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Developmental change in starch granules in sweetpotato callus

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Abstract

Developmental change in starch granules formed in sweetpotato callus was examined. The starch content increased from nearly 0 to 10.6% of the dried calli over one week. The chain-length distribution of the debranched starches determined by gel-permeation chromatography revealed that no amylose was present in the one-day sample. During development, amylose fraction increased continuously for seven days to 12.3% of total starch. On the other hand, the ratio of two unit-chain fractions from amylopectin was nearly identical, and the short-chain distribution (DP = 6–35) was also similar throughout the culture period. Furthermore, all starch granules showed an A-type crystallite. These results suggest that the amylopectin molecules were completed as a granule as early as the first day before the appearance of the amylose fraction. With respect to the structural change of starch granules, the transcript of the GBSSI gene was detected in a constant level during the culture for one month. These results indicate that in spite of the occurrence of the GBSSI transcript at the start of starch accumulation, the structural change from waxy to normal feature of starch granule occurred during the culture of sweetpotato calli.

Keywords: Starch; Amylose; Starch synthase; Sweetpotato callus

1. Introduction

Starch components, amylose and amylopectin, are packaged in a specific structure in an insoluble granule. Changes as the starch granule develops are interesting from the viewpoint of synthetic mechanisms and molecular construction in plants. It has been reported that the amylose content of starch increased with granule development in some plants, such as cereals (Shannon & Garwood, 1984) and potato (Geddes, Greenwood & Mackenzie, 1965; Sugimoto, Yamashita, Hori, Abe & Fuwa, 1995). These results suggest a time lag between amylose synthesis and amylopectin synthesis and that amylose synthesis occurs after amylopectin synthesis (Ball, van de Wal & Visser, 1998). It has been further suggested that amylose synthesis occurs inside the crystalline amylopectin matrix of the granule (Tatge, Marshall, Martin, Edwards & Smith, 1999). However, contrary to the observations mentioned above, no increase in the amylose content has been reported in cassava (Asaoka, Blanshard & Rickard, 1991; Ketiku & Oyenuga, 1972) and sweetpotato (Noda, Takahata & Nagata, 1992) starches.

We previously examined some properties of starch granules that had formed in suspension-cultured cells of a wild relative of sweetpotato (*Ipomoea cordatotriloba* Denn.) (Kitahara, Imamura, Omae & Suganuma, 1998). Since during the development of the cultured cells, the starch granule content increased from almost zero, new information concerning the structural change of starch granules at the early stage of development is expected from such a study. Moreover, constant culture conditions eliminate the influence of some environmental factors on starch development.

In this paper, further attention was given to the developmental change of sweetpotato starch. For this purpose, we used embryogenic callus of sweetpotato [*Ipomoea batatas* (L.) Lam.], and we attempted to find out the nature of the changes in starch granules during development.

2. Experimental

2.1. Materials

Embryogenic calli were induced from shoot meristems of *Ipomoea batatas* (L.) Lam. cultivar Koganesengan on Murashige–Skoog salts (Murashige & Skoog, 1962) containing

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1.7 mg/l thiamin hydrochloride, 1.2 mg/l nicotinic acid, 1.0 mg/l pyridoxine hydrochloride, 2 mg/l glycine, 90 mg/l myo-inositol, 1.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 1.0 mg/l 1-naphthaleneacetic acid (NAA), 30 mM potassium chloride, 3% sucrose, and 0.8% agar at pH 5.8. After induction, the embryogenic calli were proliferated by subculture for two weeks in a liquid medium whose composition is the same as above, except for 0.5 mg/l 2,4-D and 0.5 mg/l NAA. These calli were maintained at 27°C in the dark under rotary shaking. All cultures were done under aseptic conditions.

Distilled water was passed through a Milli-Q Labo (Nihon Millipore Ltd, Tokyo, Japan) before use. All reagents and solvents, unless otherwise specified, were obtained from Wako Pure Chemical Industries (Osaka, Japan) and were of analytical grade.

2.2. Methods

2.2.1. Growth of cultured calli and their starch contents

Embryogenic calli collected on a Nalgene filter holder (Inox Toei Co., Tokyo, Japan) with a 42-mesh screen were washed with 3% sucrose solution. The calli (3–4 g, wet basis) were inoculated into 90 ml of the above-mentioned subculture medium in a 300 ml Erlenmeyer flask and cultured at 27°C in the dark under rotary shaking for 1–5 weeks. The cultured calli for each period were collected by the filter holder, washed with distilled water, and lyophilized. The change in dry matter of the calli was expressed as a ratio of the dry weight of inoculated calli to that of grown calli (Kitahara et al., 1998).

Carbon sources in the medium were determined by using a high-performance liquid chromatograph (Tosoh Corporation, Tokyo, Japan) with a column of Shodex Ionpak KS-801 (Shoko Co., Ltd, Tokyo, Japan). The column was kept at 40°C and eluted at a flow rate of 1.0 ml/min with distilled water containing 0.02% sodium azide. The eluate was monitored by an RI detector (RI-8022, Tosoh).

The starch content of the calli was measured as a glucoamylase-digestible polysaccharide, as previously reported (Kitahara et al., 1998).

2.2.2. Chain-length distribution of starch

Starch granules were prepared from lyophilized calli by the procedure previously reported (Kitahara et al., 1998). Amylopectin was prepared from the supernatant after the removal of amylose by butanol complexing (Takeda, Hizukuri, & Juliano, 1986).

After debranching by isoamylase (Nacalai Tesque Inc., Kyoto, Japan), the chain-length distribution of the debranched starch was determined by both gel-permeation chromatography (Kitahara, Suganuma & Nagahama, 1994), using two linked columns of Superose 6 and Sephadex G25SF (Amersham Pharmacia Biotech, Tokyo, Japan), and high-performance anion-exchange chromatography (HPAEC-PAD, Dionex DX-500, Nippon Dionex K.K., Osaka, Japan) (Kitahara et al., 1998).

2.2.3. Preparation of RNA competitor for GBSSI

Complete (Wang, Yeh & Tsai, 1999) or partial sequences of sweetpotato granule-bound starch synthase I (GBSSI) gene were obtained from GenBank (Accession numbers U44126, AB009299, and AB009300). Two primers were used for amplifying a part of sweetpotato GBSSI gene; the sequences of sense and antisense primers were [5′-GTGTGTCCCCGTTATGATCAG-3′] and [5′-ACGGATTCCAGCTTTCATCCA-3′], respectively. The predicted size of PCR product was estimated to be 576 pb from the known cDNA sequence.

The quantitative evaluation for a transcript of the GBSSI gene was done by using a heterologous RNA competitor as an internal standard. At first, DNA competitors were constructed by using a Competitive DNA Construction Kit (Takara Shuzo Co., Ltd, Kyoto, Japan), which supplied the reagents and tools for PCR and for purification of the PCR products. The DNA competitor was prepared by PCR amplification on λDNA with composite primers, in which the sense primer was engineered to contain a λDNA specific sense sequence with the GBSSI specific sense primer sequence, followed by an SP6 promoter sequence flanking its 5′ ends; the antisense primer contained a λDNA specific antisense sequence with the GBSSI specific antisense sequence. The RNA competitor was then transcripted from the DNA competitor by SP6 RNA polymerase supplied in a Competitive RNA Transcription Kit (Takara). The RT-PCR product from the RNA competitor using the same primers as GBSSI was 442 bp, which could be separated from the target DNA product on electrophoresis. The concentration of the competitors were measured by spectrophotometry (absorbance at 260 nm), which was confirmed by electrophoresis.

2.2.4. Extraction of total RNA and RT-PCR

Total RNA was isolated from the calli by the procedure of Chang, Puryear, and Cairney (1993) with minor modification. A sample of the callus (200 mg) was ground with 2 ml of extraction buffer (2% cetyltrimethylammonium bromide, 100 mM Tris-HCl [pH 9.5], 20 mM ethylendiamintetraacetate (EDTA), 1.4 M sodium chloride, 5% 2-mercaptoethanol), and 2 ng of the RNA competitor (8×10^9 copies) in liquid nitrogen. The homogenate was transferred in a centrifuge tube and incubated at 65°C for 10 min. After the addition of an equal volume of chloroform:isoamylalcohol (CIA, 24:1, v/v), the tube was shaken for 10 min and centrifuged at 15,000 rpm for another 10. The upper layer was repeatedly washed with CIA three times, then mixed with an equal volume of 8 M lithium chloride. The mixture was kept at −20°C for at least 2 h. After centrifugation at 15,000 rpm for 10 min at 4°C, the pellet was dissolved in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The solution containing RNA was washed successively with

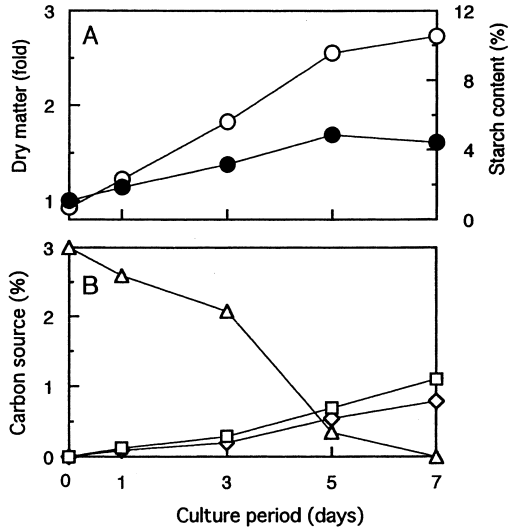

Fig. 1. Changes in dry matter and starch content of sweetpotato calli (A) and changes in carbon-source concentration in the medium (B) during seven days of culture. (A) ●: dry matter; ○: starch content, (B) △: sucrose; ◇: glucose; □: fructose. The calli cultured for four weeks were inoculated in a fresh medium.

phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) and CIA. After centrifugation, RNA was precipitated in lithium chloride solution in the same manner as above. The RNA pellet was washed with 70% ethanol, then dissolved in diethylpyrocarbonate-treated water.

RT-PCR was carried out on the extracted RNA with the GBSSI specific primers by using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech) according to the manufacturer's instructions. PCR was carried out for 20 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 60 s. An aliquot of the RT-PCR reaction was analyzed by agarose gel (2%) electrophoresis by using ethidium bromide to visualize the amplified products under UV light. The band intensity on a digital image of the gel was estimated by using a NIH image soft (Wayne Rasband, National Institute of Health, USA). The PCR product was verified by direct sequencing with one side primer, using a Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech). The sequence was determined by using an ABI PRISM 310 genetic analyzer (PE Biosystems Japan Ltd, Chiba, Japan).

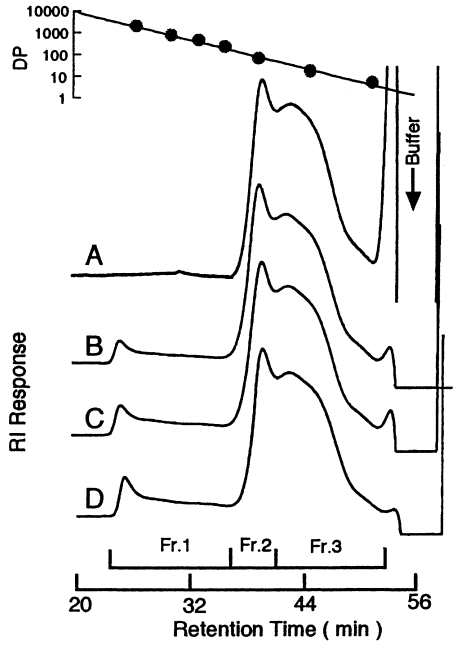

Fig. 2. Chain-length distributions of debranched starches from sweetpotato calli: (A) starch from calli cultured for one day; (B) cultured for three days; (C) cultured for five days; (D) cultured for seven days.

3. Results and discussion

3.1. Formation of starch granules in sweetpotato callus

The calli cultured for two weeks were inoculated in a fresh medium. The change in starch content of the grown calli was examined during five weeks. It was observed that the starch content of the dried calli increased up to 10.6% in a week, then decreased (data not shown). The maximal starch content at one-week culture agreed with the results for the suspension-cultured cells of *I. cordatotriloba* (Kitahara et al., 1998). The starch granules almost disappeared at four weeks. To describe their changes at the early stage of culture, the calli cultured for four weeks and containing less than 1% starch were inoculated into fresh media, and the starch formation in the calli was examined. As shown in Fig. 1, the calli was resulted in 1.5-fold dry matter for one week. A gradual decrease in sucrose following an increase in glucose and fructose in the medium were observed, suggesting that the sucrose was cleaved to monosaccharides. As no invertase activity was detected in the medium (data not shown), the sucrose might be hydrolyzed by extracellular invertases, known as cell wall invertases. Although the specific locations and functions of several sugar transporters have been shown (Williams, Lemoine & Sauer, 2000), the transport mechanism of sucrose or monosaccharides, or both, into cells of sweetpotato callus was unclear. In this study, this aspect of the work was not pursued further. On the other hand, the starch content linearly increased up to 10.5% during seven days. Thus, the formation of starch granules was confirmed in the embryogenic callus of sweetpotato.

3.2. Structural changes of starch granules in callus

Starch granules were prepared from the calli cultured for 1, 3, 5, and 7 days. A scanning electron microscopic observation showed that they were spherical and very small (1–3 μm), like those of *I. cordatotriloba* (Kitahara et al., 1998). The chain-length distributions of the debranched starches were examined by gel-permeation chromatography. As shown in Fig. 2, the elution profiles can be divided into

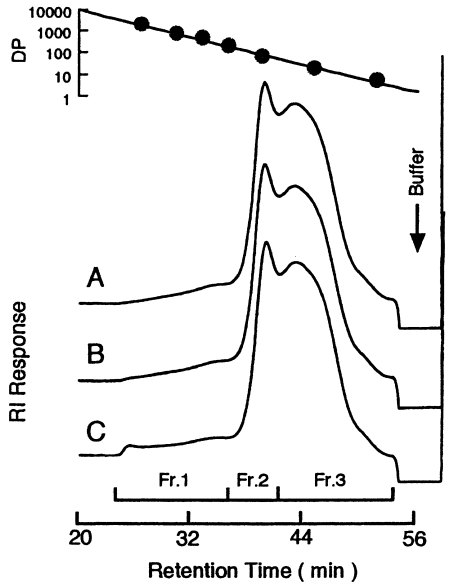

Fig. 3. Chain-length distributions of debranched amylopectins from sweetpotato calli: (A) amylopectin from calli cultured for three days; (B) cultured for five days; (C) cultured for seven days.

high molecular weight fraction (Fr. 1) and two lower molecular weight fractions (Fr. 2 and Fr. 3), which are generally referred to as amylose fraction and unit-chain fractions of amylopectin, respectively (Ikawa, Glover, Sugimoto & Fuwa, 1978). The Fr. 1 content was not detected in the one-day sample, but it rapidly increased to 7.5% for 3 days, to 10.1% for 5 days, then to 12.3% for 7 days. On the other hand, the ratio of Fr. 3 to Fr. 2, which is a structural index of the amylopectin, was nearly identical (2.1–1.8) among the starches. On the chromatograms, an unusual peak was detected at the end of the chain-length distribution. As the short chains with less than DP 5 was scarcely detected by HPAEC-PAD (data shown in Fig. 4), the peak was not derived from short chains constituting starch molecules. To determine the component that increased in Fr. 1, the amylose was removed from the whole starch by butanol complexing, and the chain-length distribution of the amylopectin alone was examined. Amylopectin could not be prepared from the one-day sample because of its paucity. As seen in Fig. 3, most of the Fr. 1 disappeared after a removal of amylose, indicating that the component that increased in Fr. 1 was an independent molecule of the amylose. It is interesting that the amount of long chains eluting at Fr. 1 of amylopectin also slightly increased with the development of starch granules. Fig. 4 shows the short-unit chains of the debranched starch analyzed by HPAEC-PAD. The distributions of the callus starches peaked at DP 13 with a trough at DP 8, which are common in sweetpotato root starch (Hanashiro, Abe & Hizukuri, 1996; Koizumi, Fukuda & Hizukuri, 1991; Noda, Takahata & Sato, 1995) and also in the starch from suspension-cultured cells of *I. cordatotriloba* (Kitahara et al., 1998). Although minor changes in the distribution were found through the culture period for seven days — a decrease of the chains at DP 9-17 and an increase of the chains at more than DP 22 — it was concluded that the distribution of the short-unit chains was fixed on the first day.

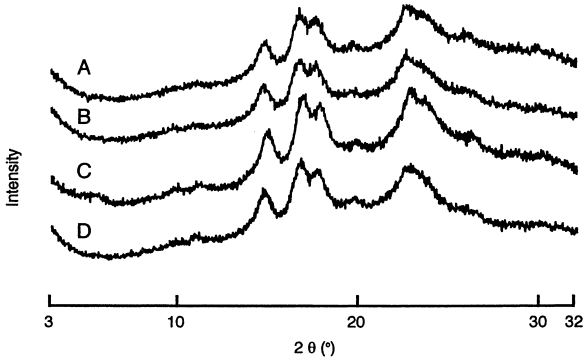

Fig. 5. X-ray diffractograms of starches from sweetpotato calli: (A) starch from calli cultured for one day; (B) cultured for three days; (C) cultured for five days; (D) cultured for seven days.

Fig. 5 shows X-ray diffractograms of the callus starches. All starches had the same crystalline organization as that of root starch (A-type). The result demonstrates that the starch granules are packed in a crystallite organization as early as

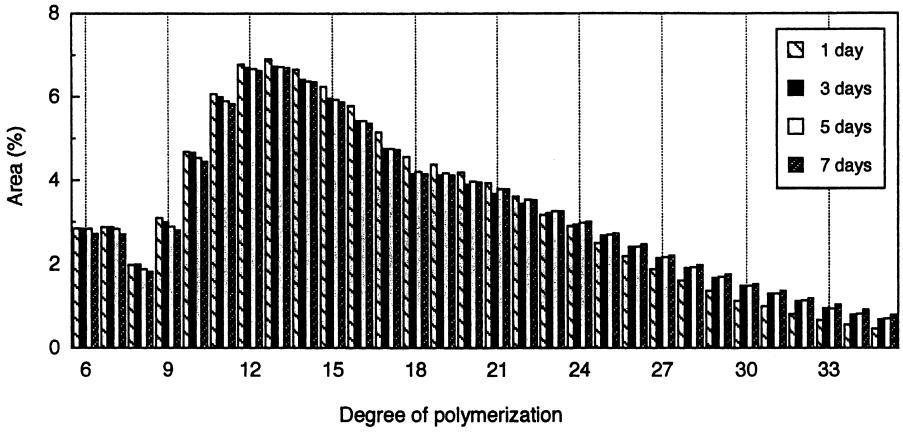

Fig. 4. Short-chain distributions of debranched starches from sweetpotato calli.

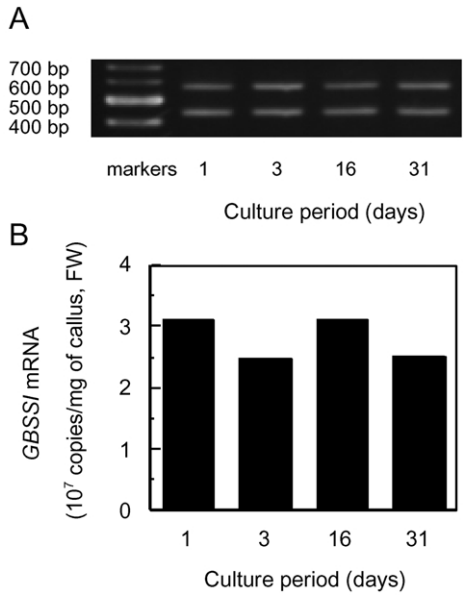

Fig. 6. Quantitative analysis of GBSSI mRNA levels in sweetpotato calli at different culture periods: (A) ethidium bromide staining of RT-PCR products separated in 2% agarose gel; (B) calculated copy numbers of GBSSI mRNA on the basis of the amount of added competitor.

the first day of culture. This result confirms that the amylose molecule is not essential in the formation of a starch granule. It is of course that waxy starches are organized into crystallline granules.

3.3. Transcription of the GBSSI gene in calli at different culture periods

During the culture of sweetpotato calli, the structural change of starch granules formed in the calli was found especially in the synthesis of an amylose molecule. It has been known that the synthesis of amylose is primarily responsible for GBSSI (for review, Ball et al., 1998; Martin & Smith, 1995; Nelson & Pan, 1995; Smith, Denyer & Martin, 1997). The transcript of the GBSSI gene in the calli was quantitatively compared at different culture periods by using an RT-PCR technique (Wang, Doyle & Mark, 1989). The fragment size of the RT-PCR product from GBSSI agreed with the predicted size, and the sequence showed a high homology (99.7%) to the corresponding parts of the known sweetpotato GBSSI (Wang et al., 1999). Thus, the RT-PCR product was confirmed to be derived from GBSSI mRNA. For a comparison of the amount of the GBSSI transcript, the simultaneous PCR amplification of both GBSSI and competitor was done under nonsaturating conditions. Fig. 6 shows the electrophoretogram on an agarose gel and its quantified copies of GBSSI mRNA on the basis of the amount of the added competitor. The PCR products of 576 and 442 bp were from GBSSI and competitor, respectively. There was a constant level of transcript of GBSSI for one month during the culture of calli. This result suggests that no correlation exists between the accumulation of GBSSI transcript and the calli development. Moreover, the constant level of GBSSI transcript was not related to the change in starch content. Wang et al. (1999) reported that the amount of GBSSI transcript increased with the development of tuberous root of sweetpotato, but that in leaf it was regulated by a circadian rhythm. The circadian regulation of GBSSI transcription in leaf, but not root, was also observed in snapdragon (Mérida, Rodríguez-Galán, Vincent & Romero, 1999). Thus, an interesting difference in the regulation of the GBSSI transcription was found among root, leaf, and callus, which should help to elucidate the organ-specific regulation of gene expression.

In this study, the dynamic metabolism of starch granules, i.e. their synthesis and degradation, was observed in the sweetpotato calli. The calli cultured for four weeks, having less than 1% starch content, corresponded to the start material of this experiment when they were inoculated in a fresh medium. Therefore, the change in starch structure at a very early stage of the development could be observed. It was interesting to note that no amylose fraction was detected from the chain-length distribution of debranched starch for a one-day culture, and the amylose fraction increased during the succeeding culture for seven days (Fig. 2). During further advanced development of the calli, the amylose fraction of the starch amounted to 16.8% for 2 weeks and to 17.8% for 3 weeks (chromatogram not shown), whose distribution was similar to that of the tuberous root starch (Kitahara, Ooi, Mizukami, Suganuma & Nagahama, 1996). The increase in amylose fraction with the calli development appeared to be roughly saturated after two weeks. It has been suggested that amylose content may be limited by space availability within the crystalline amylopectin matrix of the granule (Flipse, Keetels, Jacobsen & Visser, 1996). Although our observation may be consistent with the proposal, it could not be concluded whether the apparent saturation of the increase in the amylose fraction was due to the space limitation or to the selective degradation of amylose molecules because a decrease in the starch content had already been observed after seven days. On the other hand, the structural index of amylopectin, Fr. 3/Fr. 2, was nearly identical (Figs. 2 and 3), and the short-chain distribution included in Fr. 3 was also similar through the culture (Fig. 4). Furthermore, all starch granules showed an A-type crystallite (Fig. 5). These results suggest that the amylopectin molecules were completed as a granule as early as the first day of culture. Thus, the structural change from waxy to normal amylose containing starch was demonstrated in vivo through the culture of sweetpotato callus. Although no significant change in the amylose content was reported in starch granules during development of the tuberous roots of sweetpotato (Noda et al., 1992), it is considered that our results showed the changes in the amylose content occur at a very early stage of the development.

With respect to the structural change of starch granules, the transcript of the GBSSI gene, whose enzyme is involved

in amylose synthesis, was detected at a constant level for a month during the culture (Fig. 6). The result indicates that the GBSSI transcript would exist before the starch accumulation because the calli cultured for four weeks were the start material of this experiment. Thus, both the occurrences of GBSSI transcript at the start of culture and of the absence of amylose fraction of starch formed on the first day also support the concept that the amylose synthesis occurs after the synthesis of the amylopectin matrix (Ball et al., 1998; Tatge et al., 1999). It is assumed, as suggested by Wang et al. (1999), that a certain regulatory mechanism exists between the transcription of the GBSSI gene and the amylose synthesis by GBSSI.

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