

Characterization of Starch Synthetic Genes and Starch Granule during Seeds Development between Synthetic Hexaploid Wheat and its Parents

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To study the development of starch granules in polyploid wheats, we investigated the expression of starch synthetic genes between the synthetic hexaploid wheat SHW-L1, its parents *T. turgidum* AS2255 and diploid *Ae. tauschii* AS60. The synthetic hexaploid wheat SHW-L1 showed significantly higher starch content and grain weight than its parents. Scanning electron microscopy (SEM) showed that SHW-L1 rapidly developed starch granules than AS2255 and AS60. The amount of B-type granule in AS60 was less than that in SHW-L1 and AS2255. RT-qPCR result showed that the starch synthetic genes *AGPLSU1*, *AGPLSU2*, *AGPSSU1*, *AGPSSU2*, *GBSSI*, *SSIII*, *PHO1* and *PHO2* expressed at earlier stages with larger quantity in SHW-L1 than in its parents during wheat grain development. The expression of the above mentioned genes in AS60 was slower than in SHW-L1 and AS2255. The expression pattern of starch synthase genes was also associated with the grain weight and starch content in all three genotypes. The results suggested that the synthetic hexaploid wheat inherited the pattern of starch granule development and starch synthase gene expression from tetraploid parent. The results suggest that tetraploid wheat could play more important role for starch quality improvement in hexaploid wheat.

Keyword: starch synthase, starch granule, seed, synthetic hexaploid wheat, tetraploid

Introduction

Grain yield is largely determined by starch accumulation during the grain filling phase in cereals. Wheat grains mainly consist of starch (60–80%) and proteins (10–15%), the remains include cellulose, fat, and mineral substances (Smith et al. 1995). Starch of the wheat endosperm consists of amylose (20–30%) and amylopectin (70–80%) (Hurkman et al. 2003; Sestili et al. 2010). Amylose is synthesized by ADP glucose pyrophosphorylase (*AGPase*) and granule-bound starch synthase (*GBSS*), while amylopectin is synthesized by the *AGPase*, starch synthases (*SS*), starch branching enzyme (*SBE*), and starch debranching enzyme (*DBE*). Disproportionating enzyme (*DPE*) and phosphorylase (*PHO*)

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are considered to be involved in starch biosynthesis and degradation (Colleoni et al. 1999; Ball and Morell 2003; Tetlow et al. 2004). *GBSS* is essential for amylose synthesis and exclusively bound to the starch granule. *SSI*, *SSII*, *SSIII*, and *SSIV* are responsible for amylopectin chain elongation with their distribution between the granular and soluble fractions (Ball and Morell 2003; Li et al. 2003). Two types of starch granules, type A granules ($>15\ \mu\text{m}$) and type B granules ($<15\ \mu\text{m}$), exist in wheat endosperm. The content and ratio of amylose and amylopectin, as well as the granule type and ratio, strongly affect grain yield and the end product utilization of wheat. New germplasm resource with diverse starch properties is very valuable for wheat improvement. Therefore, identification of new alleles of starch synthetic genes can help to improve wheat grain quality. Recently, some alleles of starch synthetic genes have been identified in *Triticum* and *Aegilops* species. Two types of *waxy* gene in *T. urartu* and *Ae. tauschii*, respectively (Murai et al. 1999), alleles *Wx-Am1^a* and *Wx-Am1^{a'}* in Spanish *T. monococcum* (Guzmán et al. 2009), null *SGP-A1* alleles (gene *SSIIa*) (Hogg et al. 2013) and alleles *Wx-B1c'* and *Wx-B1f* in durum wheat (Nieto-Taladriz et al. 2000), were observed. The variation of SNPs (single nucleotide polymorphisms) and InDels (insertions or deletions) of *waxy* gene produced new alleles in *Triticum* and *Aegilops* (Li et al. 2012; Yamamori and Guzman 2013). The significant differences of *Wx-A1* and *Wx-B1* between diploid and hexaploid wheat also have been reported (Huang and Brûlé-Babel 2010). Phylogenetic analysis suggested that *Wx-A^u1* and *Wx-A^m1* of diploid wheat were clearly different from the *Wx-A1* loci of tetraploid and hexaploid wheat (Li et al. 2015). These findings suggested that the starch synthetic genes had diversified variation in *Triticum* and *Aegilops* species. These alleles have potential useful value for starch improvement of wheat breeding.

Synthetic hexaploid wheat provides a good strategy to evaluate the change of genes during polyploidization in wheat. The differences of protein composition and quality between synthetic wheats and their parents have been reported (Pena et al. 1995). It has been suggested that the tetraploid parents often exert greater influence on the properties of the synthetic hexaploid than diploid parents (Gororoet al. 2001). The little difference in the amylose contents and amylose-lipid dissociation peak temperatures between synthetic hexaploid wheat and lower ploidy wheats was also observed (Konik-Rose et al. 2009). However, there are only a few reports on the starch properties of synthetic hexaploid wheat and their parents (Gororoet al. 2001; Konik-Rose et al. 2009).

The aim of this study was to evaluate the performance of starch granule development and the expression of starch synthetic genes in synthetic hexaploid wheat and its parents.

Materials and Methods

Plant materials

Synthetic hexaploid wheat SHW-L1 ($2n = 6x = 42$, AABBDD), and its parents, *T. turgidum* landraces AS2255 ($2n = 4x = 28$, AABB) and *Ae. tauschii* AS60 ($2n = 2x = 14$, DD), used in this study were provided by Triticeae Research Institute, Sichuan Agricultural University (Zhang et al. 2004). All the plants were grown during 2014–2015 in a field

(longitude 103.88 E, latitude 30.73 N) of Sichuan Agricultural University, Chengdu city, Sichuan Province, China.

Determination of grain weight and starch concentration

The mature seeds were collected from all the plants. The 1000-grain weight was determined using an electric balance (BS200S-WE1, Sartorius, Gottingen, Germany) in three replications for each accession. The starch content was determined in triplicate using total starch assay kit (Megazyme, Ireland). Mature seeds were cut into halves and stained in 0.1% (w/v) iodine solution (1.0 g of potassium iodide and 0.1 g of iodine diluted to 100 mL with distilled water). Amylose content of samples was measured by the dual-wavelength colorimetric method (Zhu et al. 2008). Amylopectin content was calculated through starch content minus amylose content. Data was analyzed by ANOVA and the means were compared by the least significant difference (LSD) test.

Scanning electron microscopy (SEM) analysis

During the flowering period, the pollinating spikelets were tagged in SHW-L1, AS2255, and AS60, respectively. The kernels in the middle of spike were collected at 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 DAP (days after pollinated), respectively. These kernels were transversely sectioned across the centre by knife and mounted on circular aluminum specimen holders with double-sided sticky tape. The newly broken surface of kernels was directly viewed and photographed with a FEI Quanta 450 Environmental SEM (FEI Company, Hillsboro, Oregon State, USA).

RNA extraction, cDNA synthesis and real-time quantitative PCR (RT-qPCR)

The kernels of the spikelets located on the middle spike of SHW-L1, AS2255, and AS60 were collected at 6, 12, 18, 24, and 30 DAP, respectively. Total RNAs were extracted from these kernels with RNAPrep pure Plant Extraction Kit (DP432) (Tiangen Biotech Co. Ltd, Beijing, China). The cDNA was synthesized from reverse transcription using a PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). The primers of RT-qPCR (Table 1) were designed based on the sequences DQ839506 (*AGPLSU1*), DQ406820 (*AGPLSU2*), EF405961 (*AGPSSU*), AY050174 (*GBSSI*), AF258608 (*SSIII*), EU595762 (*PHO1*), AF275551 (*PHO2*) from GenBank (<http://www.ncbi.nlm.nih.gov>). The PCR products were sequenced to confirm the amplification (Sangon Biotech Co., Ltd. Shanghai, China). Each PCR reaction in a total volume of 20 µl contained 9 µl SYBR Green Master Mix (Tiangen Biotech Co. Ltd., Beijing, China), 1 µl of cDNA and 100 nM of both forward and reverse primers. The RT-qPCRs were performed in the BIO-RAD MyiQ Real Time PCR (BIO-RAD, Hercules, California, USA). The data were analyzed via the Bio-Rad iQ5 software. The *ACTIN* gene (AB181991) was used as an internal reference and control for expression analysis. The RT-qPCR program included an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 15 s,

Table 1. The primers of starch synthetic genes used in RT-qPCR

Gene name	NCBI number	Name of primer	The sequence of primer (5'-3')	Amplified region (bp)	Product of PCR (bp)	T _m (°C)
<i>AGPLSU1</i>	DQ839506	F1	CACATTCACCGCACCTAC	573–720	148	52.8
		R1	CCTCAAGCACCCAGATAA			
<i>AGPLSU2</i>	DQ406820	F2	CATCACGCAGAAACCTACC	1094–1217	124	49.8
		R2	GAGAACGGATTCCAACGA			
<i>AGPSSU</i>	EF405961	F3	TACGATGTTACTGGAAGAT	887–1030	144	49.2
		R3	TGAAGGAGGCAAGTGTCG			
<i>GBSSI</i>	AY050174	F4	CCTCAACAACAACCCACA	766–913	148	53.7
		R4	ATTGGACTGGTAGTTGCTCT			
<i>SSIII</i>	AF258608	F5	ACGGTCGCACGGTCTATGAG	2460–2638	178	52.3
		R5	CTTTCTTCGCCGCTGTTCTT			
<i>PHO1</i>	EU595762	F6	GATGTTCTTATCCTGGCTAC	912–1097	186	50
		R6	CTCTGATGATTCGTCCCCT			
<i>PHO2</i>	AF275551	F7	CAGAGATGGAACGACACCT	232–353	152	56.5
		R7	AACTTCTTCAGGGCGTCA			
<i>ACTIN</i>	AB181991	F	TACTCCCTCACAAACCG	367–508	142	52.3
		R	CCATCAGGCATCTCATAGC			

and 72 °C for 15 s, and a melt curve stage. Relative expression levels of all genes were calculated using the Pfaffl method (Pfaffl 2001).

Results

Thousand-grain weight and starch content

The average 1000-grain weight in AS2255, SHW-L1 and AS60, were 25.63 g, 24.35 g and 5.42 g, respectively. AS2255 had significantly higher 1000-grain weight than SHW-L1, while SHW-L1 had significantly higher value than AS60 by Duncan multiple comparisons. SHW-L1 (52.7%) showed slightly higher value of starch content than that of AS2255 (51.8%) and AS60 (44.7%). The amylose content of SHW-L1 (15.6%) was higher than that of AS2255 (14.7%) and AS60 (12.8%), whereas the amylopectin content of SHW-L1 (37.0%) and AS2255 (37.1%) were significantly higher than that of AS60 (31.9%). These results show that the synthetic hexaploid wheat SHW-L1 had similar performance of grain weight and starch content to the tetraploid parent.

Development of starch granules

Scanning electron microscopic (SEM) analysis revealed that at three DAP, most of A type starch granules ($>10\ \mu\text{m}$) enriched in the amyloplast were observed in all the three genotypes (Fig. 1). The producing of A type starch granules from the amyloplast was observed in AS2255. At six DAP, the membrane of amyloplast was disappearing and most of

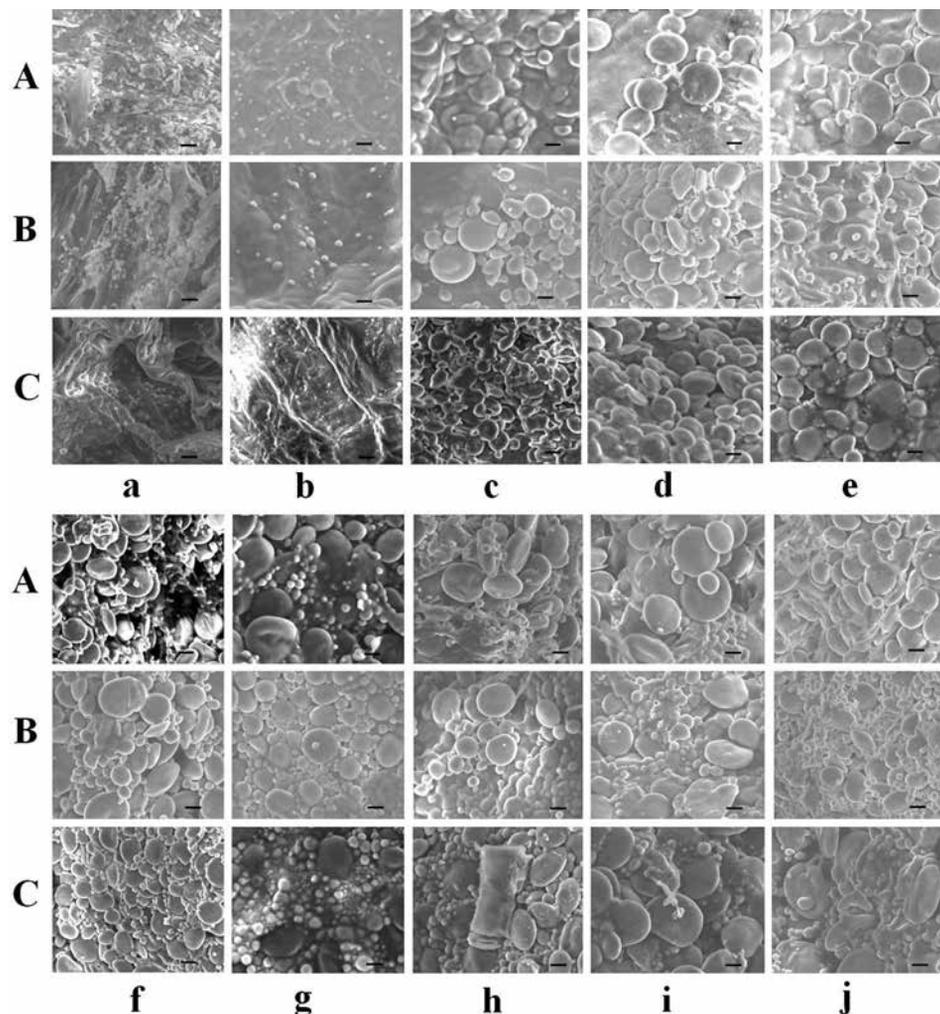


Figure 1. Starch granule of diploid *Ae. tauschii* AS60(A), tetraploid *T. turgidum* AS2255(B), and synthetic hexaploid wheat SHW-L1(C) visualized by SEM. These images of transverse grain sections represent 10 developmental stages, including at 3 DAP (a), 6 DAP (b), 9 DAP (c), 12 DAP (d), 15 DAP (e), 18 DAP (f), 21 DAP (g), 24 DAP (h), 27 DAP (i), and 30 DAP (j), respectively. For each accession and stage, at least three seeds were examined and similar results were observed. Magnification is $\times 3000$. Scale bars represent $10\ \mu\text{m}$

A-type granules were dissociating from the amyloplasts. AS2255 and SHW-L1 showed faster dissociating process than AS60. At nine DAP, both of A-type and B-type starch granules were observed. AS2255 and SHW-L1 showed faster appearance of granules than AS60. At 12 DAP, the B-type starch granules were observed in all accessions. At 15 DAP, the amount of B-type granules had been increased rapidly in all the three genotypes, but AS2255 and SHW-L1 showed larger number of B-type granules than AS60. At 18 DAP, the B-type granules were observed clearly and distributed evenly in AS2255 and SHW-L1. At 21 DAP, the amount of B type granule had the first peak value, and the smaller size (<5 µm) C-type starch granule appeared. At 30 DAP, the quantity of A type granule did not change and reached a stable stage. Although the amount of B type granules had increased in three genotypes compared with the last stage, the less quantity of B type granules in AS60 was observed compared with AS2255 and SHW-L1.

Expression patterns of starch synthase genes

DNA sequencing confirmed that the fragments amplified with the designed primers were the expected genes (Table 1). All primers can be used to detect the expression of these genes in RT-qPCR. The expression of *AGPLSU1*, *AGPLSU2*, *AGPSSU*, *GBSSI*, *SSIII*, *PHO1*, and *PHO2* showed significant differences between the synthetic hexaploid wheat SHW-L1 and the tetraploid wheat AS2255 and diploid AS60 at five developing stages of seed by RT-qPCR (Fig. 2). At six DAP, the expression of five genes, except *AGPLSU2* and *GBSSI* in AS60, were detected in all three accessions. *AGPLSU1* and *AGPSSU* had the highest expression in AS60 than in AS2255 and SHW-L1. *GBSSI* and *PHO1* had the highest expression in AS2255, followed by SHW-L1 and AS60. *SSIII* and *PHO2* had the highest expression in SHW-L1, followed by AS60 and AS2255. *AGPLSU2* had higher expression in SHW-L1 than in AS2255. *AGPLSU2* and *GBSSI* had higher expression level than the other genes at this stage. At 12 DAP, the expression of *AGPLSU1* and *AGPLSU2* in SHW-L1, and *AGPLSU2* and *GBSSI* in AS60, were not detected. The expression of *AGPLSU1*, *SSIII*, and *PHO2* had the highest level in AS60, followed by AS2255 and SHW-L1. *AGPLSU2* and *GBSSI* had the highest expression in AS2255. *PHO1* had the highest expression in SHW-L1, implied that the degradation of starch had been activated in SHW-L1. The expression of gene *AGPSSU* had insignificant difference among AS60, AS2255, SHW-L1. At 18 DAP, *AGPLSU1* had the highest expression in AS2255 than in AS60 and SHW-L1. *AGPLSU2*, *SSIII*, *PHO1*, and *PHO2* had the highest expression level in AS60, compared with AS2255 and SHW-L1. The expression of *GBSSI* was not detected in AS60. Otherwise, *AGPSSU* and *GBSSI* had the highest expression in SHW-L1, and followed by AS2255 and AS60. At 24 DAP, the expression of *AGPLSU1*, *AGPLSU2*, and *GBSSI* were not detected in AS60. SHW-L1 showed very low level expression of *AGPLSU1*, but significantly higher expression level of *AGPSSU* and *GBSSI* than in AS2255 and AS60. At 30 DAP, only the expression of *AGPLSU1* was not observed in AS2255. *AGPLSU1*, *AGPLSU2*, *PHO1*, and *PHO2*, had the highest expression in AS60. *AGPSSU2* and *GBSSI* had higher expression in AS2255 and SHW-L1 than that in AS60. *SSIII* had the highest expressing level in AS2255 than that in AS60 and

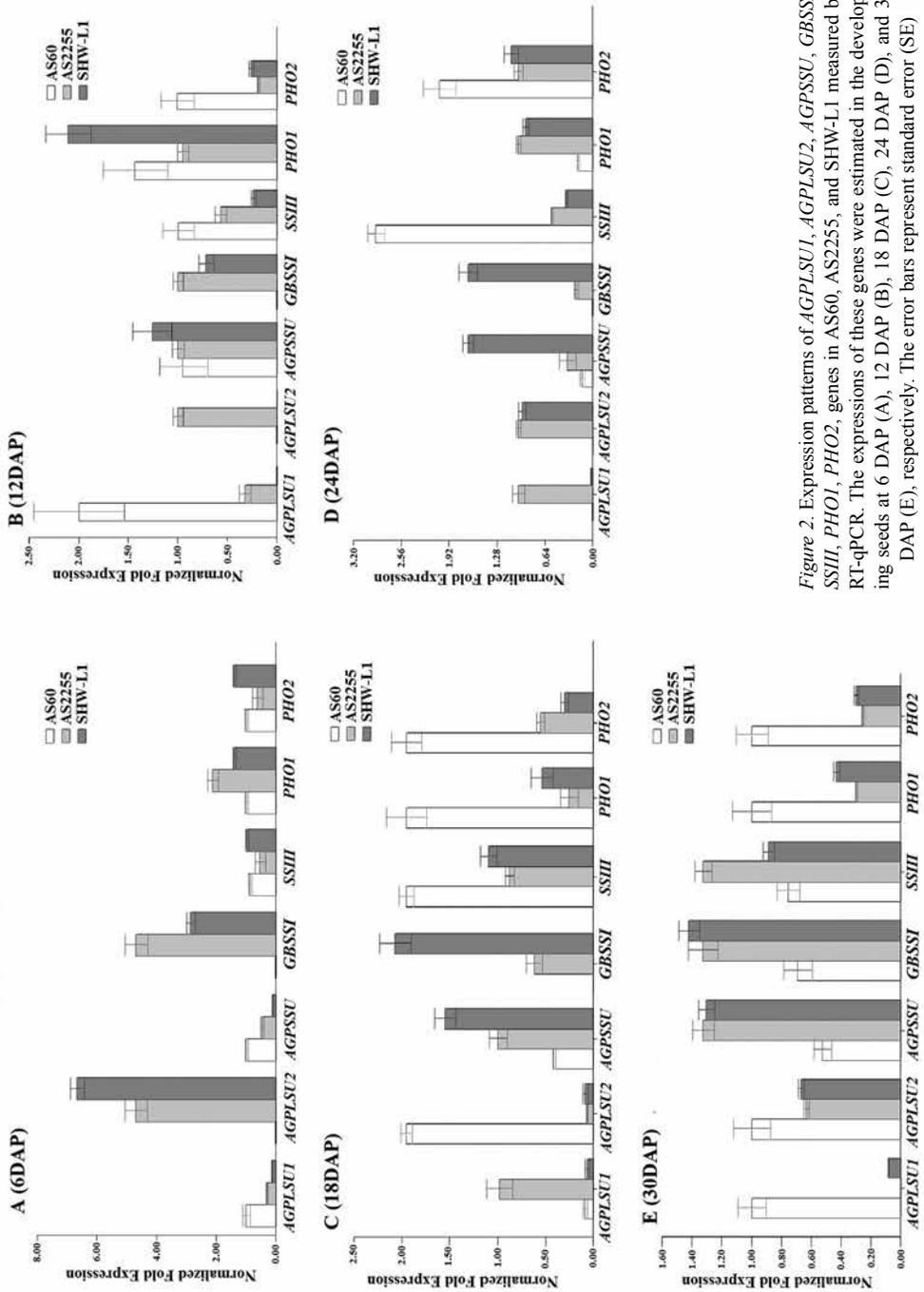


Figure 2. Expression patterns of *AGPLSU1*, *AGPLSU2*, *AGPSSU*, *GBSSI*, *SSIII*, *PHO1*, *PHO2*, genes in AS60, AS2255, and SHW-L1 measured by RT-qPCR. The expressions of these genes were estimated in the developing seeds at 6 DAP (A), 12 DAP (B), 18 DAP (C), 24 DAP (D), and 30 DAP (E), respectively. The error bars represent standard error (SE)

SHW-L1. These results showed that most of starch synthase genes had higher and earlier expression in synthetic hexaploid and tetraploid wheat. But *PHO1* and *PHO2* had been keeping higher activity at the whole stages of seed development in diploid AS60.

Discussion

The utilization of novel alleles of starch synthase genes in germplasm resources is very valuable for wheat breeding (Konik-Rose et al. 2009; Guzmán et al. 2011). These alleles could strongly affect the characterizations of starch for use in both food and non-food industries (Martin and Smith 1995). A total of 12 accessions with amylose contents above 40% were reported in *T. durum* and *Ae. tauschii* (Watanabe et al. 1998). The previous research showed that the synthetic hexaploid wheats had intermediate swelling power values, and tetraploid durum wheat had the highest swelling powers, and the diploid *Ae. tauschii* had the highest gelatinisation peak temperatures (Konik-Rose et al. 2009). In the present results, the synthetic hexaploid wheat SHW-L1 had higher grain weight and starch content, higher amount of B-type granules, and stronger expression of starch synthetic genes than its tetraploid and diploid parents. As wheat grains mainly consist of starch (60–80%) (Smith et al. 1995), the change of starch content might be one of reasons for the increasing of seed production of synthetic hexaploid wheat.

In wheat, the large granules appear at about 4–7 DAA (days after anthesis) and continue to increase in size throughout the grain filling stage, while small granules were initiated at about 12–14 DAA and remain considerably small at final maturity (Bechtel and Wilson 2003). The development of granules was well consistent with starch synthase activities and relative gene expression (Zhang et al. 2010). The *durum* wheat had higher B granule contents than the *Ae. tauschii* accessions, but not as high as the synthetics (Konik-Rose et al. 2009). In the present result, at three DAP (Figure 1a), most of A-type starch granule enriched the amyloplast, and at nine DAP (Figure 1c), the small granule appeared among three accessions. The granules in *Ae. tauschii* AS60 developed slowly and showed less quantity compared with the synthetic hexaploid and tetraploid wheat. These results suggested that the development of starch granules is species specific. For the developing pattern and the quantity of granules, hexaploid SHW-L1 were more similar to tetraploid AS2255, suggesting that synthetic hexaploid wheat could be inherited more genetic characterizations of starch from tetraploid wheat than diploid. The similar performance that the tetraploid parents had greater influence on the traits of the synthetic hexaploid than the diploid parent had been reported (Flood et al. 1992; Limin et al. 1995; Gororo et al. 2001).

The small and large subunits of ADP glucose pyrophosphorylase (*AGPase SSU* and *LSU*) were the rate-limiting enzyme of starch synthesis in cereal endosperm (Tomlinson and Denyer 2003). *AGPase* activities in wheat grain are mainly relative to the activities of cytosolic *SSUI* (Kang et al. 2010). *AGPase LSU II* showed the highest expression levels from 6 to 11 DPA, and was then down-regulated to trace levels at grain desiccation (Guo et al. 2012). In the present results, the expression of *AGPase LSU* and *SSU* in all materials showed the similar developing trend compared to the previous researches, while

the expression of these genes in AS60 lagged behind in SHW-L1 and AS2255. *AGPLSU1* and *AGPSSU* had the highest expression in AS60 at 6 DAP, implied that the expression of *AGPase* in synthetic hexaploid wheat and *T. turgidum* could be activated early.

GBSS and *SS* are responsible for the synthesis of amylose and amylopectin, respectively (Ball and Morell 2003; Li et al. 2003). *GBSSI* transcripts were low at the beginning, and dramatically increased at 5 DAF (days after florescence) and reached peaks at 24 DAF and then remained abundantly expressed until 32 DAF at the end of endosperm development (Zhang et al. 2010). In the present study, at most of developing stages, SHW-L1 showed higher expression of *GBSSI* than AS2255 and AS60, suggesting that the synthetic hexaploid wheat had more activity of *GBSSI* compared with its parents. The expression of *GBSSI* in diploid AS60 was only detected at 30 DAP, implied that *GBSSI* could be lagged and had weak expression and short duration in diploid *Ae. tauschii*.

SSIIIa produces longer length chains extending between clusters in cereal endosperm (James et al. 2003) and also influences indirectly the final molecular architecture of the starch (Gao et al. 1998). The suppression of *SSIII* activity reduced amylopectin synthesis and the long chains length of the amylopectin in rice (Dian et al. 2005). The *SSIII* expressed very high at four DAF (days after florescence) in the superior grains while eight DAF in the inferior grains, and then rapidly reached peaks between 12 and 16 DAF and gradually decreased thereafter (Zhang et al. 2010). In the present result, *SSIII* had higher expression at six DAP in AS2255 and SHW-L1, and showed the similar expressing pattern with the previous research. But diploid *Ae. tauschii* AS60 lagged the expression of *SSIII* at 12 DAP compared with synthetic hexaploid and *T. turgidum*.

Plant *PHO*, including plastidial *PHO1* and cytosolic *PHO2*, was involved in the process of starch degradation and starch biosynthesis in seeds (Colleoni et al. 1999; Critchley et al. 2001; Satoh et al. 2008). *PHO1* transcripts were numerous at the onset of seed development, rapidly increased to peak at 5 DAF, and diminished to a low but significant level until 15 DAF. The transcripts of *PHO2* were scarce from the start and dwindled from 5 DAF (Tickle et al. 2009). In our results, the expression of *PHO1* and *PHO2* in *Ae. tauschii* AS60 also lagged behind those in synthetic hexaploid and *T. turgidum*. But *PHO2* showed the highest expression in AS60 at 12 DAP. The results suggested that *PHO* genes were activated in the early developing stages of seed, and the synthase and degradation of starch might be activated at the same time during seed development.

Hexaploid wheat *T. aestivum* (AABBDD) was obtained by the hybridization between a tetraploid *T. turgidum* (AABB) and a diploid *Ae. tauschii* (DD) (Huang et al. 2002). The new synthetic hexaploid wheat provides a unique system for the investigation of the polyploidization process. A series of studies have demonstrated that allopolyploid formation or speciation in *Triticum* and *Aegilops* species could induce directional sequence elimination, random structural changes, epigenetic modifications, DNA methylation, and gene silencing in gene expression (Liu et al. 1998; Ozkan et al. 2001). Our present results indicated that the synthetic hexaploid wheat had a different expression pattern of starch synthetic genes compared with its tetraploid and diploid parents. The similar results for other traits were also reported (Flood et al. 1992; Gororo et al. 2001; Konik-Rose et al. 2009; Guzmán et al. 2011). Synthetic hexaploid wheat could be used for wheat starch

improvement. Furthermore, the pattern of gene expression of starch synthases in the synthetic hexaploid SHW-L1 was mainly similar to *T. turgidum* AS2255, suggested that tetraploid wheat could be play a more important role in the improvement of the starch quality of hexaploid wheat.

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