xu55-2 modified with the *SBD2* gene have been obtained in a previous study. The presence of the *SBD2* gene in the genomic DNA of 12 transgenic clones has been verified by PCR amplification and confirmed by Southern blot analysis (Xing et al., 2008). The resulting transgenic plants are referred to as 55-2-SSxx (where 55-2 represents sweet potato cultivar Xu55-2, SS represents the *SBD2* gene and xx represents the clone in a series of transformant). Untransformed control plants are referred to as 55-2-UT. In this study, the transgenic plants were clonally propagated and five plants of each transgenic clone were transferred to the greenhouse for tuberous-roots development. In addition, 10 untransformed controls were grown in the greenhouse.

Isolation of tuberous-root starch

All tuberous-roots from the five plants of each clone were combined, and the peeled roots were cut into small pieces and homogenized in a blender. The homogenate was filtered through a cheese-cloth to remove particulate material and allowed to settle for 20 min at 4°C. Subsequently, the root juice was collected and stored at -20°C for later use. The starch sediment was washed three times with distilled water, and air-dried at room temperature.

Western dot blot analysis

The amount of SBD2 proteins accumulated in transgenic starches was estimated with a Western dot blot procedure as described by Ji et al. (2003). 20 mg of (transgenic) starch was boiled for 5 min with 200 µL of a 2× sodium dodecyl sulphate (SDS) sample buffer (Laemmli, 1970). After cooling to room temperature, the SBD2 proteins in the transgenic starch gels were electro-blotted onto the polyvinylidene-fluoride (PVDF) membrane (Roche, Germany) using a Fastblot B33 (Whatman Biometra, Germany; 150 mA, 4°C, 5 h). A 1:500 dilution of antiSBD antibody (Ji et al., 2003) was used as the primary antibody, and a 1:1000 dilution of Goat Anti-Rabbit IgG horseradish peroxidase (HRP) conjugate (Boster, China) was used as the secondary antibody for detection. The blot was stained with a 3-amino-9-ethylcarbazole (AEC) solution.

To investigate whether SBD2 protein was present in the solution fraction of tuberous-root, A 500 µL of juice sample was dried in a freeze-drier (LNG-T98, China). The resulting material was dissolved in 200 µL of a 2× SDS sample buffer. In order to make the sample suitable for the Western dot blot procedure, the mixture was boiled for 5 min in the presence of 20 mg starch from the untransformed control (Ji et al., 2004). The rest of the procedure was conducted in the same way as described above for root starch analysis.

Analysis of physicochemical properties of starch granules

Starch granule morphology was observed by light microscopy (LM; BX41-32H02, Japan) and scanning electron microscopy (SEM; HITACHI S-3000N, Japan). For light microscopy, starch granules were stained with a 20× diluted 1% I_2/KI solution. For scanning electron microscopy, starch samples were dried and coated with gold on the ion sputter (E-1010, Hitachi, Japan). Average granule size distribution of the transgenic starches was determined in triplicate with a particle size analyzer (CIS-50, Ankersmid, The Netherlands) according to the manufacturer's instructions. A 10 mg of starch sample was suspended in 100 µL of 20% iso-propanol. The apparent amylose content was determined in triplicate following the method as described by Hovenkamp-Hermelink et al. (1989).

Gelatinization properties of starch were determined by using a differential scanning calorimetry (DSC; DSC 27, PerkinElmer, USA). A 2.5 mg starch sample (dry basis) was weighed in aluminum sample pans and mixed with 7.5 µL of distilled water. The pan was sealed and the sample was equilibrated for 24 h at room temperature. The samples were heated from 30 to 110°C at a scanning rate of 10°C/min. An empty sample pan was used as a reference. For each endotherm, the onset temperature of gelatinization (T_0) and the difference in enthalpy (ΔH) were computed automatically. Crystallinity of starch granules was analyzed by an X-ray diffractometer (XRD; Scintag XDS 2000, USA). Prior to X-ray diffraction, the water content of the starch was equilibrated at 100% of relative humidity at room temperature in presence of iso-propanol. A 20 mg of starch sample was then sealed between two tape foils to prevent any significant change in water content during the measurement. X-ray diffraction patterns were obtained by the XRD working at 35 kV, 20 mA and producing Cu-k_α radiation at a wave-length of about 0.15405 nm. Starch

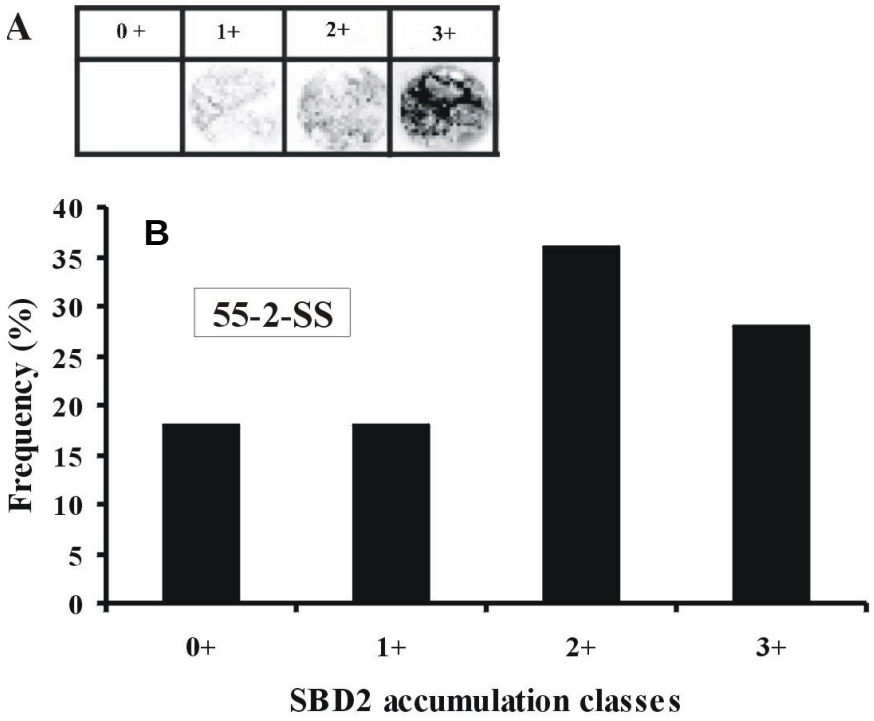

Figure 1. Accumulation levels of SBD2 in transgenic sweet potato starch granules. (A) The classes of SBD2 accumulation in starch granules, where the 0+,1+,2+ and 3+ classes represent no, low, intermediate and high levels of SBD2 accumulation, respectively. (B) The distribution of sweet potato transformants over the classes of SBD2 accumulation in the 55-2-SS series.

samples were scanned from 4 and 40º (2θ) at increments of 0.04º.

RESULTS

Preparation of transgenic plants

The 12 transgenic sweet potato clones obtained in a previous study have been analyzed to confirm the integration of the *SBD2* gene into the genomic DNA by PCR amplification and confirmed by Southern blot analysis (Xing et al., 2008). Five transgenic plants from each clone were multiplied and grown in the greenhouse to generate tuberous-roots. The morphology of plants and roots, as well as the root-yield, revealed no consistent differences between transformed and control plants (results not shown).

Western dot blot analysis

The levels of SBD2 accumulation in transgenic granules were analyzed by Western dot blot. The SBD2 accumulating clones were divided into four classes (ranging from 0+ to 3+) based on the amount of SBD2 protein associated with the starch granules, in which 0+, 1+, 2+ and 3+ represent no, low, intermediate and high accumulation levels, respectively (Figure 1A). SBD2 accumulation levels in the transgenic sweet potato starches are summarized in Figure 1B. The results clearly show that the highest level of SBD2 accumulation in sweet potato starch granules was the 3+ class. However, transformants belonging to the 2+ class were more abundant than in other classes. The results demonstrate that SBD2 protein could be accumulated in sweet potato starch granules during the biosynthesis process. The SBD2 protein concentration in the soluble fraction of roots from each class was also determined by Western dot blot. Table 1 shows the analysis results from one representative of each class. For comparison, the levels of SBD2 accumulation in relative transgenic granules were also indicated in the table. It appeared that SBD2 was only found in the root juices of transformants belonging to the 3+ class (the highest accumulator). The amount of unbound SBD2 in the root juice was 2+, corresponding to the dot with an intensity of 2+ in Figure 1A. This suggested that the amount of SBD2 in the 3+ class in starch granules was saturating.

Table 1. Accumulation levels of SBD2 protein in granules and juice of selected 55-2-SS transgenic roots, as estimated with Western dot blot analysis.

Clone	Amount of SBD2	
	In granules	In juice
55-2-SS8	0+	n.d[a]
55-2-SS7	1+	n.d
55-2-SS2	2+	n.d
55-2-SS4	3+	2+

[a]Not detected. The number representation (1+, etc) is according to the dot intensities in Figure 1A.

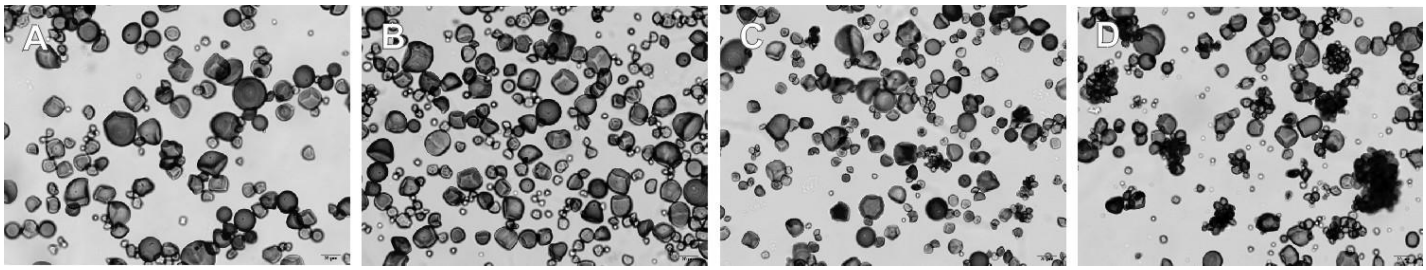

Figure 2. Light micrographs of 55-2-SS transgenic and untransformed starch granules: **(A)** 55-2-UT. **(B)** 55-2-SS7, 1+. **(C)** 55-2-SS2, 2+. **(D)** 55-2-SS4, 3+. Magnification of the starch granules: 200x.

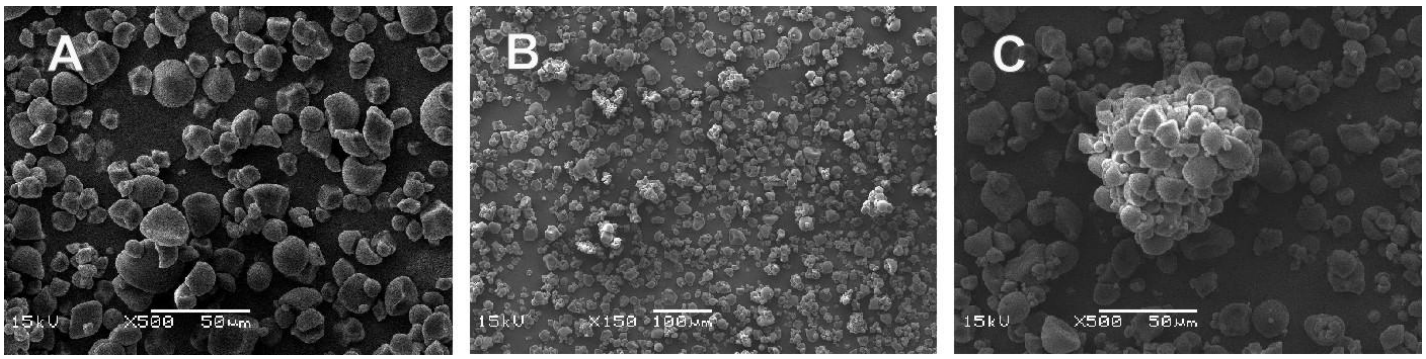

Figure 3. Scanning electron micrographs of transgenic and untransformed starch granules. **(A)** 55-2-UT. **(B)** and **(C)** 55-2-SS4, 3+.

Granule morphology

Starch granule morphology of each 55-2-SS transformed clone in the series was investigated by light microscopy. The micrographs of transgenic granules from one representative of each class and their control are shown in Figure 2. It can be seen that the morphology of 55-2-UT and 55-2-SS7 (1+) starch granules was more or less similar (Figures 2A and B), whereas higher SBD2 accumulation levels (2+ and 3+) in transgenic starch granules showed altered morphology in comparison with the control. Granules were sometimes organized in large clusters of many small ones (further referred to as “large clusters”) (Figures 2C and D). It seemed that this phenomenon was most pronounced in 55-2-SS4 (3+), although it could also be observed in 55-2-SS2 (2+). Based on the LM results, starches from 55-2-SS4 and the control were further investigated by scanning electron microscopy (Figure 3). The scanning electron micrographs of 55-2-SS4 granules (Figures 3B and C) revealed that large clusters were more loosely associated, suggesting that SBD2 accumulation could affect morpho-

Table 2. Gelatinization characteristics (T_0, ΔH), apparent amylose content AM (%), crystallinity (%) and starch granule size (d_{50}) measurements of starches from transgenic clones and the control.

Clone	AM (%)	T_0 (°C)[a]	ΔH (kJ/g)[b]	Crystallinity (%)	d_{50} (µm)[c]
55-2-UT	22.4 (±2.4)	68.1	9.9	31.2	15.3 (±0.4)
55-2-SS7(1+)	23.1 (±0.9)	66.9	9.6	30.5	14.1 (±1.1)
55-2-SS2(2+)	21.6 (±1.2)	68.5	10.0	31.9	13.7 (±1.4)
55-2-SS4(3+)	22.6 (±1.5)	67.4	9.9	30.9	14.8(±0.6)

[a]Temperature at onset of starch gelatinization, [b]enthalpy released, [c]median value of the granule size distribution. Data (±SD) are the average of three independent measurements.

logy of starch granule, at least when it was present in a high level.

Characterization of transgenic starches

The impact of SBD2 accumulation in granules on the physicochemical properties of starch was also investigated. Within the transgenic series, one transformant in each SBD2 accumulation class (0+, 1+, 2+ and 3+) was selected for further study. The untransformed starch was used as a control. Table 2 summarizes the results on starch granule size (d_{50}), granule-melting behaviour (T_0 and ΔH), crystallinity, and apparent amylose content (AM%).

From the table, it can be seen that no consistent difference was observed between transgenic starches and their control, sug-gesting that the altered granule morphology did not seem to lead to different physico-chemical properties of the granules. However, it should be noted although 55-2-SS4 and 55-2-UT starches gave similar results with respect to granule size (d_{50}), the granule size distribution of 55-2-SS4 (3+) was relatively wide compared to that of 55-2-UT (result not shown), which was in accordance with the SEM results (Figure 3B). Although it seemed that the large cluster granules of 55-2-SS4 dominated the SEM at first sight, their abundance was low compared with the small granules, and consequently they contributed relatively little to the granule size distribution.

DISCUSSION

In this study, the transgenic sweet potato modified with the engineered tandem-repeat starch-binding domain (SBD2) was investigated to determine if the SBD2 could be used as an anchor for the modification of starch. Our data shows that high levels of SBD2 protein could be accumulated in granules during starch biosynthesis, indicating that SBD2 could be used as tools to anchor effector proteins in sweet potato starch granules. In our previous study, the *SBD2* gene has been introduced into the amylose-containing potato genotype Kardal, and the highest levels of SBD2 accumulation in starch granules is 4+ (Firouzabadi et al., 2007). Sweet potato cultivar Xu55-2 used in this study is also an amylose-containing genotype. However, the highest level of SBD2 accumulation (3+) in transgenic sweet potato starch was lower than that in potato Kardal. This might be caused by using the potato GBSS I promoter and transit peptide sequence, which might not be suitable for expression of foreign genes in sweet potato starch granules.

The expression of SBD2 in sweet potato plants resulted in some clones containing starch with “large clusters” granules. Its occurrence seemed to be correlated with the amount of SBD2 accumulated in the granule, because they were most pronounced in 55-2-SS4 (3+ class). Interestingly, the “large clusters” granules is not observed when SBD2 is expressed in potato Kardal background, but large “amalgamated clusters” ones are encountered in transformants belonging to the 3+ class (Firouzabadi et al., 2007).

We know that both sweet potato Xu55-2 and potato Kardal are amylose-containing genotype and that there is presence of another granule-bound protein GBSS I (granule-bound starch synthase I) in the granules. When SBD2 is expressed in starch granules, SBD2 and GBSS I proteins are simultaneously present. We postulate that, in this situation, they might bind similar sites of the growing starch granule and the SBD2 has a higher affinity for starch granules than GBSS I.

As a consequence, GBSS I can be out-competed by the SBD2 protein in the granules. Additionally, it is known that there is a difference in size between potato and sweet potato starch granules. The mean granule size of potato Kardal is 27.9 µm (Ji et al., 2003), whereas that of sweet potato Xu55-2 was 15.7 µm. The former is approximately two times bigger than the latter. Thus, when SBD2 accumulation level in both starch granules is the same (3+ class), the surface of potato starch granules has more sites for both SBD2 and GBSS I binding. In this case, the surface of some granules is mostly covered by SBD2, in which due to the limitation of surface area, both SBD domains can not be accommodated on the same surface anymore.

Consequently, the SBD2 proteins may have only one SBD attached to a binding site, whereas the exposed SBD of SBD2 is available for interaction with amylose-like molecules produced by GBSS I, and/or with different growing granules (Firouzabadi et al., 2007). In this situation, the enzymes involved in starch biosynthesis are not hindered greatly in attaching material from the stroma to the growing granule. If the exposed SBD of SBD2 binds amylose-like molecules from a different nucleation sits, amylose may effectively fill the gap between loosely associated small granules, then the large “amalgamated clusters” granules may be formed in potato Kardal starch (Firouzabadi et al., 2007).

For sweet potato starch, the surface of granules is smaller and has fewer sites for both proteins binding. We speculate that, at the 3+ class of SBD2 accumulation, the surface of some granule might be fully covered by SBD2, in which also only one of SBD2 attached to a binding site. In this case, the SBD2 might hinder synthases from elongation amylopectin side-chains at the granule surface, and/or synthases from attaching side chains to the growing granule. Thus, small granule formation and additional nucleation sites for granule formation might be enforced by SBD2 (Ji et al., 2004), but it is possible to produce smaller starch granules without accompanying changes in contents of amylose and amylopectin. If the exposed SBD of SBD2 stuck to different growing granules, which might facilitate cross-linking of the small granules, the loosely associated “large clusters” might be formed (Figures 3B and C). This is also the reason why the analysis results of apparent amylose in the 55-2-SS4 starch did not reveal a reduction in the content in comparison with the control.

Except for the alteration of transgenic starch granule morphology, no consistent differences in the melting temperature of the granules, granule size distribution, crystallinity and apparent amylose content between transgenic SBD2 starches and the control were found, suggesting that SBD2 could be used as an anchor for targeting starch-modifying proteins to granules without having other side-effects. This study provides good perspectives for the applicability of SBD technology for sweet potato starch bioengineering. Our future research will focus on developing a number of applications in which SBD2 is used as an anchor to incorporate effector proteins in sweet potato starch granules.

ACKNOWLEDGEMENTS

This work was supported by National 863 Project (No. 2009AA10Z102), National Natural Science Foundation of China (No. 30771367), Jiangsu Education Department (No.JHB04-043), and Huaiyin Normal University (06HSJS025). The authors wish to thank the Laboratory of Plant Breeding, Department of Plant Sciences, Wageningen University for providing the pBIN19/SBD2 plasmid.

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