Theor Appl Genet (2000) 101:21–29

M. Yamamori · S. Fujita · K. Hayakawa · J. Matsuki
T. Yasui

Genetic elimination of a starch granule protein, SGP-1, of wheat generates an altered starch with apparent high amylose

Received: 30 April 1999 / Accepted: 9 November 1999

Abstract A starch granule protein, SGP-1, is a starch synthase bound to starch granules in wheat endosperm. A wheat lacking SGP-1 was produced by crossing three variants each deficient in one of three SGP-1 classes, namely SGP-A1, -B1 or -D1. This deficient wheat (SGP–1 null wheat) showed some alterations in endosperm starch, meaning that SGP-1 is involved in starch synthesis. Electrophoretic experiments revealed that the levels of two starch granule proteins, SGP-2 and -3, decreased considerably in the SGP-1 null wheat though that of the waxy protein (granule-bound starch synthase I) did not. The A-type starch granules were deformed. Apparent high amylose level (30.8–37.4%) was indicated by colorimetric measurement, amperometric titration, and the concanavalin A method. The altered structure of amylopectin was detected by both high-performance size-exclusion chromatography and high-performance anion exchange chromatography. Levels of amylopectin chains with degrees of polymerization (DP) 6–10 increased, while DP 11–25 chains decreased. A low starch crystallinity was shown by both X-ray diffraction and differential scanning calorimetry (DSC) analyses because major peaks were absent. Abnormal crystallinity was also suggested by the lack of a polarized cross in SGP-1 null starch. The above results suggest that SGP-1 is responsible for amylopectin synthesis. Since the SGP-1 null wheat produced novel starch which has not been described before, it can be used to expand variation in wheat starch.

Key words Common wheat · Starch synthase · Amylose · Amylopectin

Communicated by L. Willmitzer

M. Yamamori (✉)
National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305–8602, Japan

S. Fujita
Tsu City College, Tsu, Mie 514-0112, Japan

K. Hayakawa
Nisshin Flour Milling Co, Ohimachi, Iruma, Saitama 356–8511, Japan

J. Matsuki · T. Yasui
National Agriculture Research Center, Tsukuba, Ibaraki 305–8666, Japan

Present address:
M. Yamamori, Tohoku National Agricultural Experiment Station, Morioka, Iwate 020-0824, Japan

Introduction

Starch is the major component of wheat (*Triticum aestivum* L.) endosperm and consists of amylose and amylopectin. The amylose to amylopectin ratio of normal (non-waxy) wheat is around 1:3. Amylose is a relatively less-branched (essentially a linear chain) α(1,4)-linked glucose polymer, and amylopectin is a branched glucose polymer in which α(1,4)-linked polymers are connected by α(1,6)-linkages. Starch synthase, which produces the (1,4)-linkage, is essential for synthesizing linear glucose polymers while the branching enzyme generates the (1, 6)-linkage in amylopectin.

Enzymes for starch synthesis, e.g., starch synthase (SS) and the branching enzyme (BE), are found in soluble fractions of storage organs, for example, the endosperm of seed. These enzymes are present in the amyloplast stroma where starch is deposited (Yu et al. 1998). Studies in pea, maize, and wheat (Denyer et al. 1993, 1995; Echt and Schwartz 1981; Mu et al. 1994) have shown that some enzymes for starch synthesis are tightly bound to starch granules from seed endosperms of maize and wheat or from pea embryos. These enzymes exist as both soluble and granule-bound forms or as exclusively granule-bound. However, detailed knowledge of the binding mechanism is still unavailable. In wheat, at least four kinds of proteins, i.e., waxy protein and three starch granule proteins (SGP-1, -2, -3), are tightly bound to starch granules and are responsible for starch synthesis. Waxy protein or granule-bound starch synthase I (GBSS I) is responsible for amylose synthesis and is the product of the *waxy* gene (Ainsworth et al. 1993). SGP-1, -2 and -3

(Yamamori and Endo 1996) correspond to the 100- to 105-kDa, 90-kDa, and 77-kDa granule-bound isoforms, respectively, reported by Denyer et al. (1995). Immunoblotting, amino acid sequencing, and the detection of starch synthase or branching activities (Denyer et al. 1995; Rahman et al. 1995; Takaoka et al. 1997) suggest that SGP-2 is a homolog of maize branching enzyme IIb (Fisher et al. 1993) and that SGP-3 is a homolog of maize starch synthase I (Knight et al. 1998). Internal amino acid sequences of SGP-2 (Takaoka et al. 1997) show homology to a cDNA of wheat branching enzyme II (Nair et al. 1997).

Immunoblotting studies of the 100- to 105-kDa proteins (SGP-1), have shown that SGP-1 is exclusively bound to starch granules (Denyer et al. 1995; Rahman et al. 1995), and is also present in the soluble fraction at early stages of endosperm development (Li et al. 1999). This protein is assumed to be a starch synthase based on results from studies of antiserum recognition, its enzymatic activity, and its homology in peptide sequences (Denyer et al. 1995; Takaoka et al. 1997). Activity staining of renatured SGP-1 on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels and amino acid sequence data from cDNA indicate that SGP-1 is a starch synthase or a homolog to class II starch synthase (SS II) and is more similar to maize SSIIa (Li et al. 1999). To understand the function of SGP-1 and learn whether it is necessary for amylose and amylopectin synthesis, we should investigate wheat whose SGP-1 is genetically nullified.

Wheat has three isoforms of SGP-1, SGP-A1, -B1, and -D1. The gene coding for SGP-A1, *Sgp-A1*, is located on chromosome arm 7AS, *Sgp-B1* is on 7BS, and *Sgp-D1* is on 7DS (Denyer et al. 1995; Yamamori and Endo 1996). Using SDS-PAGE, Yamamori and Endo (1996) found that a few wheat cultivars lacked either SGP-A1, -B1 or -D1, but no wheats lacked more than one SGP-1. This finding enabled us to produce a wheat with no SGP-1 (SGP-1 null wheat) by crossing three variant wheats. This report describes the production of the SGP-1 null wheat and characterization of its starch. Results showed that SGP-1 is involved in starch (most likely amylopectin) synthesis and that the lack of SGP-1 causes pleiotropic effects on starch.

Materials and methods

Plant material

To produce a wheat which has no SGP-1 (SGP-1 null wheat), we used the following four parental cultivars: Chousen 30 (C 30) and 57 (C 57) lacking the SGP-A1, Kanto 79 (K 79) lacking the SGP-B1, and Turkey 116 (T 116) lacking the SGP-D1. F_2 plants which lacked both SGP-D1 and -B1 from a cross T 116/K 79 were selected by electrophoretically analyzing SGPs from F_2 seeds. These F_2 plants were pollinated by C 30 or C 57 lacking the SGP-A1. Consequently, from (K 79/T 116)F_2//C 30 or C 57, the variant progeny having no SGP-1 was selected. For the characterization of starch, cvs. Chinese Spring or Norin 61 having SGP-A1, -B1 and -D1 were used as controls.

Starch granule preparation

Starch granules were prepared according to Echt and Schwartz (1981). Flours were homogenized in a protein extraction buffer (55 m*M* TRIS/HCl, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol and 10% glycerol). The starch pellet was suspended three times in extraction buffer; the starch was then washed twice with distilled water and twice with acetone and air-dried. Starches from the distal half of F_2 seeds were prepared according to Sulaiman and Morrison (1990) using 80% CsCl.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE of starch granule-bound proteins (SGPs) was performed as described by Yamamori and Endo (1996). Five milligrams of starch prepared from ten mature grains or starch from a distal half of F_2 grain was gelatinized in 70 µl of the protein extraction buffer by boiling water. After centrifugation, the supernatant (15 µl) was subjected to electrophoresis. Proteins were visualized by silver staining (Silver stain kit, Wako Pure Chemical Industries, Japan).

Light and scanning electron microscopy (SEM)

Starch granules suspended in 50% glycerol were observed by light and polarizing microscopes. For SEM (JSM-5800LV, Jeol, Japan), dried starch granules were fixed onto stubs with double-sided tape and coated with gold.

Starch content, blue value (BV), λmax, and amylose measurement

Total starch content (%, dry wt. basis) was measured using a total starch assay kit (Megazyme Int Ireland, Ireland). About 3 g of matured seeds from two SGP-1 null lines, cvs. Chinese Spring and Norin 61, was milled and used.

The absorbance at 680 nm (BV) and maximum absorbance (λmax) of the iodine-starch complex were determined according to Konishi et al. (1985). Ten milligrams of starch was gelatinized in 1 ml of 1 *N* NaOH for 1 h at 40°C and then neutralized by 9 ml of 0.11 *M* acetic acid. One milligram of gelatinized and neutralized starch was mixed with 2 mg of I_2 and 20 mg of KI, and distilled water was added to make a 25-ml solution. Absorption curves of the starch-iodine complexes were measured at 500–700 nm.

Colorimetric measurement of the iodine binding capacity was according to the method of Kuroda et al. (1989) using an auto-analyzer (Bran Lubbe Co). Thirty-five milligrams of starch was gelatinized in 5 ml of 0.75 *M* NaOH and 25% ethanol overnight and then neutralized by acetic acid. Absorbance of the starch iodine complex was measured at 600 nm by colorimetry. For control, two wheat starches were used. A wheat starch purchased from Wako Pure Chemicals (Japan) contained 31.2% amylose, which was determined by the auto-analyzer using potato amylose and amylopectin as control, and waxy wheat starch contained 0.6% amylose.

Amperometric titration (Fukuba and Kainuma 1977) was carried out using defatted starch with an iodine amperometric titration device (Model 3-05, Mitamura Riken Kogyo, Japan). Amylose content of the starch was determined based on the assumption that 20 mg of iodine can bind to 100 mg of pure wheat amylose. The starch concentration of the solution used was determined by the phenol-sulphuric acid method (Dubois et al. 1956) with glucose as a standard.

Concanavalin A (Con A) method was according to the manufacture's protocol using an amylose/amylopectin assay kit (Megazyme Int Ireland, Ireland).

High-performance size-exclusion chromatography (HPSEC) and high-performance anion exchange chromatography (HPAEC)

HPSEC was according to the method of Hayakawa et al. (1997). Starch was first treated with methanol for 2 h at room temperature, then with ethanol, and 20-mg aliquots digested with isoamylase from *Pseudomonas amyloderamosa* (600 U, Sigma Chemical Co, Mo.). The HPSEC system included a differential refractometer (Shodex RI se-61, Showa-Denko, Japan) and two columns (G3000PW-TSK, Tosoh, Japan). As molecular-weight standards, a solution of the TSK standard, poly- (ethylene oxide) (Showa-Denko), of different molecular weights ($2.1–16.0\times10^4$) was used.

HPAEC (DX-300) with a pulsed amperometric detector (HPAEC-PAD, Dionex, Calif. USA) was used according to the method of Nagamine and Komae (1996). Five milligrams of starch was gelatinized in 3 ml of pure water for 60 min at 100°C. For debranching, the gelatinized starch solution (930 µl) was treated in a reaction solution [10 µl (700 U) isoamylase (Hayashibara Biochemical Lab, Japan), 50 µl 0.5 *M* acetate buffer (pH 4.5), and 10 µl 2% sodium azide) for 24 h at 40°C. After 60 µl of a 6% ammonium solution was added to the debranched starch solution, the isoamylolyzate was reduced by adding 300 µl of 1% sodium borohydride. After standing overnight at room temperature, the products were lyophilized, dissolved in 0.1 *M* NaOH and used for HPAEC.

X-ray diffraction

X-ray diffraction was done as described in Fujita et al. (1998) using a Shimadzu X-ray diffractometer XD-D1W equipped with the computer application software DP-D1 (Shimadzu Co, Japan). The starch, saturated with water, was layered on a sample holder, then scanned from 4° to 30°. The relative crystallinity (degree of crystallization) of the starch was calculated as follows: crystallinity (%)=(crystallized area on diffractogram)/(amorphous plus crystallized area).

Differential scanning calorimetry (DSC)

DSC measurement was according to the method of Kohyama and Nishinari (1991) using a differential scanning calorimeter SSC 5300 with a DSC 120U module (Seiko Instruments, Japan). The instrument was calibrated with indium. Five milligrams of dried starch in 45 µl water (10% starch solution) was weighed into a silver pan and sealed. Scans were run at 1°C/min from 40° to 120°C. The transition of onset (*T*o), peak (*T*p) and completion (*T*c) temperatures, and gelatinization enthalpy (ΔH) were measured for the major peak around 55°C which derives from amylopectin gelatinization, and *T*p(AM) was recorded for the second major peak around 90°C, which derives from the amylose-lipid complex.

Results

SGP-2 and -3 decreased in the SGP-1 null wheat

SDS-PAGE analysis of 968 F_2 seeds obtained from the cross (Kanto 79/Turkey 116)F_2//Chousen 30 or Chousen 57 revealed that 4 seeds yielded no SGP-1. F_2 seeds can be classified into eight categories based on *Sgp-1* alleles or the presence or absence of SGP-A1, -B1, and -D1. Since the three genes *Sgp-A1*, *-B1* and *-D1* are situated on different chromosomes, the expected ratio for the eight categories is 27:9:9:9:3:3:3:1 (Table 1). However, the observed number did not fit the expected ratio ($\chi2=14.26$, $P<0.05$). The deviation was mainly caused by fewer than expected F_2 progeny carrying no SGP-1 (*Sgp-A1b*, *-B1b*, *-D1b*). Seed fertility of the F_3 plants (SGP-1 null) was 94% (cv. Chinese Spring 97%), which shows that the fertility of the SGP-1 null wheat was normal and, consequently, that the zygote of the SGP-1 null will develop normally. Therefore, the significantly fewer F_2 progeny with no SGP-1 can not be explained by its lethality, and the reason for fewer F_2 is unknown. The SGP-1 null wheat used in this study was obtained from (Kanto 79/Turkey 116)F_2//Chousen 57.

In addition to SGP-1, wheat starch granules retain three granule-bound proteins, i.e., the waxy protein, SGP-2, and SGP-3. In the SGP-1 null wheat (F_2–F_4 seeds), the levels of SGP-2 and -3 decreased considerably, while that of the waxy protein did not, as determined on a gel of SDS-PAGE (Fig. 1). To examine how

Table 1 Segregation of F_2 from (Kanto 79/Trukey 116)F_2//Chousen 30 or Chousen 57

Alleles of *Sgp-1*[a]			Number of F_2 seeds observed	Expected ratio	$\chi2$ value
-A1	*-B1*	*-D1*			
a	*a*	*a*	424	27	0.60
b	*a*	*a*	150	9	1.41
a	*b*	*a*	125	9	0.91
a	*a*	*b*	143	9	0.35
a	*b*	*b*	47	3	0.06
b	*a*	*b*	38	3	1.20
b	*b*	*a*	37	3	1.55
b	*b*	*b*	4	1	8.18
		Total	968	64	14.26*

* Significant difference from the expected ratio at the 5% level by the $\chi2$ test

[a] *Sgp-A1a*, *-B1a* and *-D1a* are standard alleles in cv. Chinese Spring and cv. Norin 61. *Sgp-A1b*, *-B1b* and *-D1b* are null alleles, each lacking SGP-1. *Sgp-1* alleles of Kanto 79 are *Sgp-A1a*, *Sgp-B1b*, *Sgp-D1a*; those of Turkey 116 are *Sgp-A1a*, *-B1a*, *-D1b*; those of Chousen 30 and Chousen 57 are *Sgp-A1b*, *-B1a*, *-D1a*

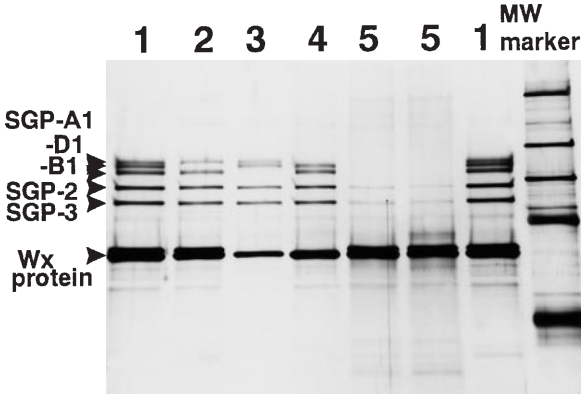


Fig. 1 Electrophoregram (SDS-PAGE) of starch granule proteins (SGPs) in the SGP-1 null wheat (F_4 seeds), its parents, and cv. Chinese Spring. Proteins were visualized by silver staining. *Lane 1* Chinese Spring, *2* Turkey 116, *3* Kanto 79, *4* Chousen 57, *5* SGP-1 null (F_4 seeds). Molecular weight markers are 205, 116, 97, 66, 45 kDa from *top* to *bottom*. Turkey 116, Kanto 79, and Chousen 57 are parents of the SGP-1 null wheat, and cv. Chinese Spring is a control

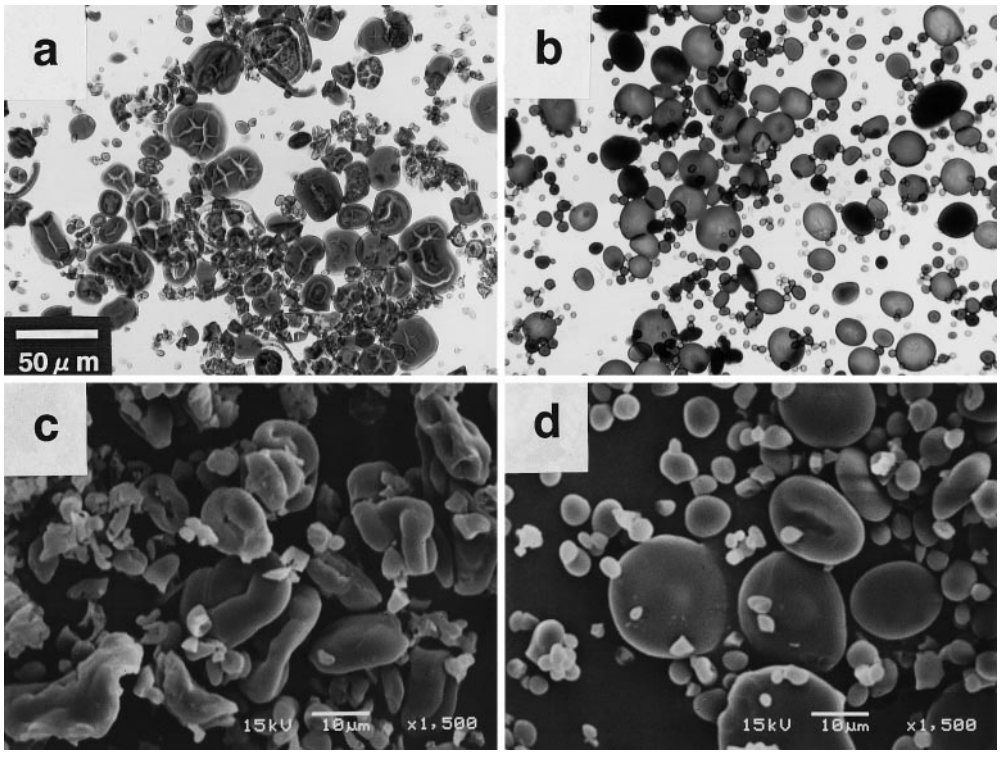


Fig. 2a–d Starch morphology. Starch granules isolated from endosperms of the SGP-1 null wheat (**a**) and cv. Chinese Spring (**b**) were stained with a KI/I_2 solution and observed under light microscopy. Starch granules from SGP-1 null (**c**) and cv. Chinese Spring (**d**) were coated with gold and observed under scanning electron microscopy

Table 2 Maximum absorbance (λmax) and absorbance at 680 nm (blue value) of the starch-iodine complex and amylose content of wheats (F_4 seeds harvested from F_3 lines)

Wheat[a]	λmax (nm)	Blue value	Amylose content (%)		
			Colorimetric	Titration	Con A
SGP-1 null (F_4)	602±6	0.485±0.023	37.3±0.8	37.3±0.8	30.8±1.3
Turkey 116	589±5	0.370±0.011	30.3±0.2	29.8±0.5	–
Kanto 79	565±0	0.307±0.004	23.9±0.5	23.5±0.1	–
Chousen 57	591±1	0.365±0.003	29.4±0.1	28.1±0.2	–
Chinese Spring	586±3	0.358±0.013	29.6±0.1	29.3±0.3	27.0±0.4
Norin 61	–[b]	–	28.2±0.1	26.6±0.2	26.6±0.2

[a] One sample was independently prepared from five or four SGP-1 null (F_3) lines, Turkey 116–Norin 61. Values in Turkey 116–Norin 61 are means±SD from 3 measurements per sample. For SGP-1 null, values in λmax and blue value are from 15 measurements (3 measurements per sample), values in colorimetric are from 10 measurements (2 measurements per sample), and values in titration and con A are from 8 measurements (2 measurements per sample)
[b] Not examined

much the levels of SGP-2 and -3 decreased in SGP-1 null wheat, we subjected 1, 1/2, 1/4, 1/8, 1/16, and 1/32 sample volumes of cv. Chinese Spring to electrophoresis, and the thickness of the SGP-2 and -3 bands detected by silver staining were compared to 1 volume from the SGP-1 null wheat. The results showed that the lack of SGP-1 lowered the levels of SGP-2 and -3 to about 1/16 of that of cv. Chinese Spring.

Deformed starch morphology and apparent high amylose content

Normal wheat starch granules consist of disk-shaped large granules and globular small ones, which are termed A and B granules, respectively. Light microscopic observation showed that in the SGP-1 null wheat, A granules were mostly deformed, and their hila appeared to be cracked. Scanning electron microscopy showed that the hila of the deformed granules were hollow (Fig. 2). Though the shape of the starch granules was abnormal in the SGP-1 null, its seed appearance was not.

Starch content was 49.4% in SGP-1 null, 60.7% in cv. Chinese Spring, and 61.4% in cv. Norin 61, which showed that the starch content of SGP-1 null seeds was about 20% lower than that of normal wheat.

To characterize the starch components, we measured the blue value (absorbance at 680 nm) and maximum absorbance (λmax) of the iodine-starch complex of the SGP-1 null wheat, its parents and cv. Chinese Spring (Table 2). The higher blue value shows that the apparent amylose content of the SGP-1 null wheat was higher

than those of the others. To confirm high amylose, we examined amylose content by three methods, i.e., colorimetric measurement, amperometric titration, and the Con A method. The amylose content of SGP-1 null starch was about 8% more than that of cv. Chinese Spring when determined by both the colorimetric measurement and amperometric titration analyses and about 4% more by the Con A analysis (Table 2). These results show an apparent high amylose content in the SGP-1 null wheat starch. Since Kanto 79 lacks the Wx-A1 and -B1 proteins responsible for amylose synthesis, λmax, blue value, and amylose content of Kanto 79 were lower than those of the other wheats (Turkey 116–Norin 61).

It is known that amylopectin structure influences apparent amylose content. For example, *amylose extender* (*ae*) starch of maize shows a high apparent amylose percentage and it contains abnormally (loosely) branched amylopectin (Boyer et al. 1976). The long external chains of loosely branched amylopectin may form a complex with more iodine than does normal amylopectin, which can cause high apparent amylose (Shannon and Garwood 1984). Thus, amylopectin structure was examined.

Altered structure of SGP-1 null starch (amylopectin) revealed by HPSEC and HPAEC with a pulsed amperometric detector (PAD)

The HPSEC of debranched starch produced three peaks or fractions. In the SGP-1 null, the fraction I (Fr. I) with the highest molecular weight increased, the ratio of Fr. III to Fr. II (Fr. III / Fr. II) was greater than in cvs. Chinese Spring and Norin 61, and a shoulder was present in Fr. III (Table 3). Compared to the controls, the reduction in Fr. II caused an increase in both Fr. I and ratio of Fr. III/Fr. II in SGP-1 null. Hayakawa et al. (1997) reported that Fr. II and Fr. III derive from amylopectin and that Fr. III contains shorter chains than Fr. II. Therefore, in the SGP-1 null starch, the ratio of shorter chains of amylopectin increased. The additional shoulder in Fr. III indicated an altered structure of amylopectin shorter chains. Though the amount of Fr. I (area %) in Table 3 differed from the amylose content in Table 2, the increased Fr. I in SGP-1 null would be responsible for apparent high amylose.

To examine the chain length distribution of amylopectin in more detail, we subjected debranched starch to HPAEC-PAD. Because the HPAEC profile of starch was reported to be the same as that from purified amylopectin, the results obtained were considered to be chain length distributions of amylopectin (Nagamine and Komae 1996). DP 6–54 chains were individually compared to those of cv. Chinese Spring. The results showed that DP 6–10 chains increased (0.3–3.2% difference), DP 11–25 decreased (0.1–1.1%), DP 29–36 increased (0.1%), and DP 42–54 decreased (<0.1%) in the SGP-1 null wheat (Fig. 3). A wavy increase-decrease pattern similar to Fig. 3 was also obtained when the SGP-1 null was compared to cv. Norin 61 and three parental wheats (Turkey 116, Kanto 79, Chousen 57). The HPAEC detected increased short chains but could not detect an increased long chain (Fr. I) assumed from HPSEC. Consequently, HPAEC-PAD analysis demonstrated that the amylopectin structure of SGP-1 null wheat had changed; the percentage of DP 6–10 chains increased at the expense of a reduction in DP 11–25.

Table 3 Three fractions (Fr.) of debranched starch analyzed by HPSEC

Wheat[a]	Fractions of debranched starch[b] (%)			Ratio of Fr. III/II[c]
	Fr. I	Fr. II	Fr. III	
SGP-1 null (F_4)	21.7±6.0	23.6±2.8	54.7±4.9	2.3
Chinese Spring	8.5±0.3	36.4±0.1	55.0±0.4	1.5
Norin 61	7.9±0.1	42.0±0.2	50.1±0.3	1.2

[a] One sample was independently prepared from three SGP-1 null (F_3) lines, Chinese Spring, and Norin 61. Values in SGP-1 show mean±SD from 8 measurements (2 measurements per sample and 4 measurements per sample), values in cvs. Chinese Spring, and Norin 61 are from 3 measurements per sample

[b] Percentages of each fraction areas to total area are shown

[c] Ratio of Fr. III/II indicates (shorter chain of amylopectin)/longer chain of amylopectin

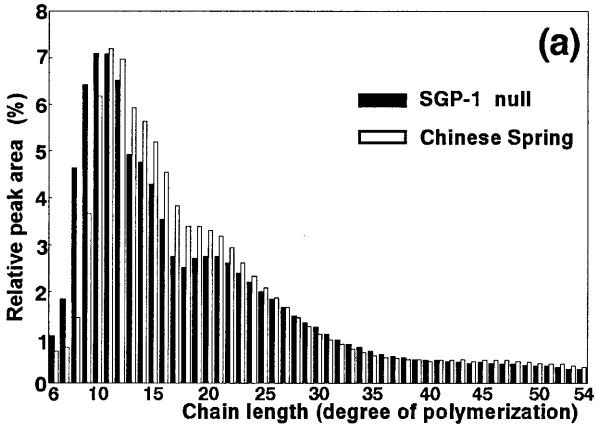
Lack of crystallinity detected by X-ray diffraction and polarizing microscope

The X-ray diffractograms of cvs. Chinese Spring and Norin 61 showed an A-type pattern which has peaks at 2θ values of 15°, 17°, 18° and 23° (Fig. 4). Their crystallinities (degree of crystallization) were 24.1% and 27.7%, respectively. However, we could not determine the type of diffraction pattern in the SGP-1 null starch because its diffractogram did not show any major peaks.

The SGP-1 null starches did not show polarized crosses while starch granules from cv. Chinese Spring did (Fig. 5). Polarized crosses in starch are generated by birefringence of crystallinity. The results from X-ray diffraction and the polarizing microscope suggest an absence of crystallinity in the SGP-1 null.

Absence of endothermal peaks in DSC thermogram

The DSC thermogram of SGP-1 null starch did not produce the sharp endothermal peak around 55°C that is caused by gelatinization of amylopectin or disruption of double-helical order (Cooke and Gidley 1992), while three parental wheats and cvs. Chinese Spring and Norin 61 had the endothermal peak (Table 4, Fig. 6). Thus, the corresponding gelatinization enthalpy (*ΔH*), onset (*T*o), peak (*T*p), and completion (*T*c) temperatures could not be determined in the SGP-1 null (Table 4). On the other hand, a very broad peak might have been present around 65°C in the SGP-1 null starch. However, its *ΔH*, *T*o, *T*p and *T*c were impossible to determine because of the broadness.



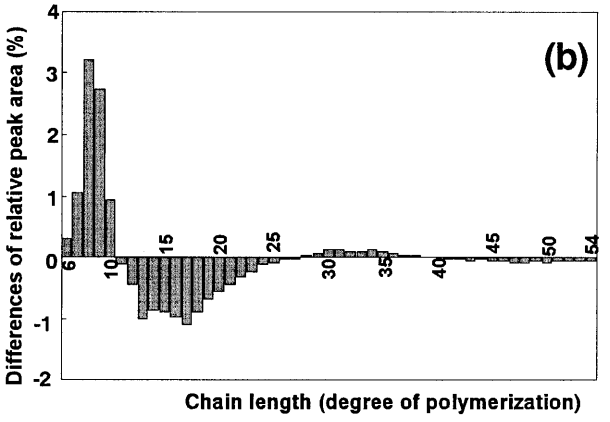


Fig. 3a, b Chain-length distributions of starch from the SGP-1 null and cv. Chinese Spring analyzed by high-performance anion exchange chromatography. Endosperm starches debranched by isoamylase were subjected to HPAEC-PAD. **a** Relative peak area of each DP 6–54 chain to total area (%). **b** Differences in relative peak area between SGP-1 null and a control, cv. Chinese Spring. The value of SGP-1 null minus cv. Chinese Spring is shown for each chain of DP 6–54

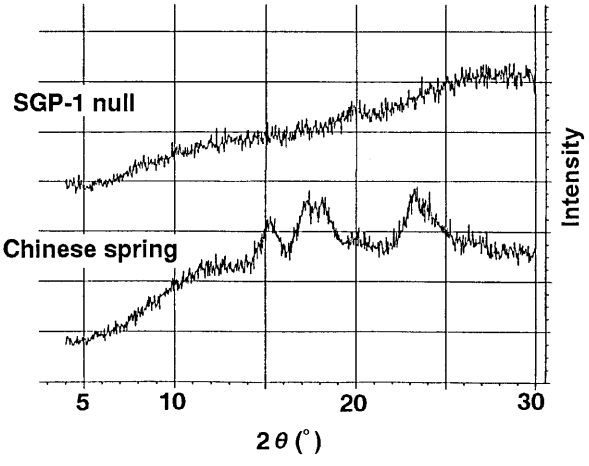


Fig. 4 X-ray diffractograms of SGP-1 null and cv. Chinese Spring. Water-saturated starches were scanned from 4° to 30°

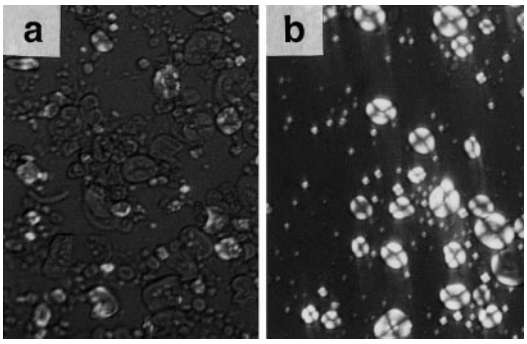


Fig. 5 Starch photographed with polarized light. Starches from the SGP-1 null starch (**a**) and cv. Chinese Spring (**b**) were suspended in 50% glycerol solution and observed by polarizing microscope

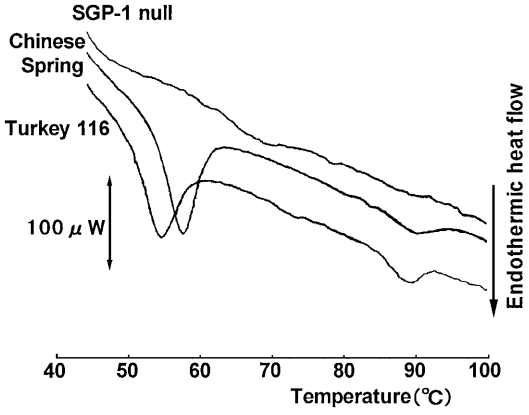


Fig. 6 DSC thermograms of SGP-1 null and cvs. Chinese Spring and Turkey 116

Table 4 DSC analysis of starch gelatinization[a]

Wheat[b]	*To*(°C)	*Tp*(°C)	*Tc*(°C)	*ΔH*(J/g)	*Tp*(AM)(°C)
SGP-1 null (F_4)	ND[c]	ND	ND	ND	ND
Turkey 116	50.5	54.8	59.2	7.9	89.9
Kanto 79	54.1	57.3	61.1	10.3	90.8
Chousen 57	53.2	56.7	61.1	9.6	89.3
Chinese Spring	54.5	57.7	61.3	8.8	89.9
Norin 61	54.4	58.0	61.4	8.8	91.8

[a] The parameters (*To*–*Tp*(AM)) were determined from DSC thermograms (Fig. 6)
[b] One sample was independently prepared from three SGP-1 null (F_3) lines, Turkey 116–Norin 61. Values are averages from 2 measurements per sample for Turkey 116 to cv. Norin 61, and 6 measurements (2 measurements per sample) for the SGP-1 null
[c] These values could not be determined because no major peaks were detected

A second smaller endothermal peak around 90°C, which was attributable to dissociation of the lipid-amylose complex, was not clearly visible in the SGP-1 null (Fig. 6). This small peak was observable in the other five wheats. In the SGP-1 null starch, amylose was present because the waxy protein for amylose synthesis was normal on the gel of SDS-PAGE and HPSEC detected an

amylose peak (Fr. I). Therefore, the lack of the second peak does not mean an absence of amylose. At present, we can not explain why the second peak disappeared.

Discussion

The present study revealed that SGP-1 deficiency generates pleiotropic effects on wheat starch. The possibility of an SGP-1 (SSII) homolog being present in rice and maize starches was suggested by the electrophoregrams of starch granule proteins because three protein bands were detected above the waxy proteins in rice and maize (Yamamori and Endo 1996; Hylton et al. 1996).

The mechanism for decreased level of SGP-2 and -3 in the SGP-1 null wheat (Fig. 1) should be investigated in future studies. Though we presently do not have any data to explain the mechanism, one possible hypothesis holds that an abnormal starch crystalline order or amylopectin structure in the SGP-1 null may decrease the efficiency of trapping SGP-2 and -3 during the growth of starch granules. Starch crystallization may be necessary for the entrapment of SGP-2 and -3. Another possibility is that a lack of SGP-1 may decrease both SGP-2 and -3 in the soluble fractions or amount of mRNAs coding for the two proteins. In this case, total activities of starch synthase and branching enzyme would be altered.

Matheson and Welsh (1988) suggested that the differences in amylose content of high-amylose pea as measured by iodine amperometric titration and the Con A method could be explained by intermediate material or material with an atypical structure having (1, 6) branches (amylopectin) present in high-amylose pea. Con A can precipitate an atypical amylopectin which can bind more iodine than normal amylopectin. Similar differences detected in the SGP-1 null starch (6.6% difference between Con A method and amperometric titration) (Table 2) suggest the presence of atypical amylopectin. The increased Fr. I detected in HPSEC (Table 3) might be indicative of longer chains of amylopectin, resulting in high levels of apparent amylose. The increased short chains (DP 6–10) detected in HPAEC (Fig. 3) could not be responsible for the apparent high amylose because these chains bind comparatively less iodine. Any increase in the percentage of long chains responsible for apparent high amylose was not detected by HPAEC (Fig. 3). The presence of intermediate material which binds more iodine should be studied further in SGP-1 null starch. For this purpose, amylose and amylopectin will be separated and analyzed.

Sasaki and Matsuki (1998) reported a narrow diversity in the distribution profiles of amylopectin side chains among six wheat cultivars: 1.7% range for DP 6–12 chains, 1.1% difference in DP 13–34 chains, and 2.2% in DP 35-. This is consistent with our results among five cultivars of Turkey 116–Norin 61 (data not shown). In addition, analyzing debranched amylopectin from ten wheats using HPSEC, Kobayashi et al. (1986) did not find any detectable differences in their structure. These researchers reported little if any variation in the amylopectin structure of wheat. Consequently, we conclude that the SGP-1 null starch has a significantly altered structure of amylopectin. According to Hanashiro et al. (1996), DP 6–12 and DP 13–24 chains probably correspond to A and B_1 chains, respectively. Thus, the result that the level of DP 11–25 chains decreased and that of DP 6–11 increased in the SGP-1 null (Fig. 3) suggests that SGP-1 synthesizes B_1 chains using A_1 chains.

Amylopectin is considered to be responsible for the crystalline structure of starch (Manners 1989). This crystalline structure consists of an ordered arrangement of double helixes formed by short chains of amylopectin. The crystalline order in the starch can diffract X-rays. However, the SGP-1 null starch in this study did not diffract X-rays (Fig. 4), due to the altered amylopectin structure. The absence of both the major peak in the DSC thermogram and polarized crosses also shows disrupted starch crystallinity.

Amino acid sequences from cDNAs of SGP-1 (Li et al. 1999) showed that SGP-1 proteins are members of the starch synthase II (SS II) group. A lesion in SSII has been reported in pea, *Chlamydomonas reinhardtii*, and potato. A pea of the *rugosus* (*rug*) *5* genotype lacks starch synthase II (Craig et al. 1998), *Chlamydomonas reinhardtii* of the *sta3–1* genotype is missing soluble starch synthase II (Fontaine et al. 1993; Buléon et al. 1997) and transgenic potato with reduced SSII was produced by antisense inhibition (Edwards et al. 1995, 1999; Lloyd et al. 1999). These mutant starches show characteristics similar but not identical to SGP-1 null starch. Like the SGP-1 null seeds, starch content of *rug5* seeds (30–40%) decreased compared to that of wild type. However, SSII reduced potato produced starch with normal level in developing tubers (Edwards et al. 1995, 1999). Mutant *rug5* pea and *sta3–1 Chlamydomonas* have apparent high amylose or high blue values, while high amylose content was not detected in transgenic potato (Edwards et al. 1995). The *rug 5* pea generated an abnormal starch shape (Craig et al. 1998), and the potato transformant has more apparent cracks on starch hilum (Edwards et al. 1999). As in the SGP-1 null, HPAEC, gel electrophoresis and gel-permeation chromatography detected increased levels of short chains of amylopectin in 3 plants. X-ray diffraction and DSC analyses were carried out on *sta3–1 Chlamydomonas:* the *sta3–1* mutant showed a B-type X-ray diffractogram with small peaks and low crystallinity; 5% crystallinity in the mutant and 30% in the wild type (Buléon et al. 1997). It showed a broad endothermal peak of amylopectin gelatinization in the DSC thermogram, which resulted in low gelatinization enthalpy (ΔH). These two points are similar to our results with the SGP-1 null. Consequently, this defect in SSII that we observed in 4 plants influences amylopectin structure; in particular it increases the levels of short chains at the expense of the intermediate chains, which should cause pleiotropic effects on starch properties, e.g., morphology, apparent amylose content, gelatinization.

The possibility has to be considered that abnormal wheat starch is not due solely to a SGP-1 deficiency but may also be caused by a decrease in SGP-2 and –3 within the starch granule, or by a combination of SGP-1, -2 and -3 reductions. As described above, the amounts of SGP-2 and -3 in the soluble fraction or the activities of enzymes related to starch synthesis (e.g., ADP-glucose pyrophosphorylase, starch synthase, branching enzyme) in the soluble fraction has to be examined. Apparent high amylose (high blue value), abnormal starch shape, and anomalous amylopectin structure (increased long chains) result from a lack of BEIIb (SGP-2 homolog) in maize of the *amylose-extender* genotype (Shannon and Garwood 1984; Boyer and Preiss 1978). Because there are no maize or rice mutants for SSI (SGP-3 homolog), it is not yet known how the lack of SSI affects starch, but it is probably that a decrease in SSI will cause abnormal starch. Thus, if the levels of SGP-2 and -3 in the soluble fraction decrease in SGP-1 null wheat, abnormalities may be caused by combination of reductions in SGP-1, -2, and -3. Even if SGP-2 and -3 in the soluble fraction are at normal levels, the decrease in SGP-2 and -3 bound to starch may influence starch structure. Namely, SGP-2 and -3 within starch may be necessary for normal starch. In the above suggested mechanisms, it is clear that novel starch is primarily caused by genetic elimination of SGP-1.

This is the first report of high amylose mutants in wheat. We have shown that the lack of SGP-1 enhances apparent amylose content and generates altered amylopectin, indicating that the SGP-1 null mutant can expand starch variation in wheat.

References

Ainsworth C, Clark J, Balsdon J (1993) Expression, organisation and structure of the genes encoding the waxy protein (granule-bound starch synthase) in wheat. Plant Mol Biol 22:67–82

Boyer CD, Preiss J (1978) Multiple forms of starch branching enzyme of maize: evidence for independent genetic control. Biochem Biophys Res Commun 80:169–175

Boyer CD, Garwood DL, Shannon JC (1976) The interaction of the amylose-extender and waxy mutants of maize (*Zea mays* L.). Stärke 28:405–410

Buléon A, Gallant DJ, Bouchet B, Mouille G, D'Hulst C, Kossmann J, Ball S (1997) Starch from A to C: *Chlamydomonas reinhardtii* as a model microbial system to investigate the biosynthesis of the plant amylopectin crystal. Plant Physiol 115: 949–957

Cooke D, Gidley MJ (1992) Loss of crystalline and molecular order during starch gelatinisation: origin of the enthalpic transition. Carbohydr Res 227:103–112

Craig J, Lloyd JR, Tomlinson K, Barber L, Edwards A, Wang TL, Martin C, Hedley CL, Smith AM (1998) Mutations in the gene encoding starch synthase II profoundly alter amylopectin structure in pea embryos. Plant Cell 10:413–426

Denyer K, Sidebottom C, Hylton CM, Smith AM (1993) Soluble isoforms of starch synthase and starch-branching enzyme also occur within starch granules in developing pea embryos. Plant J 4:191–198

Denyer K, Hylton CM, Jenner CF, Smith AM (1995) Identification of multiple isoforms of soluble and granule-bound starch synthase in developing wheat endosperm. Planta 196:256–265

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Echt CS, Schwartz D (1981) Evidence for the inclusion of controlling elements within the structural gene at the *waxy* locus in maize. Genetics 99:275–284

Edwards A, Marshall J, Sidebottom C, Visser RGF, Smith AM, Martin C (1995) Biochemical and molecular characterization of a novel starch synthase from potato tubers. Plant J 8:283–294

Edwards A, Fulton DC, Hylton CM, Jobling SA, Gidley M, Rossner U, Martin C, Smith AM (1999) A combined reduction in activity of starch synthases II and III of potato has novel effects on the starch of tubers. Plant J 17:251–261

Fisher DK, Boyer CD, Hannah LC (1993) Starch branching enzyme II from maize endosperm. Plant Physiol 102:1045–1046

Fontaine T, D'Hulst C, Maddelein M-L, Routier F, Pépin TM, Decq A, Wieruszeski J-M, Delrue B, Van den Koornhuyse N, Bossu J-P, Fournet B, Ball S (1993) Toward an understanding of the biogenesis of the starch granule: evidence that *Chlamydomonas* soluble starch synthase II controls the synthesis of intermediate size glucans of amylopectin. J Biol Chem 268: 16223–16230

Fujita S, Yamamoto H, Sugimoto Y, Morita N, Yamamori M (1998) Thermal and crystalline properties of waxy wheat (*Triticum aestivum* L) starch. J Cereal Sci 27:1–5

Fukuba H, Kainuma K (1977) Quantification of amylose and amylopectin. In: Nakamura M, Suzuki S (eds) Starch science handbook. Asakura Shoten, Tokyo, pp 174–179

Hanashiro I, Abe J, Hizukuri S (1996) A periodic distribution of the chain length of amylopectin as revealed by high-performance anion-exchange chromatography. Carbohydr Res 283: 151–159

Hayakawa K, Tanaka K, Nakamura T, Endo S, Hoshino T (1997) Quality characteristics of waxy hexaploid wheat (*Triticum aestivum* L): properties of starch gelatinization and retrogradation. Cereal Chem 74:576–580

Hylton CM, Denyer K, Keeling PL, Chang M-T, Smith AM (1996) The effect of waxy mutations on the granule-bound starch synthases of barley and maize endosperms. Planta 198: 230–237

Knight ME, Harn C, Lilley CER, Guan H, Singletary GW, Mu-Forster C, Wasserman BP, Keeling PL (1998) Molecular cloning of starch synthase I from maize (W64) endosperm and expression in *Escherichia coli*. Plant J 14:613–622

Kobayashi S, Schwartz SJ, Lineback DR (1986) Comparison of the structures of amylopectins from different wheat varieties. Cereal Chem 63:71–74

Kohyama K, Nishinari K (1991) Effect of soluble sugars on gelatinization and retrogradation of sweet potato starch. J Agric Food Chem 39:1406–1410

Konishi Y, Nojima H, Okuno K, Asaoka M, Fuwa H (1985) Characterization of starch granules from waxy, nonwaxy, and hybrid seeds of *Amaranthus hypochondriacus* L. Agric Biol Chem 49:1965–1971

Kuroda A, Oda S, Miyagawa S, Seko H (1989) A method of measuring amylose content and its variation in Japanese wheat cultivars and Kanto breeding lines. Jpn J Breed 39 [Suppl 2]: 142–143

Li Z, Chu X, Mouille G, Yan L, Kosar-Hashemi B, Hey S, Napier J, Shewry P, Clarke B, Appels R, Morell MK, Rahman S (1999) The localization and expression of the class II starch synthases of wheat. Plant Physiol 120:1147–1156

Lloyd JR, Landschütze V, Kossmann J (1999) Simultaneous antisense inhibition of two starch-synthase isoforms in potato tubers leads to accumulation of grossly modified amylopectin. Biochem J 338:515–521

Manners DJ (1989) Recent developments in our understanding of amylopectin structure. Carbohydr Polym 11:87–112

Matheson NK, Welsh A (1988) Estimation and fractionation of the essentially unbranched (amylose) and branched (amylopectin) components of starches with concanavalin A. Carbohydr Res 180:301–313

Mu C, Harn C, Ko Y-T, Singletary GW, Keeling PL, Wasserman BP (1994) Association of a 76 kDa polypeptide with soluble starch synthase I activity in maize (cv B73) endosperm. Plant J 6:151–159

Nagamine T, Komae K (1996) Improvement of a method for chain-length distribution analysis of wheat amylopectin. J Chromatogr 732:255–259

Nair RB, Båga M, Scoles GJ, Kartha KK, Chibbar RN (1997) Isolation, characterization and expression analysis of a starch branching enzyme II cDNA from wheat. Plant Sci 122:153–163

Rahman S, Kosar-Hashemi B, Samuel MS, Hill A, Abbott DC, Skerritt JH, Preiss J, Appels R, Morell MK (1995) The major proteins of wheat endosperm starch granules. Aust J Plant Physiol 22:793–803

Sasaki T, Matsuki J (1998) Effect of wheat starch structure on swelling power. Cereal Chem 75:525–529

Shannon JC, Garwood DL (1984) Genetics and physiology of starch development. In: Whistler RL, BeMiller JN, Paschall EF (eds) Starch, chemistry and technology, 2nd edn. Academic Press, New York, pp 25–86

Sulaiman BD, Morrison WR (1990) Proteins associated with the surface of wheat starch granules purified by centrifuging through caesium chloride. J Cereal Sci 12:53–61

Takaoka M, Watanabe S, Sassa H, Yamamori M, Nakamura T, Sasakuma T, Hirano H (1997) Structural characterization of high molecular weight starch granule-bound proteins in wheat (*Triticum aestivum* L). J Agric Food Chem 45:2929–2934

Yamamori M, Endo TR (1996) Variation of starch granule proteins and chromosome mapping of their coding genes in common wheat. Theor Appl Genet 93:275–281

Yu Y, Mu HH, Mu-Forster C, Wasserman BP (1998) Polypeptides in the maize amyloplast stroma. Plant Physiol 116:1451–1460