JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 4193–4201 4193
DOI:10.1021/jf903861q

Selected Carbohydrate Metabolism Genes Show Coincident Expression Peaks in Grains of *In Vitro*-Cultured Immature Spikes of Wheat (*Triticum aestivum* L.)

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An *in vitro* culture system is useful to study grain development under defined conditions to minimize confounding effects associated with whole plant studies and metabolite movement into the developing grains. The objective of this study was to monitor the expression patterns of carbohydrate metabolism genes during grain development in an *in vitro* wheat spike culture system. Immature spikes were cultured prior to anthesis, and grains were collected at various days postanthesis (DPA). Grains from cultured spikes showed maximum expression of starch metabolic genes by 10 DPA, with a rapid decline thereafter. The rapid increase and decrease in expression rate in the *in vitro* system was thought to be due to *fructan exohydrolase* (*1-FEH* and *6-FEH*) or *sucrose transporter 1* (*SUT1*) and *sucrose synthase* (*SuSy*) genes being highly expressed. *SUT1* reached peak expression at 8 DPA, two days earlier than the other genes, and may account for the rapid early stage trigger in expression of the other genes. However, expression of *1-FEH* and *6-FEH* genes in *in vitro*-cultured spikes peaked at 12 DPA, two days later than the other genes, and could indicate that fructan catabolism was not a factor in the rapid accumulation of starch in the *in vitro*-cultured spikes. Accumulation of GBSSI polypeptides generally showed similar patterns in both systems, with the maximum amount in the *in vitro* system observed four days later than in the *in planta* spikes, reflecting different turnover controls of *GBSSI* transcripts. The *in vitro* system offers opportunities for further refinement and detailed grain development studies.

KEYWORDS: Grain development; quantitative gene expression; starch

INTRODUCTION

Cereal grain development is a complex and coordinated process requiring a concerted interaction of several metabolic processes. Cereal grains are a very important food/energy source, and therefore extensive efforts have been made to understand grain development to improve their productivity and end-use quality. Although over the years there have been comprehensive studies of the grain development processes, further refinement in our understanding is required to enhance yield to meet the needs of the growing world population and also to alter grain composition to develop specialty grains and diversify cereal grain utilization. In wheat grain, starch is the most abundant storage carbohydrate, accounting for two-thirds to three-quarters of grain weight (*1*). Starch is made up of two glucan polymers: amylose (25%) is a predominantly linear chain of α-1,4 glucans, and amylopectin (75%) has a linear chain that is branched by α-1,6-linkages. The fine structure of these polymers, determined to a large extent by their degree of branching, and the ratio of amylose to amylopectin, influences the physicochemical properties of starch and therefore its end uses. Thus, control over the biogenesis of these glucan polymers would enable a more targeted approach to enhance grain yield, as well as to develop specialty novel starches *in planta* (*2*).

The starch accumulated in wheat leaves during the day is hydrolyzed at night and re-synthesized in the endosperm of the grains for storage. Wheat storage starch biosynthesis involves a minimal subset of 14 conserved starch biosynthetic enzymes (*3*). ADP-glucose pyrophosphorylase (AGP), the first committed enzyme of the pathway, combines ATP and glucose-1-phosphate to produce ADP-glucose, the donor for both amylopectin and amylose synthesis (**Figure 1**). AGP is a heterotetrameric allosteric enzyme composed of two subunits: a large (AGPL) subunit and a small (AGPS) subunit (for review see ref *4*). Starch synthases (SS) elongate glucan chains by adding ADP-glucose units through α-1,4-linkages. Several isoforms of SS have been identified in the plant kingdom. SSI and SSII are primarily involved in amylopectin synthesis, whereas granule-bound SS (GBSSI) is the only enzyme committed to amylose synthesis in wheat (**Figure 1**). Branches in amylose and amylopectin are introduced by starch

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Published on Web 03/17/2010

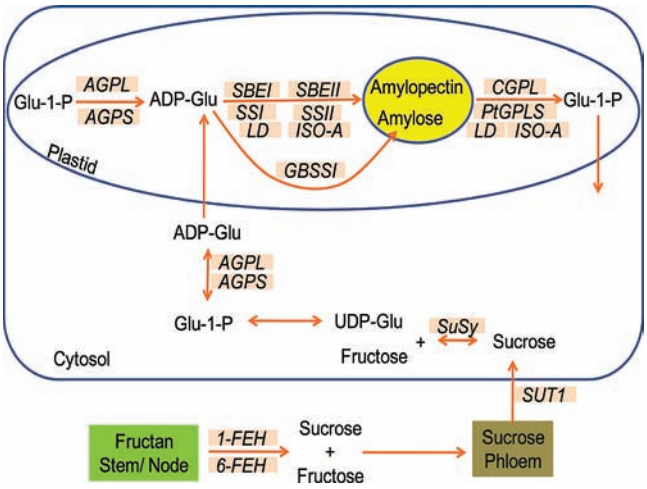

Figure 1. Schematic representation of metabolic processes in endosperm cells showing selected genes used in this study.

branching enzymes (SBE) that catalyze cleavage of α-1,4-linkages and attach the released chains through α-1,6-linkages to new sites on the glucan molecule (**Figure 1**). Starch debranching enzymes (DBE) have been primarily implicated in the complete hydrolysis of starch during seed germination. However, in recent years DBE have been studied for their roles in starch debranching and starch biosynthesis (*5*−*8*). The two types of DBE include limit dextrinase (LD) and isoamylase (ISO-A) (**Figure 1**). The starch phosphorylases, another group of enzymes, have also been associated with starch metabolism in wheat (**Figure 1**), with their physiological roles still requiring further elucidation (*9*).

The complexity of this biosynthetic pathway is further increased by environmental factors that can seriously affect the enzyme activities and consequently the accumulation and composition of starch in the grain (*10*). For example, factors such as water availability, fertilizer, and light quality and quantity during grain filling have all been implicated in final grain quality (for reviews see refs *11*−*14*). These factors are often difficult to precisely control in the field for grain development studies. It has therefore been an arduous task to study starch biosynthesis due to the confounding effects of the environment. Particularly, it has been suggested that the lack of control over supply and composition of assimilates from different parts of the plant further compounds understanding of grain development processes (*15*, *16*). In this context, an *in vitro* culture system based on excised wheat spikes would be highly valuable. Studies have indicated that, in general, physiological and biochemical processes proceeded normally in detached cultured wheat ears (*17*, *18*). Therefore, the objective of this study was to test if an *in vitro* wheat spike culture system could be used to further refine understanding of wheat grain development. Expression patterns of selected metabolic genes (**Figure 1**) were monitored during grain development *in vitro* and compared to patterns in grains developing in growth chamber-grown plants under similar conditions. Detached wheat ears have previously been used to study grain development (*15*, *17*, *19*, *20*). However, unique to the present study is the ability to monitor grain development from anthesis to maturity under *in vitro* culture conditions. We speculate that since anthesis is occurring during the *in vitro* culture process, the grain development *per se* under such conditions is less likely to be perturbed than when starting the culture after anthesis has occurred. Essentially, the timing of anthesis and onset of grain development in growth chamber-grown plants and in *in vitro*-cultured spikes would be a common denominator for grain development.

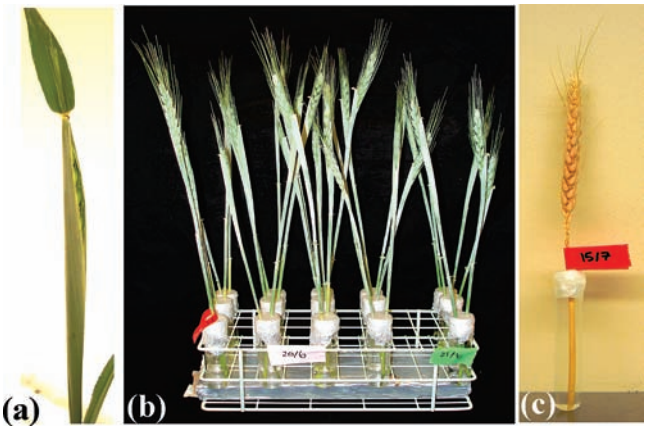

Figure 2. Establishment of spike culture. (**a**) Stage at which spikes were collected for culture; (**b**) spikes in culture medium; (**c**) mature spike after 30 days of culture.

MATERIALS AND METHODS

Plant Growth and Spike Culture Establishment. The soft white spring wheat cv. Fielder was used for *in planta* and *in vitro* grain development studies. Plants were grown in a growth chamber in pots containing Redi-earth (W. R. Grace & Co. of Canada, Ontario, Canada) at 23 °C/16 h light (350 μmol m^{-2} s^{-2} PPFD) and 19 °C/8 h dark. Plants were fertilized every 3 weeks with slow release fertilizer, Nutricote-14-14-14:N-P-K (Plant Products Co. Ltd., Brampton, ON, Canada). Spikes were tagged at anthesis, collected and frozen in liquid N_2 at the selected days postanthesis (DPA), viz., 2, 5, 8, 10, 12, 15, 20, 25, and 30 days, and stored at −80 °C for starch, RNA and protein extraction. Spikes were collected in the midafternoon consistently for all DPA. Three replicate spikes were collected for *in planta* as well as *in vitro* experiments.

For spike culture establishment, immature spikes were cut above the soil surface when awns started to emerge from the leaf sheath. All the leaves and flag leaves were removed (**Figure 2a**). The sheaths were left intact and undisturbed. Excised spikes were placed in tubes containing 4 mL of culture medium and incubated for approximately 40 days in a growth chamber under identical conditions as donor plants (**Figure 2b**,**c**). The spikes were transferred to fresh culture medium every 4 days, after excising a few millimeters to a centimeter from the bottom of the peduncle. Thus, during the course of an experiment, spike length was reduced. Furthermore, if browning of the peduncular tissues occurred in the medium (generally after 15 days in culture), these were promptly excised. The culture medium consisted of 50 g L^{-1} sucrose and 0.4 g L^{-1} L-glutamine, buffered with 0.5 g L^{-1} morpholinoethanesulfonic acid (*21*). Medium pH was adjusted to 6.2.

Determination of Total Starch Concentration. For measurement of total starch concentration during grain development, the Megazyme method based on the amyloglucosidase/α-amylase procedure was used (Megazyme International Ireland Ltd., Bray Business Park, Ireland). Briefly, 3−6 seeds from the middle region of the spike from each of the three biological replicates were ground to a fine powder, and a 100 mg flour sample was transferred into 0.2 mL of 95% (v/v) ethyl alcohol. Thermostable α-amylase was added to partially hydrolyze the starch. Amyloglucosidase was added, and samples were incubated at 50 °C for 30 min. After treatment with GOPOD (glucose oxidase, peroxidase and 4-aminoantipyridine) reagent, the amount of glucose was measured. Starch concentration was calculated as % dry weight basis as described by McCleary et al. (*22*).

Determination of Amylose Concentration. For determination of amylose concentration, starch was extracted as for starch concentration determination (see above) using a cesium chloride method (*23*) with modifications. Amylose concentration was determined as described by Demeke et al. (*24*). Briefly, 4−6 mg of starch was weighed and the concentration adjusted to 1 mg mL^{-1} in a glass test tube. The samples were incubated at 130 °C for 15 min, vortexed and incubated for another 15 min. To 1 mL of sample in a 1.5 mL microcentrifuge tube, 55 μL of 1 M sodium acetate (pH 4.0) and 3 μL of isoamylase (200 U mL^{-1}) were added, and the sample

Table 1. Sequences of Oligodeoxynucleotide Primers Used for Real-Time PCR Amplification, GenBank Accession Numbers of the Genes Used, the Corresponding Amplification Product Sizes and Amplification Efficiencies

gene	GenBank accession no.	forward (F) and reverse (R) primers	primer sequence 5′ ... 3′	amplicon size (bp)	efficiency (%)
ADP-glucose pyrophosphorylase (*large subunit*)	AJ563452	F/AGPL	TCTTCCCACTCACAAGCACAAG	162	102
		R/AGPL	TGCGGTGAATGTGGCGATTAAG		
ADP-glucose pyrophosphorylase (*small subunit*)	AF244997	F/AGPS	AATGTTCAAGAAGCGGCAAGGG	125	101
		R/AGPS	TTGGCACACATTTCACACCTGC		
starch branching enzyme I	Y12320	F/SBEI	TTTGGCCACCCAGAATGGATTG	152	100
		R/SBEI	TTGTCGTCGAGCGCATTCATTG		
starch branching enzyme IIa	Y11282	F/SBEII	AGAGCACATGAGCTTGGTTTGC	94	100
		R/SBEII	TGCCATCGAAACCATTCAAGCC		
starch synthase I	AJ292522	F/SSI	ACACAGTCGAGACCTTCAACCCTT	177	103
		R/SSI	TATGGTCTTTCGTCATGCCTCGCT		
starch synthase II	AB201445	F/SSII	AGCATTTCCACCTGCCACAAACA	143	99
	AB201446	R/SSII	AACGTGCCAAGGAACCTGCAAA		
	AB201447				
granule-bound starch synthase I	Y16340	F/GBSSI	TTGTTGTAGCGAAGAAGGGCCG	140	100
		R/GBSSI	ATGCAAGCACAGTTCAGCACAC		
isoamylase	AF438328	F/ISO-A	TGCTGCCTCATGACCAAATTCC	119	100
		R/ISO-A	TTCTCAGACCAATCAGGCTTCC		
limit dextrinase	AF359460	F/LD	TGCGGCTTGAATTGCATCCTGT	155	100
		R/LD	TGCGTTGAAACCAAGGAGGCAT		
cytosolic glucan phosphorylase	AY237103	F/CGPL	TGATGTTGGTGCTGTGGTGAAC	158	100
		R/CGPL	TTGTTCCACTTGCTTCCATGCC		
plastidic glucan phosphorylase	AY357118	F/PtGPLS	ATGGCAAAGTGGTTGAAGGCAC	200	99
		R/PtGPLS	TTCGTTGGCCTTGACATGATCC		
fructan 1-exohydrolase	FJ184990	F/1-FEH	TTGACCCGAACAAGAACAGGAGGA	98	101
		R/1-FEH	TACATGGAGTCCTGCCCAACCTTT		
fructan 6-exohydrolase	AM075205	F/6-FEH	AAGTTCTCATGTGCACCGACCTCA	158	100
		R/6-FEH	ACCTCCAAAGCTTTCCACTACCGA		
sucrose transporter I	AF408842	F/SUT1	TGGACCCAGTGCAGCAAATTCA	189	100
	AF408843	R/SUT1	AAACAGAAGGCCAGGAACAGCA		
	AF408844				
sucrose synthase	AJ001117	F/SuSy	TCTGTGACACCAAGGGAGCATT	99	98
		R/SuSy	TCGCAATTGTCGGCAAACCACA		
ubiquitin	DQ086482	F/UBI	CCTTGGCGGACTACAACATC	164	100
		R/UBI	GCAACGACAGACACAGACC		

was incubated at 40 °C for 4 h. The samples were then placed in a boiling water bath for 20 min, frozen at −70 °C for 30 min and lyophilized overnight. The lyophilized samples were resuspended in 200 μL of HPLC grade dimethyl sulfoxide, mixed by vortexing and allowed to stand at room temperature for 10 min. After mixing again, samples were centrifuged at 15500*g* for 10 min and 150 μL of the supernatant was used for HPLC analysis (*24*).

Total RNA Extraction, cDNA Synthesis and Real-Time PCR Analysis. Total RNA was extracted from frozen caryopses using a phenol:guanidine method, specifically optimized to maximize RNA yield and prevent starch interference during the extraction procedure. Grains (4−6) collected from the middle region of the spikes were ground in liquid N_2 in a −20 °C precooled RNase-free pestle and mortar. Ground seeds were transferred to an RNase-free container at room temperature containing 3 mL of hot (65 °C) acidic phenol:guanidine mix (50% (v/v) acidic phenol, 30% (w/v) guanidine HCl and 5% (w/v) SDS). The slurry was mixed well with a pipet, transferred to a 15 mL conical tube, vortexed and incubated at 65 °C for 10 min, with shaking performed several times during incubation. The tube was centrifuged at 15500*g* for 5 min at 2 °C. The supernatant was transferred to a new RNase-free tube, 3 mL of chloroform was added and the tube was vortexed for 15 s. The tube was again centrifuged at 15500*g* for 5 min at 2 °C, and the aqueous phase was transferred to a new RNase-free tube and re-extracted with 3 mL of chloroform. The supernatant was transferred to a new RNase-free tube and 3 mL isopropanol were added. The sample was mixed and incubated at −80 °C for 15 min. The RNA was pelleted by centrifugation at 15500*g* for 10 min at 2 °C. The RNA pellet was washed in 1 mL of 75% (v/v) ethanol, briefly air-dried and resuspended in 50−100 μL of DEPC-treated water. RNA integrity was determined on a Bioanalyzer 2100 (Agilent Technologies Canada, Inc., Mississauga, Ontario, Canada).

Total RNA (5 μg) was treated with TurboDNA-free DNase (Ambion, Inc., Austin, TX) to remove any contaminating genomic DNA. For cDNA synthesis, 1 μg of the DNase-treated RNA was reverse-transcribed using 200 U of Superscript III (Invitrogen, Inc., Burlington, Ontario, Canada) according to the manufacturer's instructions and containing 500 ng of oligod$T_{(12-18)}$ (Invitrogen, Inc., Burlington, Ontario, Canada) and 900 ng of random primer (Invitrogen, Inc., Burlington, Ontario, Canada).

Quantitative real-time PCR analyses were performed as previously reported (*25*). Briefly, 25 μL reactions containing each of the primer pairs were set up as follows: 3 μL of 1/15 dilution of cDNA, 600 nM each of forward and reverse gene-specific oligodeoxynucleotide primers (**Table 1**), and 12.5 μL of 2× Maxima SYBR Green I master mix (Fermentas Life Sciences, Burlington, Ontario, Canada). Real-time PCR was performed in an Mx3000P (Stratagene, Cedar Creek, TX). The wheat *ubiquitin* gene was used as a reference to normalize expression of genes of interest. For each time-point, three biological replicates × two technical replicates were set up. Standard curves were run for each of the genes of interest and the reference gene to determine the amplification efficiency (**Table 1**). Furthermore, *ubiquitin* was selected based on its superior stability compared to *18S rRNA*, *28S rRNA*, *actin*, *GAPD*, *cyclophilin*, α-*tubulin* and β-*tubulin* genes using the geNorm algorithm (*26*).

Protein Extraction and Western Blotting for GBSSI Polypeptide Detection. Starch granule proteins were extracted as previously described (*27*). Briefly, 3 grains without embryos from the middle region of the spikes from the three replicates were imbibed overnight at 4 °C in 1 mL of deionized water. Following imbibition, the water was removed and the grain crushed in 300 μL of deionized water and overlayered with 1 mL of 80% (w/v) cesium chloride. The sample was then centrifuged at 15000*g* for 5 min. The starch pellet was washed twice in 1 mL of wash buffer (55 mM Tris-HCl, pH 6.8; 2.3% (w/v) SDS; 10% (v/v) glycerol;

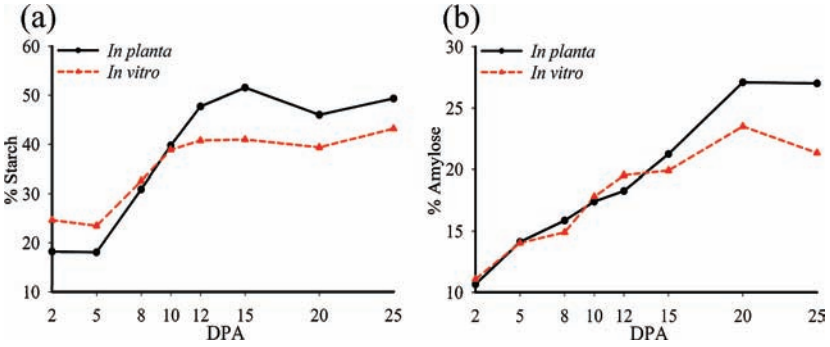

Figure 3. Starch and amylose concentrations in developing wheat grains from growth chamber-grown plants and *in vitro*-cultured spikes. (**a**) Starch concentration; SE of data points = 0.54. (**b**) Amylose concentration; SE of data points = 0.46.

5% (v/v) β-mercaptoethanol), three times with water and once with acetone and dried. Granule proteins were extracted by boiling 100 mg of starch in 100 μL of buffer (62.5 mM Tris-HCl, pH 6.8; 4% (v/v) SDS; 5% (v/v) β-mercaptoethanol; 10% (v/v) glycerol) and cooling on ice for 10 min. Samples were subsequently centrifuged and the protein concentration was determined according to the Bradford protein dye binding method (*28*). Five micrograms of proteins was loaded on an SDS−PAGE gel as described previously (*27*). Proteins were electrophoretically transferred to a 0.45 μm pore size nitrocellulose membrane (GE Healthcare, Quebec, Canada). The membrane was incubated with a 1:2000 dilution of anti-GBSSI antibody for 2 h after blocking with 4% (w/v) nonfat dry milk in TBS as described by Ganeshan et al. (*25*). Briefly, Alkaline Phosphatase Labeled Goat anti-Rabbit IgG (KPL, Inc., Gaithersburg, MD) was applied at 1:50000 dilution after washing the membrane with TBS containing 0.05% (v/v) Tween-20 and 0.2% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO). The protein:antibody complex was visualized by PhosphaGlo Reserve AP Chemiluminescent Substrate (KPL, Inc., Gaithersburg, MD). A Chemi-Doc XRS (Bio-Rad Laboratories, Mississauga, Ontario, Canada) image capture system was used to detect chemiluminescence emitted from the membrane. The Quantity One (version 4.6.3.) software (Bio-Rad Laboratories, Mississauga, Ontario, Canada) was used for densitometric quantification of the GBSSI polypeptides. Densitometric values were combined for the GBSSI polypeptides from the A, B and D genomes. To convert the densitometric values (intensity mm^{-2}) to percent expression values, the highest value was set at 100% and all other samples were considered to be expressing at a percentage relative to this sample.

Data Analyses. Real-time PCR data were analyzed as previously described (*25*), using three biological × two technical replicates. Relative expression levels were determined by the ΔΔCt method (*29*). All data generated were statistically analyzed using the Minitab software (Version 14) (Minitab, Inc., Pennsylvania). *In planta* expression data and *in vitro* expression data were separately analyzed using ANOVAs.

RESULTS AND DISCUSSION

Overall *in Vitro* Spike Culture Responses. The *in vitro* spike culture system established in this study was based on the culture of excised immature wheat spikes, prior to spike emergence from the leaf sheath (boot) or when less than a centimeter of the awns was visible (**Figure 2a**). Wheat spike in the context of this study refers to the culm and leaf sheath enclosing the spike proper. This system was developed recently to monitor grain development under defined conditions (*21*, *30*). Generally, the *in vitro* grain maturity (from anthesis to complete dry grain) was found to be expedited, reaching completion in about 30 days after anthesis as opposed to grains from growth chamber-grown plants which generally reached maturity in about 60 days. Previous studies have indicated that grains from the *in vitro*-cultured spikes weighed half as much as field-harvested grains (*21*). Our preliminary studies in attempting to mimic the intact plant by leaving the leaves on the stem during culture led to senescence of the leaves, and subsequently senescence of the stems after about 15 days. Grain development failed, resulting in empty or abortive caryopses (data not shown). No further attempts were made to optimize the culture conditions with leaves remaining on the stem. It is possible that leaving the leaves on the stem may have diverted the sucrose primarily to the leaves, thereby causing nutritional stress to the developing grains. Further studies need to be conducted using radiolabeled ^{14}C-sucrose to confirm this. Perhaps with more specialized culture medium composition, abortion of grain development could have been prevented.

Starch Accumulation. Starch accumulation during *in planta* and *in vitro* grain development followed similar trends with an exponential phase and a plateau phase, but was significantly different between the two systems ($p < 0.001$) (**Figure 3a**). The concentration of starch in grains of *in vitro*-cultured spikes was higher during the early developmental stages compared to grains developing on the growth chamber-grown plants. By 10 DPA, starch accumulation reached saturation in the grains of *in vitro*-cultured spikes and was about 10% lower at 12 DPA compared to the developing grains of growth chamber-grown plants. This rapid accumulation of starch to saturation in the grains of *in vitro*-cultured spikes is a likely reason for reduced seed weight compared to accumulation in grains developing *in planta*. Similarly, amylose concentration increased consistently during grain development in both *in planta* and *in vitro* grown spikes, with significant effects due to DPA, development condition (*in vitro* vs *in planta*) and DPA × development condition ($p < 0.001$) (**Figure 3b**). During the later phase of development at 25 DPA, the grain amylose concentration of *in vitro*-cultured spikes was about 5% lower than in grains of *in planta*-grown spikes (**Figure 3b**). It therefore appears that although grain development is expedited in *in vitro*-cultured spikes, the ratio of starch to amylose is not affected. A previous study showed that although starch concentration was reduced in grains of *in vitro*-cultured spikes, the starch:protein ratio was similar to that in grains developing *in planta* (*21*). The development *in vitro* appears to be mimicking the *in planta* grain starch accumulation pattern under high temperature (34 °C), wherein the decrease in starch content was attributed to the reduced duration of starch accumulation (*10*).

Expression of Starch Biosynthesis-Related Genes. The results presented in this study indicate differences in gene expression patterns between grains of *in vitro*-cultured spikes and grains of growth chamber-grown plants during development. A combined ANOVA for *in planta* and *in vitro* expression data indicated significant DPA ($p < 0.001$), gene ($p < 0.001$) and DPA × gene effects ($p < 0.001$). The expression peaks of the 11 starch metabolic enzyme genes ranged from the early to mid developmental stage of the spike for growth chamber-grown plants, except for *CGPL*, which showed peak expression at 20 DPA

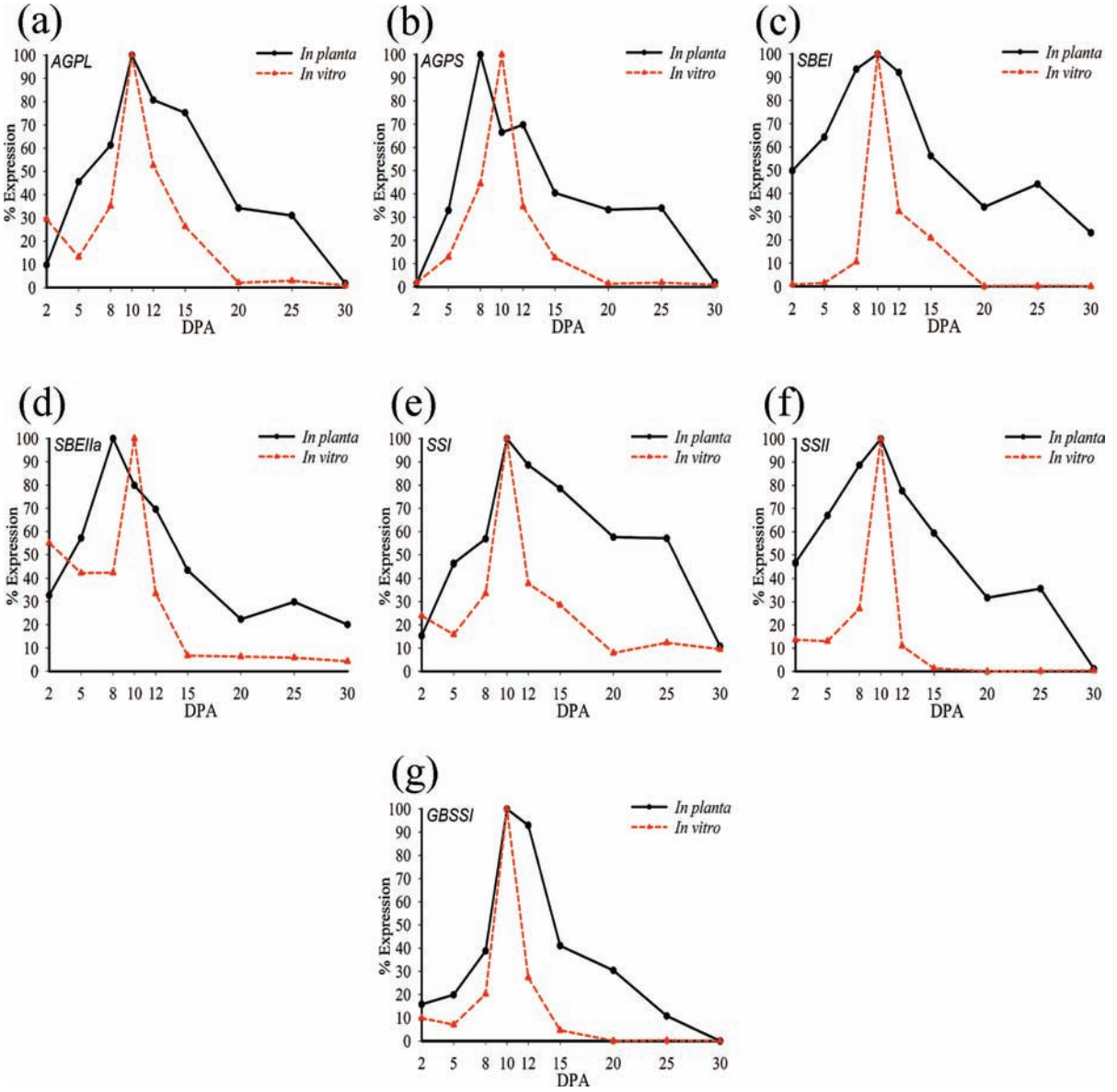

Figure 4. Quantitative expression of starch biosynthetic enzyme genes in developing grains from growth chamber-grown plants and *in vitro*-cultured spikes. (**a**) *AGPL*; SE of data points = 6.4. (**b**) *AGPS*; SE of data points = 2.0. (**c**) *SBEI*; SE of data points = 2.6. (**d**) *SBEIIa*; SE of data points = 2.5. (**e**) *SSI*; SE of data points = 2.0. (**f**) *SSII*; SE of data points = 4.6. (**g**) *GBSSI*; SE of data points = 1.2.

(**Figures 4** and **5**). *AGPL* and *AGPS* transcript accumulation in *in planta* developing grains was similar to previously reported observations (*e.g.*, ref *31*), with high expression during the early grain filling period. Similarly, levels of *SBEI* and *SBEIIa* transcripts peaked at 10 and 8 DPA, respectively (**Figure 4c**,**d**), when starch accumulation was very active, and decreased steadily thereafter. The three starch synthases, *SSI*, *SSII* and *GBSSI*, also showed increasing expression to 10 DPA, followed by a gradual decrease in expression to 30 DPA (**Figure 4e**−**g**). The debranching enzyme gene, *isoamylase*, exhibited a steady increase in transcript accumulation up to 10−12 DPA in *in planta*-developing grains, with a gradual decline thereafter (**Figure 5a**). Limit dextrinase transcripts in grains from growth chamber-grown plants exhibited peak levels at 12 DPA, with a sharp decrease to 20 DPA (**Figure 5b**).

The two other genes studied, cytosolic glucan phosphorylase (*CGPL*) and plastidic glucan phosphorylase (*PtGPLS*), have been shown to be involved in transitory leaf starch and seed starch processing, respectively (*9*). The expression of *PtGPLS* in endosperms of *in planta*-developing grains in the present study reached a maximum at 10 DPA and that of *CGPL* reached a peak at 20 DPA (**Figure 5c**). This is consistent with the role of *PtGPLS* in starch reserve accumulation in the seed (*9*). However, accumulation of *CGPL* transcripts in the developing grains has not been reported before. According to Schupp et al. (*9*), *CGPL* is involved in processing carbohydrates in the early phase of growth.

For *in vitro*-cultured spikes, expression peaks for all the starch metabolic genes were observed at 10 DPA followed by an abrupt decrease at 12 DPA (**Figures 4** and **5**). The transcript accumulation in grains of the *in vitro*-cultured spikes reflects observations on transcript accumulation reported at high temperatures during grain filling (*10*), correlating with the expedited maturation of the grains *in vitro*. In grains of *in vitro*-cultured spikes, *CGPL* showed 80% expression at 2 DPA and expression decreased sharply at 5 DPA before reaching a maximum at 10 DPA (**Figure 5c**). It appears that *CGPL* has a role in grain development, in addition to its involvement in early stages of growth, although further studies need to be conducted to confirm this hypothesis. Expression of *PtGPLS* in grains from *in vitro*-cultured spikes followed patterns

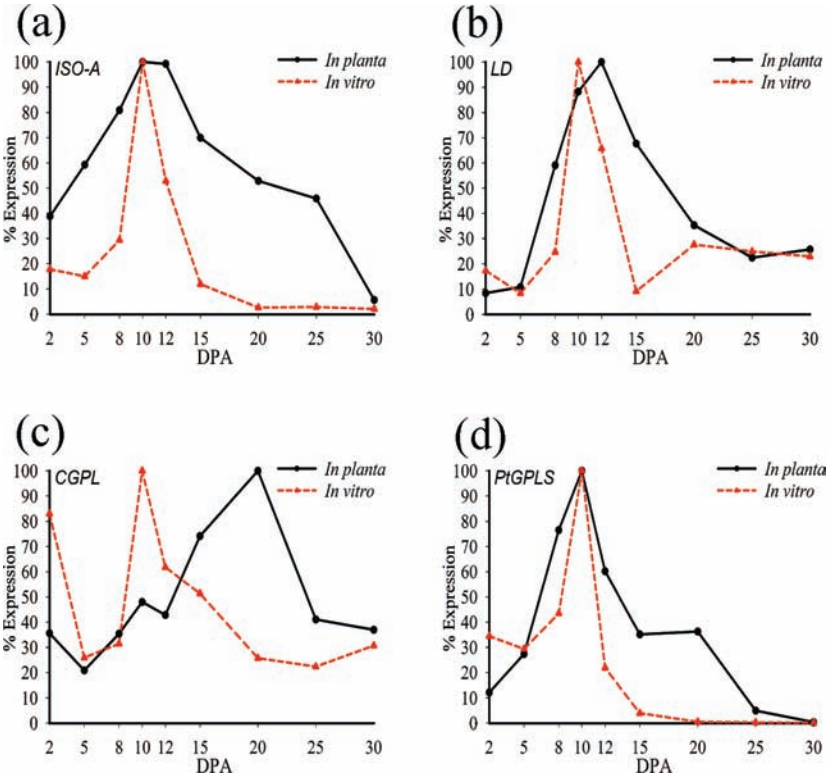

Figure 5. Quantitative expression of starch degrading enzyme genes in developing grains of growth chamber-grown plants and *in vitro*-cultured spikes. (**a**) *ISO-A*; SE of data points = 11.0. (**b**) *LD*; SE of data points = 7.5. (**c**) *CGPL*; SE of data points = 5.4. (**d**) *PtGPLS*; SE of data points = 7.2.

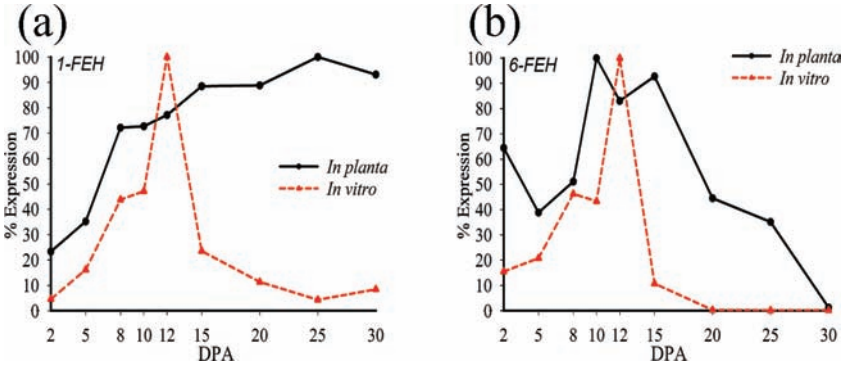

Figure 6. Quantitative expression of fructan metabolizing genes, *1-FEH* and *6-FEH*, in developing grains from growth chamber-grown plants and *in vitro*-cultured spikes. (**a**) *1-FEH*; SE of data points = 6.6. (**b**) *6-FEH*; SE of data points = 6.4.

similar to those of other metabolic genes, reaching peak expression at 10 DPA (**Figure 5d**).

Expression of *FEH*, *SUT1* and *SuSy* Genes. To further elucidate the differences observed between grains developing *in planta* and *in vitro*, expression of wheat *fructan 1-exohydrolase* (*1-FEH*), *fructan 6-exohydrolase* (*6-FEH*), *sucrose transporter* (*SUT1*) and *sucrose synthase* (*SuSy*) genes were studied. Fructans are fructose polymers serving as soluble reserve carbohydrates in many flowering plants (for a review, see ref *32*). In cereals, fructan-derived carbohydrates in the young internode are mobilized into grains during development. To test the possibility that *FEH* genes, which code for enzymes involved in the breakdown of fructans, may have contributed to a rapid influx of carbohydrates into developing caryopses, and therefore increased the rate of starch biosynthesis, expression of these genes was examined. Transcript accumulation for both these genes in *in planta*-developing grains has been reported to occur with maximum *6-FEH* expression later during grain development and *1-FEH* expression peaking in early stages of development (*33*). In the present study, *1-FEH* steadily increased during grain development and *6-FEH* showed maximum expression at 10 DPA and declined gradually thereafter (**Figure 6a**,**b**). Expression in grains of *in vitro*-cultured spikes reached a maximum at 12 DPA (**Figure 6a**,**b**). Thus, the observed increase in expression of genes in *in vitro*-cultured spikes may not be the result of an abundance of fructan-catabolized carbohydrates being available for starch biosynthesis, since the starch metabolic genes showed peak expression two days earlier in the *in vitro*-cultured spikes.

The sharp increase in expression by 10 DPA for genes in grains of *in vitro*-cultured spikes could have been due to sucrose (5%) in the culture medium. To explore this possibility, expression of a wheat *sucrose transporter* (*SUT1*) was studied. Sucrose transporters have been suggested to be involved in the postphloem sugar transport pathway during grain filling (*34*−*36*). We therefore expected an early peak in expression for *SUT1* gene in grains of *in vitro*-cultured spikes, possibly leading to increased starch

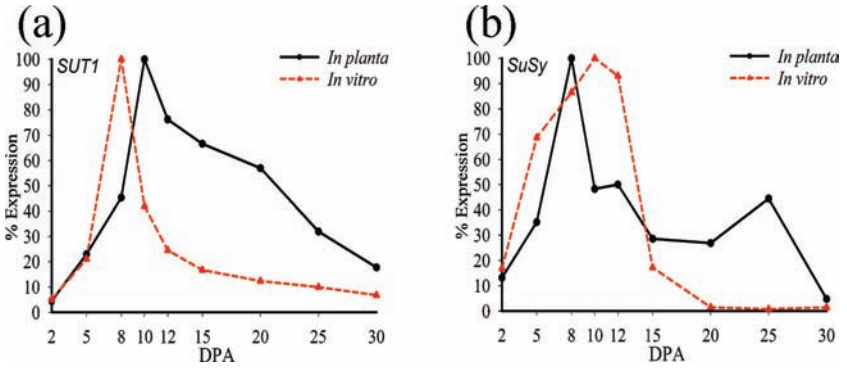

Figure 7. Quantitative expression of (**a**) *sucrose transporter* gene, *SUT1*; SE of data points = 4.5; and (**b**) *sucrose synthase* gene, *SuSy*; SE of data points = 1.3; from developing grains from growth chamber-grown plants and *in vitro*-cultured spikes.

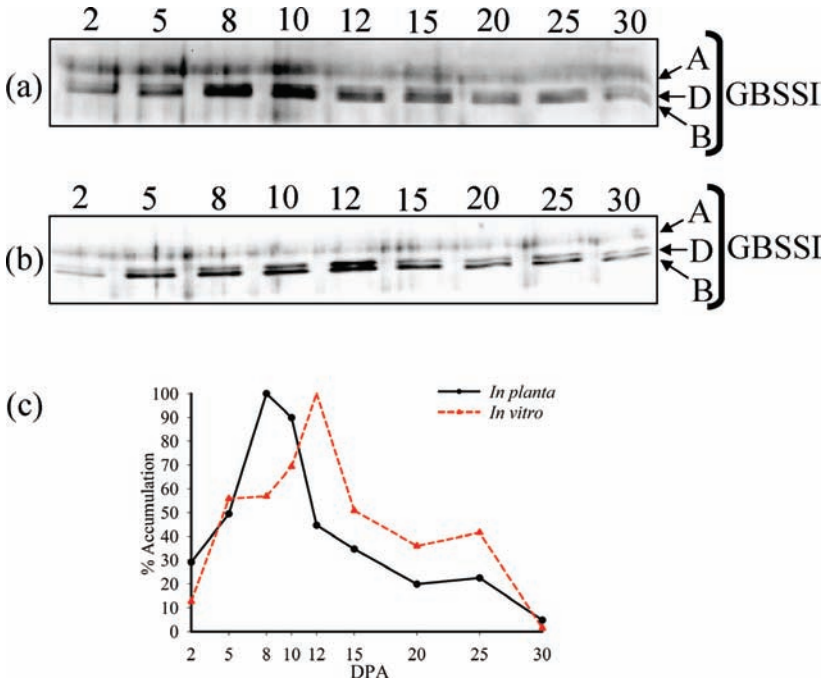

Figure 8. Accumulation of GBSSI polypeptides in developing grains from growth chamber-grown plants and *in vitro*-cultured spikes. Western blot showing the GBSSI polypeptides from the three genomes in (**a**) grains from growth chamber-grown plants and (**b**) grains from *in vitro*-cultured spikes. (**c**) Quantitative accumulation of the GBSSI polypeptides using densitometric analyses, shown as percent accumulation of the GBSSI polypeptides from Western blot data, with the highest accumulation in each development system being set at 100% and all other samples being relative to each of these two highest samples.

biosynthesis. Our data indicate that in grains from *in vitro*-cultured spikes peak expression of *SUT1* was at 8 DPA (**Figure 7a**), two days earlier than peaks observed for starch metabolic genes from grains of *in vitro*-cultured spikes. It is therefore possible that an early availability of sucrose transporters led to greater sucrose influx, which triggered expression of other metabolic genes to process sucrose influx. However, a rapid decline in expression is still intriguing. It appears that sucrose overloading may have contributed greatly to an accelerated rate of gene expression in grains of *in vitro*-cultured spikes in the early phase of grain development, but not at later stages of development.

The expression of the *sucrose synthase* (*SuSy*) gene was also assessed due to the differences observed for starch biosynthetic gene expression patterns between grains developing *in planta* and those developing on *in vitro*-cultured spikes. *SuSy* catalyzes the conversion of sucrose to fructose and uridine diphosphate-glucose (UDPG) (*37*). The UDPG produced has been suggested to play a role in starch accumulation (*38*). In the present study, expression of *SuSy* was found to rapidly reach maximum levels at 8 DPA in *in planta*-developing grains (**Figure 7b**) with an abrupt decline at 10 DPA. In grains of *in vitro*-cultured spikes, expression of *SuSy* increased steadily up to 10 DPA before abruptly decreasing after 12 DPA (**Figure 7b**). This pattern is somewhat different from other genes studied in the *in vitro*-cultured spikes. It is possible that an accumulation of SuSy may have to some extent also triggered an increased rate of starch biosynthesis in grains of *in vitro*-cultured spikes, along with help from sucrose transporters, considering that expression of *SuSy* was about 70% and 90% by 5 DPA and 10 DPA, respectively.

GBSSI Polypeptide Accumulation. The accumulation of GBSSI polypeptides was monitored to see if it reflected *GBSSI* gene expression patterns. Since the A, B and D genome GBSSI polypeptides separately showed similar accumulation patterns (data not shown), densitometry values were averaged to reflect QPCR data which were for *GBSSI* transcripts from all three genomes. In grains from growth chamber-grown plants maximum GBSSI

accumulation occurred at 8 DPA, and in grains from *in vitro*-cultured spikes maximum accumulation was observed at 12 DPA (**Figure 8**). The peaks in *GBSSI* transcript abundance in both *in vitro* and *in planta* systems were at 10 DPA. It is apparent that in the two systems *GBSSI* mRNA abundance does not reflect translational product abundance. While the accumulation patterns of the transcripts and polypeptides in the *in vitro* system would be the generally accepted trend, wherein maximum transcript levels precede protein accumulation, trends in the *in planta* system do not correspond to that notion. However, several recent reports have indicated that transcript and protein abundance may not always be correlated (*39*) due to mRNA secondary structure and translational efficiency (for review, see ref *40*). In the present study, the observation that the *GBSSI* transcript peak is two days later than the polypeptide peak may be an indication of translational efficiency and/or mRNA stability in grains from growth chamber-grown plants, considering that by 8 DPA the level of *GBSSI* transcript was at 40%. Furthermore, this is an indication that the control of transcript accumulation in the *in vitro* system is different from the one *in planta* with regard to GBSSI, which warrants further investigation.

The spike culture system presented in this study augurs well for a defined study of wheat grain development without confounding effects of extraneous factors. Furthermore, the culture medium can be variously supplemented, for example with growth regulators, to observe effects on grain development. Although at this time the *in vitro* spike culture system cannot be used as a model fully comparable to growth chamber-grown plants, it will be valuable for basic studies with regard to metabolic activities in the grain. Future research includes further defining the culture medium with respect to carbohydrate and amino acid type and concentration. In a previous study (*21*), we have shown that using Murashige−Skoog (MS) medium led to an increase in protein concentration and was attributed to the high amount of inorganic nitrogen in the MS medium. Also of relevance would be biochemical studies relating to amylopectin architecture and starch granule size distribution in the cereal grains from *in vitro*-cultured spikes.

ACKNOWLEDGMENT

The excellent technical assistance of Carli Holderness and Shuhua Zhou is greatly appreciated.

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Received for review November 3, 2009. Revised manuscript received January 27, 2010. Accepted March 08, 2010. Natural Science and Engineering Research Council, Genome Canada/Genome Prairie, Canada Research Chairs and Canada Foundation for Innovation are gratefully acknowledged for supporting research in our laboratories.