Theor Appl Genet (2007) 115:1053–1065
DOI 10.1007/s00122-007-0631-0

ORIGINAL PAPER

Effects of starch synthase IIa gene dosage on grain, protein and starch in endosperm of wheat

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Received: 9 January 2007 / Accepted: 6 August 2007 / Published online: 25 August 2007

Abstract Starch synthases (SS) are responsible for elongating the α-1,4 glucan chains of starch. A doubled haploid population was generated by crossing a line of wheat, which lacks functional *ssIIa* genes on each genome (abd), and an Australian wheat cultivar, Sunco, with wild type *ssIIa* alleles on each genome (ABD). Evidence has been presented previously indicating that the SGP-1 (starch granule protein-1) proteins present in the starch granule in wheat are products of the *ssIIa* genes. Analysis of 100 progeny lines demonstrated co-segregation of the *ssIIa* alleles from the three genomes with the SGP-1 proteins, providing further evidence that the SGP-1 proteins are the products of the *ssIIa* genes. From the progeny lines, 40 doubled haploid lines representing the eight possible genotypes for SSIIa (ABD, aBD, AbD, ABd, abD, aBd, Abd, abd) were characterized for their grain weight, protein content, total starch content and starch properties. For some properties (chain length distribution, pasting properties, swelling power, and gelatinization properties), a progressive change was observed across the four classes of genotypes (wild type, single nulls, double nulls and triple nulls). However, for other grain properties (seed weight and protein content) and starch properties (total starch content, granule morphology and crystallinity, granule size distribution, amylose content, amylose–lipid dissociation properties), a statistically significant change only occurred for the triple nulls, indicating that all three genes had to be missing or inactive for a change to occur. These results illustrate the importance of SSIIa in controlling grain and starch properties and the importance of amylopectin fine structure in controlling starch granule properties in wheat.

Communicated by B. Friebe.

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Introduction

The starch properties of naturally occurring hexaploid wheat grains do not show as wide a degree of variation compared to diploid species and this has limited the range of end uses of wheat starch. Greater genetic diversity would allow wheat to compete in new markets, including specialty foods and industrial products, currently dominated by other sources of starch. Recent advances in the study of wheat starch biosynthesis have been possible with the discovery of wheats with null alleles or mutations for the different starch biosynthesis enzymes (Nakamura et al. 1995; Zhao et al. 1998; Yamamori et al. 2000; Yamanori and Uuynh

2000; Regina et al. 2004, 2006). The generation of modified starches through genetic engineering has also been reported (Regina et al. 2006). This has extended the variation in wheat starch properties and expanded the possibilities for novel end uses.

Starch consists of two classes of polymers, amylose and amylopectin. Amylose is a mostly linear α(1,4)-linked glucose polymer with a degree of polymerization (DP) of 1,000–5,000 glucose units. Amylopectin is a much larger glucose polymer (DP 10^5–10^6) in which α(1,4)-linked glucose polymers are connected by 5–6% α(1,6)-linkages. Normal wheat starch typically contains 20–30% amylose and 70–80% amylopectin. Starch is synthesized by a complex pathway, which involves a number of enzymes including ADP-glucose pyrophosphorylase, starch synthases, branching enzymes and de-branching enzymes (See Kossmann and LIoyd 2000; Rahman et al. 2000; Smith 2001; Morell et al. 2001, 2003b, 2006; Ball and Morell 2003; James et al. 2003; Tetlow et al. 2004 for reviews). Multiple isoforms of these enzymes are found in the endosperm of higher plants and identifying the roles of each of these isoforms remains a focus of research.

Starch synthases extend α-1,4 glucan polymers through the transfer of the glucosyl moiety of ADP glucose to the non-reducing end of a pre-existing α-1,4 glucan. Five classes of starch synthases have been identified in the cereal endosperm, granule bound starch synthase (GBSS), starch synthase I (SSI), SSII, SSIII and SSIV (Li et al. 2003). The SSII class of genes contains three members in rice (Hirose et al. 2004), and the predominant SSII form in wheat starch granule is classified as an SSIIa type (Li et al. 2003). The SSIIa gene in wheat encodes the SGP-1 protein (starch granule bound protein -1) present in the wheat starch granule (Li et al. 1999).

Yamamori et al. (2000) reported a starch granule protein-1 (SGP-1) null wheat line missing activity of all three starch synthase IIa (*ssIIa*) genes (from the three wheat genomes A, B, D). The lack of starch synthase IIa activity in the SGP-1 null lines is caused by an insertion in the B genome and deletions for both A and D genomes in exon sequences (Shimbata et al. 2005). Starch from this wheat line was found to have increased amylose content (31–37%), increased proportion of amylopectin chains with DP 6–10 and a decreased proportion of amylopectin chains with DP 11–25 as well as abnormal morphology and crystallinity of granules.

An *ssIIa* mutation in barley resulted in decreased amylopectin synthesis, raising the amylose content to around 70% in which the starch granules also had abnormal morphology and crystallinity, along with an increase in the proportion of amylopectin short chains with DP 6–11 and reduced gelatinization temperature (Morell et al. 2003a). This contrasts with high amylose (>70%) phenotypes resulting from mutations in BEII, where there is a decrease in the proportion of short amylopectin chains in wheat (Regina et al. 2006) and an increase in gelatinization temperature in maize (Fuwa et al. 1999). Starches with elevated amylose contents are of interest because they provide resistant starch with positive impacts on bowel health (Regina et al. 2006; Bird et al. 2004; Topping and Clifton 2001), have glycemic index lowering capacity and manage type II diabetes with potential health benefits (Regina et al. 2006; Topping et al. 2003).

So far, the effect of gene dosage on starch properties has only been examined for granule bound starch synthase (GBSS) in wheat (Yamamori and Quynh 2000). A complete series of the eight possible homozygous genotypes were generated from a cross between a wild type line and a GBSS triple null. Amylose content was found to be around 28% for wild type (ABD), 26% for single nulls, 22% for double nulls (Abd being the lowest) and close to 0% for triple null (abd) or waxy wheat (Kim et al. 2003; Miura et al. 1999, 2002). RVA peak viscosity and breakdown were negatively correlated with amylose content and increased with increasing number of null alleles for GBSS. RVA holding strength, final viscosity and setback were positively correlated with amylose content and decreased with increasing number of null alleles for GBSS (Yamamori and Quynh 2000). Other similar experiments also found that there was no difference in granule morphology (Kim et al. 2003) or chain length distribution (Miura et al. 2002) between the eight genotypes for GBSS. Earlier work comparing waxy and non-waxy wheat found that waxy wheat had higher differential scanning calorimetry (DSC) peak gelatinization temperature and enthalpy (Yasui et al. 1996).

In this paper, we describe the characterization of grain weight, protein content, total starch content and starch properties from five wheat lines of eight different null allele combinations for *ssIIa* (ABD, aBD, AbD, ABd, abD, aBd, Abd, abd). The importance of *ssIIa* gene dosage in controlling grain weight, grain composition and starch properties has been investigated and two classes of traits identified: those that show a statistically significant gene dosage effect as the number of active genes changes, and a second class that only show a statistically significant phenotype when all SSIIa activity is removed in the triple null.

Materials and methods

Plant materials

Wheat plants, SGP-1 null (Yamamori et al. 2000) and an Australian wheat cultivar, Sunco, were grown at the CSIRO Plant Industry, Canberra, in a glasshouse, with natural light and at temperatures of 18°C (night) and 24°C (day).

A doubled haploid wheat population (100 lines) was produced by crossing between SGP-1 null wheat (abd for *ssIIa*) and Sunco (ABD for SSIIa). Chinese Spring (CS) nullisomic/tetrasomic lines for homologous group seven chromosomes, N7AT7D, N7BT7D and N7DT7B (Sears and Miller 1985) were kindly supplied by Dr E. Lagudah (CSIRO Plant Industry, Canberra, Australia).

Genotyping of a doubled haploid wheat population by SDS-PAGE gels

SDS-PAGE gels were employed for the analysis of starch granule proteins in mature seeds (from 100 doubled haploid lines) for each of the three proteins encoded by the A, B and D genomes of SGP-1 as described (Yamamori et al. 2000).

Genotyping of a doubled haploid wheat population by PCR amplification of the genome specific DNA fragments for the three genes of *ssIIa*

Young wheat leaves were collected, frozen in liquid nitrogen and stored at −80°C. Genomic DNA was purified with Fast DNA Kit (BIO101 system, Q-BIO gene).

Based on the information for the insertion or deletions for *ssIIa* genes as published by Shimbata et al. (2005), three primers were selected to give best discrimination of SGP-1 null mutation and Sunco wheat. Primers JTSS2AF (5′ TGCGTTTACCCCACAGAG CACA 3′ locating between 91 and 113 bp of AB201445, NCBI) and JTSS2AR (5′ TGCCAAAGGTCCGGAATCATGG 3′ locating between 1225 and 1246 bp of AB201445, NCBI) were used for A genome; primers JTSS2BF (5′ GCGGACCAGGTT GTCGTC 3′ locating between 5978 and 5995 bp of AB201446, NCBI) and JTSS2BR (5′ CTGGCTCACGAT CCAGGGCATC 3′ locating between 6313 and 6335 bp of AB201446, NCBI) for the B genome; and primers JTSS2DF (5′ GTACCAAGG TATGGGGACTATGAA 3′ locating between 2369 and 2392 bp of AB201447, NCBI) and JTSS2D4R (5′ GTTGGAGAGATACCTCAACAGC 3′ locating between 2774 and 2796 bp of AB201447, NCBI) were used for the D genome of wheat.

For each PCR reaction (20 µl), 100 ng genomic DNA, 1.5 mM $MgCl_2$, 0.125 mM each dNTP, 10 pmol primers, 0.5 M glycine betaine, 1 µl DMSO and 1.5–3.5 U of Hotstar Taq polymerase (QIAGEN) were used. PCR reaction was conducted using a HYBAID PCR Express (Intergrated Sciences) with 1 cycle of 95°C for 5 min, 35 cycles of 94°C for 45 s, 52°C (A genome) or 60°C (for B and D genome) for 30 s, and 72°C for 2 min 30 s, 1 cycle of 72°C for 10 min and 1 cycle of 25°C for 1 min. The PCR fragments were separated on 1 or 2% agarose gels and visualized with gel documentary (UVitec) after ethidium staining.

The DNA markers developed were used for the analysis of the 100 doubled haploid lines between SGP-1 null wheat and Sunco.

Seed weight, protein content and total starch content

Seed weight for each line was determined as the total weight of 100 seeds. Protein content was measured with nuclear magnetic resonance (NMR; Oxford 4000 NMR Magnet, Oxford Analytical Instruments Limited). Total starch content was assayed using an AACC method 76.13.

Starch extraction

Starch was extracted from 40 doubled haploid lines representing all the eight possible genotypes for SSIIa (ABD, aBD, AbD, ABd, abD, aBd, Abd, abd). Grain (20 g) was milled in a Quadramat Jnr. mill (Brabender Quadramat Jnr. Mill, Cyrulla's Instruments, Sydney, NSW Australia) after conditioning the grains to a moisture content of 14%. Starch was isolated from the five triple null samples (abd) by a protease extraction method (Morrison et al. 1984) followed by water washing and removal of the tailings. Starch was isolated from the remaining 35 samples by first making a dough and separating starch from gluten using a Glutomatic 2200 (Perten Instruments, Huddinge, Sweden), followed by extensive water washing and removal of the tailings. The difference in the methods arose because the starch from the triple null samples could not be isolated with sufficient purity by the gluten washing method. Granule size distribution was measured in the starch slurries before freeze drying (Dynavac Freeze Drier, Model FD2). The remaining analyses were performed on the freeze-dried starches.

Amylose content

Amylose content was measured using a small-scale (2 mg starch) iodine adsorption method (Morrison and Laignelet 1983) with some modifications. Approximately 2 mg of starch was weighed (accurate to 0.1 mg) into a 2 ml screw-capped tube. For defatting, 1 ml of 85% (v / v) methanol was added and incubated at 65°C for 1 h with occasional vortexing. After centrifugation at 13,000*g* for 5 min, the supernatant was removed. The defatting step was then repeated. The starch was dried at 37°C overnight, then dissolved in urea dimethyl sulphoxide (UDMSO) solution (nine parts DMSO and one part of 6 M urea) at a ratio of 1 ml of UDMSO per 2 mg of starch. The dissolution was carried out by incubating at 85°C for 2 h with intermittent vortexing. A 50 µl aliquot of the starch-UDMSO solution was treated with 20 µl of I_2–KI reagent (2 mg iodine, 20 mg potassium iodide per ml of water) and made up to 1 ml

volume with water. The optical density was read at 620 nm on 3 × 200 µl subsamples per replicate analysis and averaged. Standard samples containing amylose ranging from 0 to 100% were used to generate a standard curve. The absorbance of the test samples was converted to percentage amylose using a regression equation derived from the standard samples.

Chain length distribution

Amylopectin chain length distribution was measured by the method of O'Shea et al. (1998) using a P/ACE 5510 capillary electrophoresis system (Beckman Coulter, NSW Australia) with argon laser-induced fluorescence (LIF) detection.

Starch granule morphology, birefringence and granule size distribution

Granule morphology was examined by scanning electron microscopy (SEM) and light microscopy with polarized light. The shapes and birefringence of the starch granules were examined (Yamamori et al. 2000).

Granule size distribution (by volume) of the starch slurries was determined using a laser diffraction particle size analyzer (Mastersizer 2000, Malvern Instruments, Malvern, England). The percentage of small B-type starch granules was determined using a cut-off diameter of 6.6 µm.

Starch water content equilibration for solid state ^{13}C CP/MAS NMR and X-ray diffraction analyses

The starch samples (one sample from each genotype) were initially conditioned at a relative vapor pressure of zero to ensure a uniform starting point for all starches following the method of Spiess and Wolf (1987). The preconditioned starch powders were placed in a desiccator containing a saturated solution of K_2CO_3 at 20°C, providing an environment with a relative humidity of 44% (Greenspan, 1977). The moisture content was similar for all conditioned starch samples (ranging from 9.4 to 10.2%) as determined by the AOAC standard method of moisture content determination (AOA Chemists 1990).

Solid state ^{13}C CP/MAS NMR measurements

Solid state ^{13}C CP/MAS NMR experiments were performed at a ^{13}C frequency of 75.46 MHz on a Bruker MSL-300 spectrometer. Approximately, 200–300 mg of starch samples were packed in a 4 mm diameter, cylindrical, PSZ (partially-stabilized zirconium oxide) rotor with a KelF end cap. The rotor was spun at 5–6 kHz at the magic angle (54.7°). The 90° pulse width was 5 µs and a contact time of 1 ms was used for all starches with a recycle delay of 3 s. The spectral width was 38 kHz, acquisition time 50 ms, time domain points 2 K, transform size 4 K and line broadening 50 Hz. At least 2,400 scans were accumulated for each spectrum. Spectra were referenced to external adamantane.

Spectral analysis was performed to determine the relative proportions of the amorphous, single and double helical conformation in the granules, following a recent improvement (Tan et al. 2007) of the methods described earlier (Gidley and Bociek 1985; Bogracheva et al. 2001). The solver data analysis tool in Microsoft Excel™ software was used to carry out the first step of determining the fractional contribution of amorphous spectral features (i.e., the ratio of amorphous to combined single and double helical components). PeakFitTM software version 4.11 (SYSTAT Software Inc., CA, USA) was used to peak fit all spectra including those of crystalline components as generated from the deconvolution analysis of native starch spectra, leading to quantification of the ratio of single helical to double helical components (Tan et al. 2007).

X-Ray diffraction studies

X-ray diffraction measurements were performed on native starch powders using a Bruker axs D8 advance X-ray diffractometer operating at 40 kV and 30 mA. CuKα1 radiation ($\lambda = 0.15405$ nm) was used. The scanning region of the diffraction angle 2θ was 1–50°, which covers all the significant diffraction peaks of starch crystallites (Cheetham and Tao 1998; Gernat et al. 1993). A step interval of 0.02° and scan rate of 0.5°/min was employed for all measurements.

The degree of crystallinity of the novel starches was determined according to the general procedure of crystallinity determination for semi crystalline polymers (Cairns et al. 1997; Murthy and Minor 1990), in which the diffraction pattern is considered as a composite of the amorphous and crystalline diffraction patterns. The acquired diffraction pattern for the amorphous starch standard material was used to represent the amorphous halo of the native starch diffraction pattern. Subtracting the area underneath the amorphous "halo" from the total diffraction area of the native starch pattern yielded the area for the crystalline portion. Percentage crystallinity is determined from the ratio of the crystalline portion area to the starch total diffraction area (Cairns et al. 1997; Murthy and Minor 1990; Nara and Komiya 1983). The areas under the amorphous "halo" and native starch patterns were computed from 4° to 28°2θ using the EVA™ software by Bruker, Release 2004 Version 10.0.

Pasting properties and swelling power

Pasting properties were measured using a Rapid Visco Analyzer (RVA Model 3D+, Newport Scientific Pty Ltd.,

NSW, Australia). Starch (3.0 g) was added to distilled water (25.0 ml) in the RVA pan and the RVA run profile was: 2 min at 50°C, heat for 6 min to 95°C, hold at 95°C for 4 min, cool for 4 min to 50°C, hold at 50°C for 4 min. The measured parameters were: peak viscosity at 95°C, holding strength at the end of 95°C holding period, breakdown = peak viscosity − holding strength, final viscosity at the end of 50°C holding period, setback = final viscosity − holding strength.

The software Thermocline for Windows version 2.2 (Newport Scientific Pty Ltd., NSW, Australia) was used for collection and analysis of data.

Swelling power was measured by the 40 mg method of Konik-Rose et al. (2001).

Gelatinization and amylose–lipid dissociation properties

Gelatinization and amylose–lipid dissociation properties were measured by a differential scanning calorimeter (DSC, Pyris 1, Perkin Elmer). Starch and water were premixed in a ratio of 1:2 (dry basis), then approximately 50 mg was weighed into a DSC pan and hermetically sealed. The sealed pans were left to equilibrate overnight. The reference was an empty pan and the run profile for the test and reference samples was 30–130°C at 10°C/min. Four measurements were made for each of the two endotherms (gelatinization and amylose–lipid dissociation) using the software available with the instrument: initial temperature, peak temperature, final temperature and ΔH (enthalpy of transition).

Statistical analyses of the relationship between genotypes and starch properties

Statistical analyses were performed using Genstat version 9. Analysis of variance was performed for seed weight, protein content, total starch content and the different starch parameters to obtain the least significant difference (LSD, $P < 0.05$), looking at variation between the genotypes, and to obtain the 95% confidence interval (CI = square root of residual mean square 4) for variation between samples.

Results

Analysis of the doubled haploid wheat lines with protein profiles and PCR markers

Protein and PCR markers were scored for 100 progeny lines from the SGP-1 triple null × Sunco population. The results of this analysis showed that in all cases, there was complete linkage between the protein and PCR markers, adding further evidence to support the conclusion that the SGP-1 protein is encoded at the *ssIIa* locus. The number of lines for each of the eight genotypes for SSIIa is given in Table 1. All genotypes had a similar number of lines analyzed by both protein gel and PCR markers. Chi-square test showed that they were segregated in the expected ratio in this DH population (Table 1).

Shimbata et al. (2005) have characterized the insertion and deletion events of A, B and D genomes of SGP-1 null

Table 1 Statistical analyses of frequency of 8 SSIIa null genotypes and the relationships between SSIIa null genotypes and selected grain properties

Genotype	Genotype by protein and PCR	Expected ratio	χ^2	100 seeds weight (mg)		Protein content (%)		Total starch (%)	
				Mean	Significant difference ($P < 0.05$)	Mean	Significant difference ($P < 0.05$)	Mean	Significant difference ($P < 0.05$)
ABD	14	1:8	0.18	2.64	a	19.22	a	55.11	b
aBD	9	1:8	0.98	2.61	a	18.77	a	58.95	b
AbD	11	1:8	0.18	2.42	a	18.76	a	54.01	b
ABd	12	1:8	0.02	2.77	a	18.31	a	61.05	a
abD	11	1:8	0.18	2.46	a	18.95	a	55.00	b
aBd	15	1:8	0.5	2.78	a	18.13	a	55.45	b
Abd	15	1:8	0.5	2.34	a	18.06	a	57.03	b
abd	13	1:8	0.02	1.99	b	21.22	b	46.92	c
Total	100		2.56						
$\chi^2_{(1:1:1:1:1:1:1:1)}$ value (< 0.05)			14.1						
LSD (0.05)					0.35		1.38		4.64

$\chi^2_{(1:1:1:1:1:1:1:1)}$ value (<0.05) is 14.1 when the degree of freedom is 7. Significant difference ($P < 0.05$): mean values with the same letter are not significantly different, based on LSD

LSD least significant difference; differences greater than this are significant ($P < 0.05$)

wheat that result in loss of function. In order to achieve a clear discrimination between wildtype and null mutations of A, B and D genomes, a number of primer pairs were tested and an optimum primer set identified. The specificity of these pairs of primers for wheat chromosomes 7A, 7B and 7D was also examined using CS nullisomic/tetrasomic lines for homoeologous group seven chromosomes (data not shown). A total of 40 doubled haploid lines, representing all eight possible genotypes for SSIIa ($n = 5$ for each genotype) that were genotyped by both protein gel and PCR analysis were selected for starch analyses.

Seed weight

Seed weight of 100 seeds was between 1.99 and 2.64 mg for eight genotypes. SSIIa triple null lines gave the lightest average seed weight for five lines (1.99 mg); others (including single, double nulls and wildtype for SSIIa) gave the average seed weight between 2.34 and 2.64 mg. Only the seed weight of the SSIIa triple null lines was significantly different ($P < 0.05$) from the other SSIIa genotypes (Table 1).

Protein content

Protein content ranged from 18.06 to 21.22% in eight genotypes of SSIIa null. The protein content from single nulls and double nulls for SSIIa was similar to that of the wildtype for SSIIa, ranging from 18.06 to 19.22% (Table 1). Only the SSIIa triple null (21.2%) showed a statistically significant ($P < 0.05$) difference with the other genotypes.

Total starch content

Of the eight SSIIa genotypes, six had starch content between 54 and 59% and showed no statistically significant differences. However, wheat lines with a single d null for SSIIa contained more starch compared to the other single null, double null and willdtype genotypes for SSIIa. The statistical analysis showed that SSIIa triple null lines contained significantly less starch (46.9%, $P < 0.05$) compared to the other SSIIa genotypes (Table 1).

Amylose content

SSIIa triple null lines showed a significant increase in amylose content (44%) compared to wild type lines (34%) and other genotype lines (34–36%) (Table 2). There was no significant difference in amylose content among single null lines and double null lines and the wild type lines for SSIIa.

Debranched amylopectin chain length distribution

For SSIIa triple null lines, the proportion of amylopectin chains with degrees of polymerization (DP) 6–10 increased,

Table 2 Statistical analyses of the relationships between SSIIa null genotypes and selected starch properties

Genotype	% amylose		CE DP8		% B < 6.6 μm		RVA PV		Swell power	
	Mean	Significant difference ($P < 0.05$)	Mean	Significant difference	Mean	Significant difference ($P < 0.05$)	Mean	Significant difference	Mean	Significant difference ($P < 0.05$)
ABD	34.48	a	6.47	a	15.55	b	232.3	a	11.79	a
aBD	35.42	a	6.54	a	15.87	b	172.2	b	10.44	b
AbD	36.44	a	6.66	a	18.43	a	171.9	b	11.04	b
ABd	34.46	a	6.57	a	17.84	b	164.9	b	10.94	b
abD	34.34	a	7.35	b	16.13	b	131.7	c	8.97	d
aBd	35.46	a	7.02	b	18.21	a	118.6	c	9.8	c
Abd	35.4	a	7.71	b	17.25	b	122.7	c	9.04	d
abd	43.98	b	10.02	c	11.64	c	105.5	d	6.85	e
LSD		4.39	0.30			2.4		18.53		0.68
Significant interactions		ABD				AB,BD		ABD		AB,BD
95% CI for sample variation	13.62				14.9		115.04		5.13	

Significant difference ($P < 0.05$): mean values with the same letter are not significantly different, based on LSD

LSD, least significant difference; differences greater than this are significant ($P < 0.05$), CI, confidence interval; significant interactions: significant interactions were found in the analysis of variance for these genotypes (gene present or missing); CE DP8, percentage of amylopectin chains with DP8 from capillary eletrophoresis; %B < 6.6 μm, percentage of starch B granules that are smaller then 6.6 μm in diameter; %amylose, amylase content (%); RVA PV, RVA peak viscosity; swell power, swelling power

while the proportion of DP 11–25 chains decreased compared to the starch from the wildtype lines as described by Yamamori et al. (2000). Single and double null lines for SSIIa showed intermediate chain length distributions, with the single null lines being closer to the wild type lines and the double null lines (especially those with null b characteristic) being closer to the triple null lines (Fig. 1, Table 2).

Starch granule morphology and birefringence

Triple null lines (abd) showed altered morphology (irregular shaped granules) and loss of birefringence (loss of Maltese cross under polarized light) compared to wild type lines (ABD) as described by Yamamori et al. (2000). Single null lines (aBD, AbD, ABd) and double null lines (abD, aBd, Abd) for SSIIa showed similar morphology and birefringence to the wild type lines. This indicated that dramatic loss of radial organization of polymers within granules, as revealed by the loss of birefringence, only occurred with the loss of SSIIa from all three genomes.

Starch granule size distribution

A typical bimodal particle size distribution was observed in the starches from all eight genotypes of SSIIa. The triple null sample has a lower (12%) proportion of small B granules (<6.6 μm diameter; 6.6 μm being where the trough occurs in the bimodal particle size distribution for these samples) compared with the wild type (16%). Single and double null samples displayed results (16–18% B granules) similar to the wild type (Table 2). These results were consistent with observations from light microscopy and SEM (data not shown). The combination of null alleles for SSIIa between a and b or b and d null alleles had more significant effect on B starch granule content compared to a and d nulls (significant interactions, Table 2). The high ratio of B starch granules in b single null and ad double null SSIIa lines deserves further study.

Starch granule crystallinity and molecular order

The degree of order in starch granules can be detected using X-ray diffraction and solid state ^{13}C CP/MAS NMR spectroscopy. X-ray diffraction detects long range order or "crystallinity" in starch granules, while solid state ^{13}C CP/MAS NMR spectra provides a measure of the short range molecular order or helix content in starch granules (Tan et al. 2007). X-ray diffraction spectra showed that starches from all genotypes except the SSIIa triple null (A63) gave A-type polymorph diffraction patterns. The starch from the SSIIa triple null wheat (A63) shows a combination of B-type and V-type diffraction patterns (Fig. 2; Cheetham and Tao 1998). The solid state ^{13}C CP/MAS NMR spectra

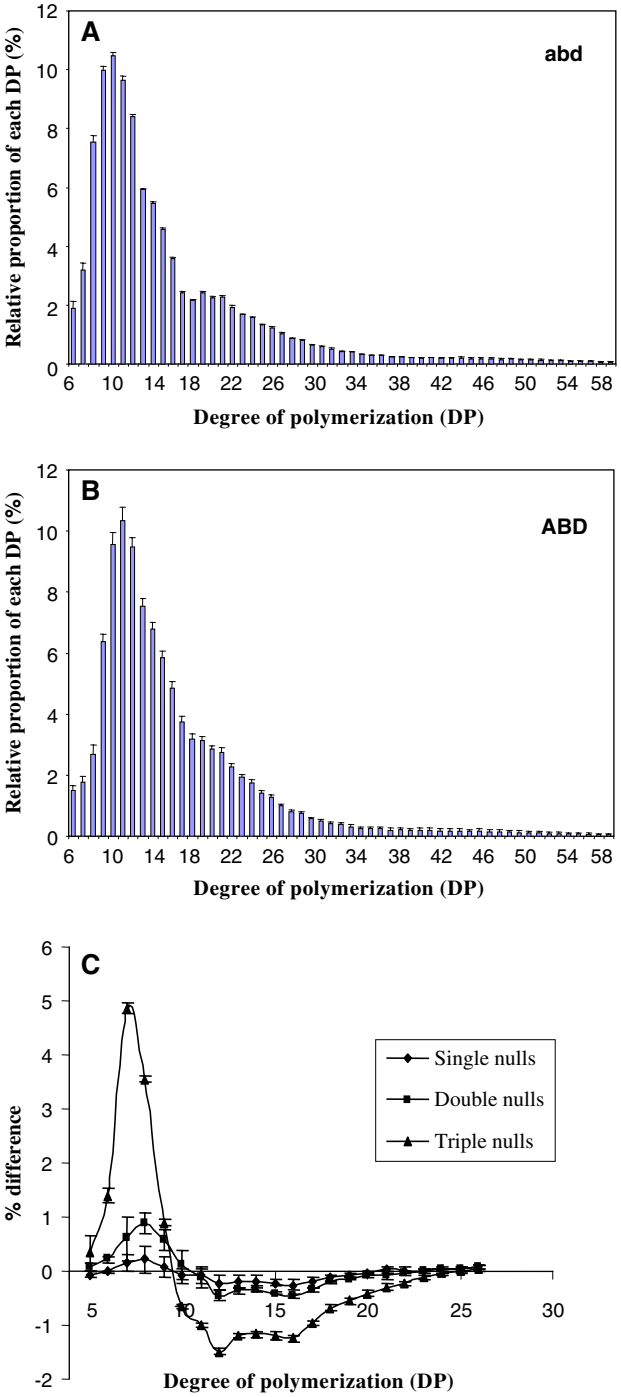

Fig. 1 Chain length distribution results of starch from different SSIIa genotypes of wheat: **a** Typical distribution profile of a triple null (abd) wheat. **b** Typical distribution profile of a wild type (ABD) wheat. **c** Comparison of single (AbD), double (abD) or triple null (abd) genotypes ($n = 5 \pm$ SE) for SSIIa with wild type. The percentage of total mass of individual oligosaccharides from starch from wild type SSIIa (ABD) lines is subtracted from the corresponding values from starches from single (AbD), double (abD) or triple null (abd) genotypes ($n = 5 \pm$ SE) for SSIIa. The *x*-axis indicates the chain length as DPs. The *y*-axis indicates the difference of chain length values when wild type values are subtracted from the average values of each mutant genotype

of the wheat starches carrying different SSIIa null alleles did not reveal any substantial differences in the C-1 peak resonances (90–105 ppm) between the different starches except for the SSIIa triple null (A63; Fig. 3). Subtraction of amorphous spectral features from these spectra (Tan et al. 2007) revealed the C-1 triplet signal characteristic of the A-type polymorphic structure in all starches except for the SSIIa triple null (A63), where the C-1 signal indicated that V-type (singlet) and B-type (doublet) polymorphs were present, consistent with X-ray diffraction data (Fig. 2).

Apart from the SSIIa triple null wheat starch (A63), the solid state ^{13}C CP/MAS NMR spectra (particularly the C-1 resonances at 90–105 ppm) and the X-ray diffraction patterns for all SSIIa null wheat starches exhibit the characteristics of the A-type polymorph packing arrangement. Therefore, the presence of single or double null alleles of the *ssIIa* genes does not induce any major variation in the packing arrangements of the double helices. Significant change in the packing arrangement of double helices only occurred in the SSIIa triple null (A63). The triple null also had the lowest degree of crystallinity (14%) among the starches from the eight SSIIa genotypes (Table 3).

The estimated values of the relative proportions of the amorphous, V-type polymorph and double helical conformations were summarized for the wheat starches carrying different SSIIa null alleles (Table 3). The relative amount of double helices inside the granules and the percentage relative crystallinity of all SSIIa null wheat starches are comparable except for the SSIIa triple null starch. A considerably higher amount of V-type polymorph (28%) was detected in the SSIIa triple null starch (A63). In addition, the SSIIa single B genome null type starch also contained an appreciable amount of the V-type polymorph. It was also observed (Fig. 3) that the C-1 peak resonances of SSIIa B genome null starch type (C3) have a more prominent triplet signal than other SSIIa null type starches, suggesting well-defined organization of A-type double helices (Gidley and Bociek 1985) and consistent with a higher crystallinity to double helix ratio than for other starches studied (Table 3).

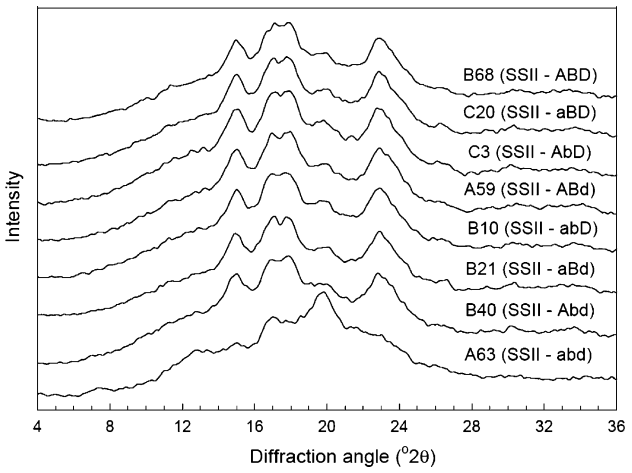

Fig. 2 X-ray diffraction for starches from different SSIIa genotypes of wheat. The wheat lines and their genotypes (*in brackets*) are labeled. Starches from all genotypes except triple null wheat give A-type polymorph diffraction pattern and starch from triple null wheat (A63) shows B-type of polymorph diffraction pattern. The scanning region between 4° and 36° of the diffraction angle 2θ is labeled underneath the diffraction pattern. SSII in *brackets* is SSIIa

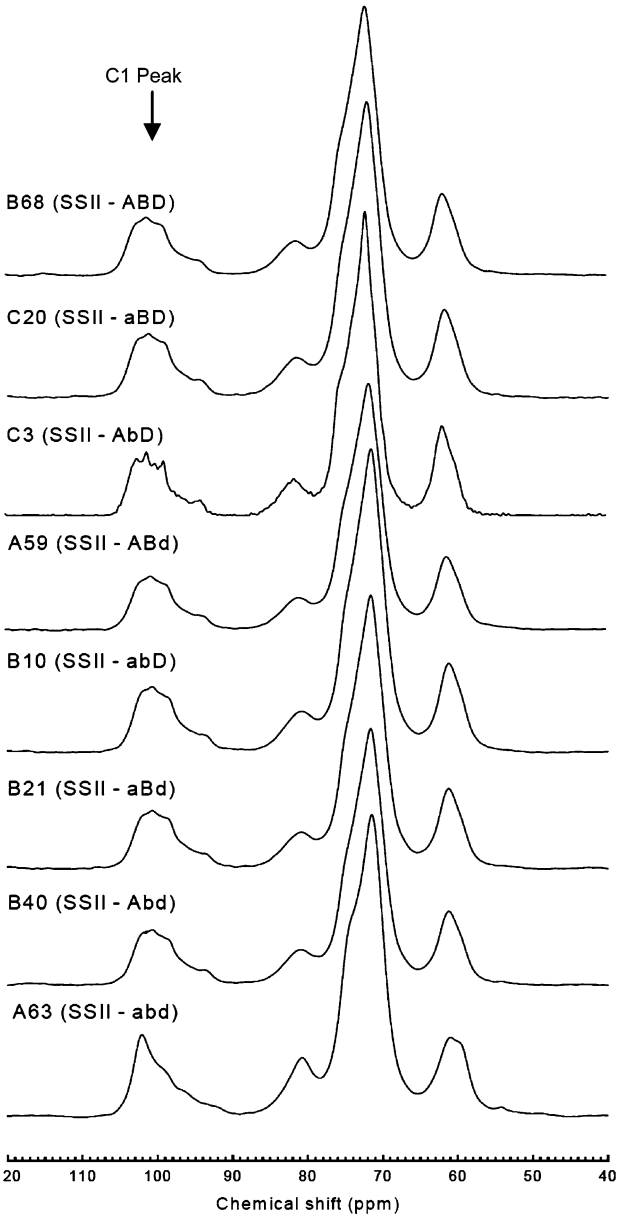

Fig. 3 Solid state ^{13}C CP/MAS solid state NMR spectra for starches from different SSIIa genotypes of wheat. Wheat lines and their genotypes (in *brackets*) are labeled. The range of the chemical shift (in ppm) is given underneath the NMR spectra. The C1 peak resonances at chemical shift of 90–105 ppm are labeled. SSII in *brackets* is SSIIa

Table 3 The estimated values for the relative proportion of the V-type polymorph, double helical and amorphous conformations, and the degree of crystallinity values for starches from different SSIIa genotypes of wheat

Starch type	V-type polymorph[a] (%)	Double helices[a] (%)	Amorphous[a] (%)	Degree of crystallinity[b] (%)
B68 (SSIIa–ABD)	0	37	63	17
C20 (SSIIa-aBD)	1	36	63	16
C3 (SSIIa-AbD)	7	31	62	18
A59 (SSIIa-ABd)	4	34	62	17
B10 (SSIIa-abD)	4	34	62	17
B21 (SSIIa-aBd)	1	38	61	18
B40 (SSIIa-Abd)	2	34	64	16
A63 (SSIIa-abd)	28	14	58	14

[a] The maximum standard deviation for the ^{13}C NMR analysis calculation was 2.2%

[b] The maximum standard error for relative crystallinity calculation was 2.3%

Starch pasting properties

Average RVA peak viscosity results for the eight genotypes are shown in Table 2. Triple null lines showed the lowest peak viscosity results (106 RVU units) and wild type lines showed the highest peak viscosity results (232 RVU units). The remaining null lines showed intermediate results, with the single null results (165–172 RVU units) being closer to the wild type, and the double null results (119–132 RVU units) being closer to the triple null results. Similar trends were observed for the other RVA parameters (holding strength, breakdown, final viscosity, setback).

Starch swelling power

Average swelling power results for five samples per genotype were measured for the eight genotypes (Table 2). Triple null lines showed the lowest swelling power (7.0) and wild type lines showed the highest swelling power (12.0). The remaining null lines showed intermediate properties, with the single nulls (11.0) being closer to the wild type, and the double nulls (9.0) being closer to the triple nulls. There are significant differences between combinations of SSIIa null alleles (a, b or d), with respect to their impact on starch swelling power, e.g., the combination of a and b or b and d null allele of SSIIa had a significant large effect on swelling power (significant interactions, Table 2).

Starch gelatinization and amylose–lipid dissociation properties

Gelatinization properties, as measured by DSC, showed differences between the *ssIIa* genotypes (Table 4). Wild type samples showed the highest gelatinization temperature (peak temperature 61°C) and the largest enthalpy (ΔH, 4.5 J/g). Triple null samples showed the lowest gelatinization temperature (peak temperature 55°C) and the smallest enthalpy (2.2 J/g). Single and double null samples gave intermediate results (Table 4), although the largest difference was between double and triple null mutants.

Amylose–lipid dissociation properties, as measured by DSC, showed no significant differences among the wild type, single null and double null samples for SSIIa (Table 4), with a peak amylose–lipid dissociation temperature range of 103–104°C and enthalpy of 0.29–0.35 J/g . However, the triple null samples for SSIIa had a slightly lower amylose–lipid dissociation temperature of 100°C and a signicantly higher enthalpy of 0.8 J/g.

Discussion

A number of starch properties have previously been reported to be modified in SGP-1 triple null wheat, including starch granule morphology, amylose content, amylopectin chain length distribution, crystallinity and starch gelatinization temperature, RVA and swelling power (Yamamori et al. 2000, 2006). In this study, we were interested in examining the gene dosage effects of SSIIa mutations on grain weight, selected grain composition and grain starch properties and in investigating whether reductions in SSIIa activity generate wheat with potentially useful application properties. In order to achieve this goal, a doubled haploid population was generated and genotyped using protein and DNA markers for eight genotypes of SSIIa that were separated at an expected ratio. Five representatives of each SSIIa genotype were selected for further analysis.

Through the analysis of the starch properties of five lines for each genotype, two groups of starch properties were observed. Group 1 showed a dose response for SSIIa activity across genotype groups (wild type, single null, double null, triple null classes) and in group 2 statistically signifi-

Table 4 Statistical analyses of the relationships between SSIIa null genotypes and DSC data

Genotype	DSCGPT		DSCGdH		DSCALPT		DSCALdH	
	Mean	Significant difference ($P < 0.05$)	Mean	Significant difference ($P < 0.05$)	Mean	Significant difference ($P < 0.05$)	Mean	Significant difference ($P < 0.05$)
ABD	61.27	a	4.54	a	103.86	ab	0.34	a
aBD	60.1	b	4.36	a	102.99	ab	0.29	a
AbD	59.95	b	4.47	a	103.96	a	0.3	a
ABd	59.44	b	4.49	a	103.47	ab	0.35	a
abD	57.99	c	3.71	b	103.33	ab	0.33	a
aBd	58.39	c	3.98	b	102.82	b	0.31	a
Abd	58.09	c	3.81	b	103.29	ab	0.35	a
abd	55.42	d	2.2	c	100.11	c	0.85	b
LSD(0.05)		0.7		0.3		1.05		0.14
Significant interactions		ABD		AB, AD, BD		ABD		ABD
95% CI for sample variation	6.12		1.88		4.61		0.63	

Significant difference ($P < 0.05$): mean values with the same letter are not significantly different, based on LSD

Significant interactions: significant interactions were found in the analysis of variance for these genotypes (gene present or missing). LSD, least significant difference, differences greater than this are significant ($P < 0.05$), CI, confidence interval, DSCALPT, DSC amylose–lipid dissociation peak temperature, DSCALdH, DSC amylose–lipid dissociation ΔH, *DSCGPT* DSC gelatinization peak temperature, DSCGdH, DSC gelatinization ΔH

cant effects were only observed for the triple null line. A subset of parameters could not be analyzed comprehensively for the five representatives of each genotype either because there was a lack of an objective quantitative measure of the trait (starch granule morphology, birefringence) or because measurement of large sample sets was impractical (X-ray diffraction and NMR). For these parameters, statistical tests were used to examine whether any of the genotypes differed from all other genotypes, but there was insufficient data to establish the statistical significance of differences between all genotypes.

Additive effects of *ssIIa* gene dosage on starch properties

Starch properties showing an SSIIa gene dosage effect included amylopectin chain length distribution, RVA peak viscosity, swelling power, gelatinization peak temperature and gelatinization enthalpy (Tables 2, 4). In triple null lines, the number of small amylopectin chains with DP 6–10 increased, while the proportion of larger amylopectin chains with DP 11–25 chains decreased (Yamamori et al. 2000, Fig. 1), which indicate that the role of SSIIa is the elongation of the short amylopectin chains (DP 6–10) to longer amylopectin chains (DP 11–25) in starch granules.

This is in agreement with the findings in wheat (Yamamori et al. 2000), barley (Morell et al. 2003) and pea (Craig et al. 1998). The single and double null lines for SSIIa showed intermediate chain length distributions, with the single null lines being closer to the wild type lines (not significant difference at $P < 0.05$, Table 2) and the double null lines (especially those with b null) being closer to the triple null lines (significant difference at $P < 0.05$, Table 2). This result contrasts with similar experiments for GBSS, where there was no difference in chain length distribution between the eight gene dosage genotypes (Miura et al. 2002). However, SSIIa has a predominant role in amylopectin synthesis, whereas GBSS has a predominant role in amylose synthesis.

All the RVA parameters (peak viscosity, holding strength, breakdown, final viscosity and setback) and swelling power were found to decrease steadily (i.e., similar differences between wild type, single, double, and triple null mutants) as the number of functional genes for SSIIa decreased. This is similar to experiments for GBSS for some parameters, as holding strength, final viscosity and setback all decreased as the number of functional genes for GBSS decreased, but different for other parameters such as peak viscosity and breakdown, which increased as the number of functional genes for GBSS decreased (Kim et al. 2003; Miura et al. 2002; Yamamori and Quynh 2000).

Gelatinization temperature also decreased as the number of functional genes for SSIIa decreased, with most changes between double and triple nulls, similar to the barley findings of Morell et al. (2003). This contrasts with mutations in BEII and GBSS, which result in increased gelatinization temperature (Fuwa et al. 1999; Yasui et al. 1996). Gelatinization enthalpy displays a similar trend to pasting parameters, swelling power and gelatinization temperature,

decreasing as the number of functional genes for SSIIa decreases. The changes in pasting properties, swelling power properties and gelatinization occur in parallel to the changes in the glucan chain distribution in amylopectin, suggesting that these properties are strongly influenced by amylopectin structure and package rather than amylose content per se. Morrison and Tester (1990) have shown that the swelling power of standard starches is correlated positively with amylopectin content. This study shows that the nature as well as quantity of amylopectin is important in determining swelling power, particularly when non-standard starches are examined.

Parameters altered only in the *ssIIa* triple nulls

Parameters that showed statistically significant changes only for the SSIIa triple null genotype include seed weight, protein content, total starch content, B granule content, amylose content, amylose–lipid dissociation peak temperature and amylose–lipid dissociation enthalpy.

SSIIa triple null lines had a lighter seed weight than that from other genotypes for SSIIa. Single null, double nulls and wildtype for SSIIa produced similar weight of seeds (Table 1). High protein content was only produced by the triple null lines for SSIIa, but not by other genotypes for SSIIa, which contained no significantly different amount of protein (Table 1). The apparent increase in protein content does not represent more protein synthesis per grain in the SSIIa triple nulls, but rather reflects the decrease in starch centent. Approximately, 10% less starch was produced by the triple null SSIIa lines than by other genotypes for SSIIa. Other genotypes for SSIIa (except SSIIa triple null lines) contained similar amount of starch in the grain. However, single d null lines for SSIIa contained more starch than that from other single null, double nulls and wildtype for SSIIa (Table 1).

For starch granule size distribution, single and double null lines showed starch granule size distribution similar to wild type lines, except that elevated amount of B starch granules were detected in b single null and ad double nulls for SSIIa (Table 2). The nature of the elevated B starch granules remains to be further studied. Then when all three *ssIIa* genes were inactivated, the granule size distribution was affected with fewer small B granules being present (Table 2). Triple null lines for SSIIa showed an increase in amylose content compared to wild type lines. This increase to 44% amylose was slightly higher than the previous wheat increase to 37% reported by Yamamori et al. (2000), but not as high as the barley increase to 70% (Morell et al. 2003). The difference in amylose contents between two types of triple null SSIIa lines (this work and Yamamori et al. 2000) could be the result of using either different genetic backgrounds or different analytical methods for amylose determination. Single and double null lines showed amylose contents similar to the wild type lines. These results contrast with similar experiments with GBSS that found an additive effect of the genes; however, this difference again reflects the differing roles of SSIIa in amylopectin synthesis and GBSS in amylose synthesis. The amylose–lipid dissociation temperature decrease and particularly the enthalpy increase also required all three *ssIIa* genes inactive in the triple null lines, consistent with these effects being linked to elevated amylose content.

Other parameters

A statistical analysis of small differences between genotypes for X-ray diffraction and NMR data was not possible, as only one representative line from each genotype was analysed. Analysis of the data to identify samples with a 95% probability of differing from the mean (i.e., greater than 2 standard deviations from the mean) demonstrated that for the X-ray diffraction parameters, V-type polymorph and degree of crystallinity, and for the NMR parameters, percentage of double helices and percentage of amorphous starch, the only genotype showing a statistically significant difference from other genotypes was the SSIIa triple null.

Triple null lines for SSIIa showed loss of birefringence and altered granule morphology, in agreement with the results of Yamamori et al.for wheat (2000), results of Morell et al. for barley (2003) and the results for pea (Craig et al. 1998). The lack of simple quantitative tests for these parameters prevented quantification of any fine differences between single and double nulls and wild type genotypes. Single, double and triple null lines for GBSS, using the current technologies, did not affect granule morphology at a detectable level (Kim et al. 2003; Miura et al. 2002). Both the above results support the view that amylopectin structure and properties, rather than amylose, are critical determinants of granule structure and properties.

While this study has focused on the SSIIa genotypes, it is important to recognize that at the biochemical level, the impact of the changes in SSIIa expression is unlikely to be the sole cause in the differences in phenotype. For example, the triple null SSIIa lines have a major reduction throughout seed development in the relative amounts of starch branching enzyme IIa, starch branching enzyme IIb and starch synthase I bound to the starch granules (Kosar-Hashemi et al. 2007). The fact that these changes are only seen in SSIIa triple null lines in the 100 progeny of the doubled haploid population argues strongly that these effects are a consequence of the SSIIa triple null state, and not a consequence of unlinked second site genes. While it is known that there is no major loss of these enzymes from the starch granule in single and double null SSIIa lines, there may be subtle effects that are yet to be quantified. The secondary

impact of mutations in starch biosynthetic enzymes may be more complicated than first throught because of the potential for starch biosynthetic enzymes in wheat to be associated in complexes (Tetlow et al. 2004).

Conclusions

The use of combinations of homeologous null mutations in hexaploid wheat is a powerful mechanism for developing a gene dosage series in target genes, allowing for the definition of relationships between phenotypic parameters. In this study, the combination of SSIIa mutations generated two classes of phenotypes, those in which an additive effect of mutations was observed, and those where an effect was only observed in the triple null. Additive effects on starch amylopectin chain length distribution, RVA peak viscosity, swelling power, gelatinization peak temperature and gelatinization enthalpy were observed. This observation adds further evidence supporting the conclusion that amylopectin chain length distribution is critical to controlling the gelatinization and swelling of the granule (Jane and Chen 1992). Effects of SSIIa mutations on seed weight, protein content, total starch content, starch granule crystallinity, B starch granule content, amylose content, amylose–lipid dissociation peak temperature and amylose–lipid dissociation delta were only observed in the triple null. The amylose–lipid dissociation peak temperature, amylose–lipid dissociation enthalpy and amylose content reflect changes in the synthesis of amylose in the triple null. The presence of only a single active SSIIa allele is able to compensate sufficiently that no statistically significant alteration in these properties can be observed in single or double nulls. It may be that only in the triple null is the granule morphology and availability of substrate for GBSS activity sufficiently altered to allow distinct differences in amylose content and amylose/lipid interactions to take place. In an ongoing study, the degree of polymerization and branching pattern of the amylose fraction of SSIIa deficient wheat is being analyzed in order to address the impacts of SSIIa activity on amylose synthesis.

Acknowledgements We wish to acknowledge the technical support of Helen Olsen in preparing the starch samples, and Russell Heywood and Mark Cmiel for growing the plants. We also wish to acknowledge the support of Dr M. Yamamori (Japan).

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