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Endosperm Carotenoid Concentrations in Wheat are Better Correlated with *PSY1* Transcript Levels than Enzyme Activities

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ABSTRACT

Phytoene synthase (PSY) catalyzes a key reaction in the biosynthesis of carotenoids, which are major contributors of grain yellow pigment content (GYPC) in wheat (*Triticum* spp.). Although quantitative trait loci (QTL) for GYPC have been linked to *PSY1* homoeologs in tetraploid and hexaploid wheat, the underlying molecular and biochemical mechanisms for such associations are not well understood. Functional characterization of wheat *PSY1* homoeologs indicated that variations in *PSY1* homoeolog enzyme activities did not correspond to different levels of GYPC in the wild-type tetraploid and hexaploid wheat genotypes analyzed. However, a correlation between *PSY1* homoeolog expression and carotenoid accumulation in grain endosperm was evident, suggesting that GYPC is at least partially modulated by transcriptional regulation of *PSY1* homoeologs in wheat grains. Further promoter analysis of *PSY-A1* homoeologs with diverse expression profiles in three wheat genotypes revealed not only potential motifs for light and hormonal responses but also new candidate *cis*-regulatory elements. Overall, the knowledge on the association of *PSY1* homoeolog expression and GYPC can be directly applied to breeding tetraploid and hexaploid wheat varieties with desirable carotenoid levels.

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Abbreviations: cDNA, complementary DNA; cRNA, complementary RNA; EMS, ethyl methanesulfonate; EMS, ethyl methanesulfonate; GGPP, geranylgeranyl pyrophosphate; GYPC, grain yellow pigment content; HPLC, high-performance liquid chromatography; LRE, light response element; PSY, phytoene synthase; qPCR, quantitative polymerase chain reaction; QTL, quantitative trait loci.

GRAIN ENDOSPERM COLOR is considered one of the important criteria for breeding tetraploid durum wheat (genomes AABB) and hexaploid common wheat (genomes AABBDD) varieties. Although yellow tetraploid wheat flour (i.e., endosperm of grains) is preferred for making pasta and couscous, white hexaploid wheat flour has traditionally been used for baking bread. Carotenoid pigments constitute a major source of GYPC in wheat (Calucci et al., 2004; Hentschel et al., 2002; Panfili et al., 2004; Pozniak et al., 2007; Zhang and Dubcovsky, 2008). Lutein is the most abundant carotenoid pigment in mature tetraploid wheat grains; mature hexaploid wheat grains, on the other hand, accumulate very low levels of total carotenoids as a result of breeding for white grain endosperm (Howitt et al., 2009; Qin et al., 2012).

In higher plants, carotenoids compete with several other plastid-localized metabolites for the common biosynthetic precursor, geranylgeranyl pyrophosphate (GGPP) (Fig. 1). Phytoene synthase

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Fig. 1. Carotenoids share the common biosynthetic precursor geranylgeranyl pyrophosphate (GGPP) with other plastid-localized metabolites. PSY, phytoene synthase.

catalyzes the first committed reaction of the carotenoid biosynthetic pathway and directs C fluxes to carotenoid production in plastids (Fig. 1). Three paralogous *PSY* genes (*PSY1-3*) have been identified in wheat and were mapped to chromosome 7 (*PSY1*) or different regions of chromosome 5 (*PSY2* and *PSY3*) (Dibari et al., 2012; Howitt et al., 2009; Pozniak et al., 2007). However, of the three wheat *PSYs*, only *PSY1* has been intensively examined for its contribution to GYPC (He et al., 2008; Howitt et al., 2009; Pozniak et al., 2007; Zhang and Dubcovsky, 2008).

Allelic variations in or near *PSY-A1* genomic DNA sequences have been associated with major QTL for GYPC on chromosome 7AL of tetraploid and hexaploid wheat varieties (Crawford et al., 2011; He et al., 2008, 2009; Howitt et al., 2009; Singh et al., 2009; Zhang and Dubcovsky, 2008). Interestingly, a low GYPC hexaploid wheat cultivar Sunco contains an insertion in the second exon of *PSY-A1*, leading to the formation of an alternative splicing site and activation of a cryptic exon in *PSY-A1* (Howitt et al., 2009). These *PSY-A1* variants resulted from alternative splicing and expression of the cryptic exon encode nonfunctional PSY proteins and were suggested to dilute the functional *PSY-A1* transcript pool (i.e., the wild-type *PSY-A1*), thus giving rise to an overall low GYPC in Sunco (Howitt et al., 2009). When *PSY-A1* protein sequences from the tetraploid wheat breeding line UC1113 (low GYPC) and cultivar Kofa (high GYPC) and the hexaploid wheat cultivar Chinese Spring (low GYPC) were compared, only a conservative amino acid substitution (Ser in UC1113 and Kofa to Thr in Chinese Spring) was identified among the three wheat genotypes, suggesting that these tetraploid and

hexaploid wheat *PSY-A1* proteins likely have comparable enzymatic activities (Zhang and Dubcovsky, 2008).

Major QTL for GYPC have also been linked to *PSY-B1* alleles located on chromosome 7BL of tetraploid and hexaploid wheat (He et al., 2009; Pozniak et al., 2007; Zhang and Dubcovsky, 2008). An unusual conversion *PSY-B1* allele was identified in the tetraploid wheat cultivar Kofa, which resulted from the introgression of Kofa *PSY-A1* into the normal *PSY-B1* allele commonly found in tetraploid wheat varieties; the *PSY-B1* conversion likely occurred in the Cappelli *ph1c* mutant (Zhang and Dubcovsky, 2008). In the recombinant inbred lines of UC1113 and Kofa, the conversion *PSY-B1* was consistently associated with a high GYPC QTL located on chromosome 7BL according to analysis using a codominant marker (Zhang and Dubcovsky, 2008). Despite many polymorphisms in the DNA sequences, the conversion *PSY-B1* differs from the normal tetraploid wheat *PSY-B1* in only two amino acids at the protein sequence level; these amino-acid changes were evaluated and predicted not to impact *PSY-B1* enzyme activities (Zhang and Dubcovsky, 2008). When protein sequences of the normal tetraploid wheat *PSY-B1* and the hexaploid wheat *PSY-B1* were compared, modifications of three amino acids were found, of which the substitution of Phe at position 324 (normal tetraploid wheat *PSY-B1*) by Val (hexaploid wheat *PSY-B1*) was suggested to affect the biochemical property of the amino acid and may alter the hexaploid wheat *PSY-B1* enzyme activities (Zhang and Dubcovsky, 2008).

Although QTL for GYPC have not been mapped to chromosome 7DL (where *PSY-D1* is located) in hexaploid wheat (Wang et al., 2009), association mapping studies revealed that *PSY-D1* plays an important role in GYPC (Ravel et al., 2012). In addition to the A-, B- and D-genome *PSY1* homoeologs, the function of *PSY1* in GYPC was also demonstrated using *PSY-E1* originated from tall wheatgrass (*Lophopyrum ponticum* (Podp.) Barkworth & D. R. Dewey; genomes AABBEE) (Zhang and Dubcovsky, 2008). It was shown that *LpPSY-E1* was completely linked to a high GYPC locus on chromosome 7EL in the recombinant chromosomes generated from 7AL (tetraploid wheat) and 7EL (tall wheatgrass) (Zhang and Dubcovsky, 2008). Agatha is a high GYPC hexaploid wheat cultivar that contains a 7DL/7EL translocation (i.e., substitution of the hexaploid wheat chromosome 7DL with the tall wheatgrass chromosome 7EL) (Zhang et al., 2005). An ethyl methanesulfonate (EMS)-induced mutant of Agatha, Agatha 28-4, has white grain endosperm and contains a missense mutation in a highly conserved amino acid (Pro at position 422 in Agatha to Leu in Agatha 28-4) of *PSY-E1*. It was suggested that such an amino-acid mutation may reduce *PSY-E1* enzyme activities and lead to decreased GYPC in Agatha 28-4 (Zhang and Dubcovsky, 2008).

Overall, the compelling genetic evidence has established the critical role of PSY1 homoeologs in controlling GYPC of tetraploid and hexaploid wheat. However, the mechanistic basis of how PSY1 homoeologs contribute differently to GYPC in various tetraploid and hexaploid wheat genotypes remains unclear. In this work, we characterized the enzymatic activities of various wheat PSY1 homoeologs using a carotenoid-accumulating *Escherichia coli* system. To evaluate the impact of PSY1 homoeolog transcript abundance on carotenoid production in the flour and the photosynthetic tissue of wheat grains, we also determined the transcript levels of PSY1 homoeologs and carotenoid concentrations in endosperm or flour and pericarp or photosynthetic tissue of developing tetraploid and hexaploid wheat grains. Furthermore, we isolated and analyzed the promoter sequences of PSY-A1 homoeologs from three tetraploid and hexaploid wheat genotypes with different PSY-A1 expression profiles.

MATERIALS AND METHODS

Plant Materials

Seedlings of tetraploid wheat breeding line UC1113 and cultivar Kofa, as well as hexaploid wheat breeding line UC1041, cultivar Agatha, and mutant Agatha 28-4, were grown under a 16-h light vs. 8-h dark cycle in a temperature-controlled growth chamber. Leaves, stems, and roots were collected from the wheat seedlings at 3 wk after seed germination. Wheat plants of the aforementioned genotypes were also grown in a temperature-controlled greenhouse for harvesting grains. Tagging of flowers and collection of grains at six defined developmental stages, corresponding to watery (Stage 1; ~5 d after flowering), early milk (Stage 2; ~10 d after flowering), late milk (Stage 3; ~15 d after flowering), soft dough (Stage 4; ~20 d after flowering), hard dough (Stage 5; ~25 d after flowering), and ripening (Stage 6; ~30 d after flowering) were performed as previously described (Qin et al., 2012). Grains of developmental Stages 3 through 5 were dissected using a sharp scalpel and the endosperm and pericarp tissues were quickly frozen in liquid nitrogen. Three biological replicates, each encompassing tissues collected from multiple grains harvested from multiple plants, were used for gene expression and metabolite analyses. The frozen wheat tissues were ground into fine powder in liquid nitrogen using mortar and pestle and stored at -80°C until further analysis.

RNA Extraction, Cloning, and Functional Characterization of Wheat PSY1 Homoeologs

Total RNA from leaf, stem, and root as well as grain endosperm and pericarp tissues was extracted using TRIzol reagent (Invitrogen), followed by clean-up with the RNeasy Plant Mini kit (Qiagen). Quantity and quality of the RNA samples were determined by Nanodrop (Thermo Fisher Scientific). RNA integrity was verified by agarose gel electrophoresis.

To clone the wheat PSY1 homoeologs, Superscript III reverse transcriptase (Invitrogen) and oligo(dT)₂₀ primers were used for synthesis of the first-strand complementary DNA (cDNA), which was then served as template and subjected to

amplification using PSY1 homoeolog-specific primers (Supplemental Table S1). Polymerase chain reaction (PCR) products of expected sizes were cloned into the pENTR/D-TOPO vector (Invitrogen), verified by DNA sequencing, and recombined into the *E. coli* expression vector pDEST17 (Invitrogen). The recombinant plasmids were transformed into *E. coli* JM109 (DE3) cells that coexpress pAC-85b. The pAC-85b plasmid contains all the genes, except PSY, required for β -carotene biosynthesis (Cunningham and Gantt, 2007). Therefore, coupled expression of a functional PSY and pAC-85b will lead to β -carotene accumulation in *E. coli* cells. The *Arabidopsis* PSY (*AtPSY*) was used as a positive control for PSY activities; the plasmid that contains all the genes for β -carotene formation, including *AtPSY*, was designated pAC-BETA (Cunningham and Gantt, 2007). pDEST17-GUS that expresses a β -glucuronidase (*GUS*) gene was used as a negative control for PSY activities.

Real-Time Quantitative Polymerase Chain Reaction Analysis

Total RNA was treated with RNase-free DNase I (Fermentas) prior to real-time quantitative PCR (qPCR) analysis. Specificity of the qPCR primers (Supplemental Table S1) was verified using nullisomic tetrasomic lines of hexaploid wheat cultivar Chinese Spring (Supplemental Fig. S1). Melting curve analysis was performed for each primer pair to ensure that only the intended amplicon (i.e., a single peak) was obtained in the qPCR reactions. The real-time qPCR products were also separated on a 1% agarose gel for visual inspection of single gene amplification.

To prepare complementary RNA (cRNA) standards, wheat PSY1 homoeologs were amplified from the recombinant plasmids (i.e., PSY1 homoeologs cloned in pENTR/D-TOPO) using primers designed for real-time qPCR analysis (Supplemental Table S1). The PCR products of expected sizes were subcloned into the pGEM-T Easy vector (Promega). The pGEM-T-wheat PSY1 homoeolog plasmids were then linearized and used as templates for synthesis of cRNAs by T7 RNA polymerase (New England Biolabs). After the transcription reactions, the DNA templates were removed by RNase-free DNase I (Fermentas). The cRNAs were quantified using Nanodrop with multiple measurements at various concentrations and with different dilutions.

For construction of cRNA standard curves, cRNA dilutions were made that correspond to 10⁵ to 10 single strand molecules and used for reverse transcription reactions. Total RNA extracted from wheat tissues (2 μ g) was also reverse transcribed using the iScript cDNA synthesis kit and random hexamers following manufacturer's instructions (BioRad). Overall, amplification efficiencies of the cRNA standards and wheat PSY1 homoeologs were in a similar range (93 to 101%).

A cRNA standard curve, with six replicates at five different concentrations, was included on each 96-well optical plate. On the same plate, for each type of wheat tissue, there were three biological replicates and each with three technical replications. The PCR mix included cDNA templates, iTaq Universal SYBR supermix, and H₂O. The cycling parameters were 1 cycle of 15 s at 95°C, followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C. The PCR products were also cloned and verified by DNA sequencing.

High-Performance Liquid Chromatography Analysis

For functional characterization of wheat *PSY1* homoeologs, four colonies were randomly picked from each transformation of pDEST17-wheat *PSY1* homoeolog or pDEST17-*GUS* plasmids into *E. coli* JM109 (DE3) cells coexpressing pAC-85b. Each colony was used to inoculate a 30-mL liquid Luria Bertani culture with 35 $\mu\text{g mL}^{-1}$ chloramphenicol and 100 $\mu\text{g mL}^{-1}$ ampicillin. Bacterial cell growth, induction of gene expression, and harvest of cells were conducted as described in Qin et al. (2012); the fresh weight of the cell pellet was recorded (UC1041 *PSY-A1*, 0.24 ± 0.02 g; UC1113 *PSY-B1*, 0.21 ± 0.01 g; Kofa *PSY-B1*, 0.22 ± 0.01 g; UC1041 *PSY-B1*, 0.22 ± 0.03 g; UC1041 *PSY-D1*, 0.22 ± 0.01 g; Agatha *PSY-E1*, 0.23 ± 0.01 g; Agatha 28-4 *PSY-E1*, 0.24 ± 0.02 g). There was no statistically significant difference in the fresh weight of cell pellets based on one-way ANOVA analysis ($p = 0.12$). Total carotenoids were extracted from bacterial pellets using 0.3 mL of formaldehyde, 0.3 of mL methanol, and 0.6 mL of ethyl acetate. Water (0.4 mL) was added to the extracts for phase separation. The upper organic phase was transferred to a new vial and analyzed by reverse-phase high-performance liquid chromatography (HPLC) (Qin et al., 2012).

Carotenoid extraction from endosperm and pericarp tissues and HPLC separation and quantification were performed as previously described (Qin et al., 2012). β -apo-carotenal was added to each sample prior to extraction and used as internal standard for calculation of recovery. Three biological replicates were analyzed for each tissue.

Promoter Analysis

Promoter sequences (~900 bp upstream of the translation initiation codon) were obtained for *PSY-A1* homoeologs of UC1113, Kofa, and UC1041 using inverse PCR (Hartl and Ochman, 1993) and DNA sequencing. The promoter sequences were aligned using multiple sequence comparison by log-expectation (Edgar, 2004). Bioinformatic analysis of putative *cis*-elements in these *PSY-A1* promoters was performed using PlantCare (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Statistical Analysis

One-way ANOVA followed by Tukey's honest significant difference post hoc test was conducted on data obtained from *PSY1* homoeolog enzyme activity assays as well as *PSY1* homoeolog or gene expression and carotenoid metabolite analyses. Technical replications of each biological replicate were averaged and used as subsamples. The statistical analysis was performed using JMP vers. 11 (SAS Institute, 2014).

RESULTS

Analysis of Carotenoid Profiles in Endosperm and Pericarp of Developing Wheat Grains

To determine carotenoid composition and content in tetraploid and hexaploid wheat genotypes with different GYPC, total carotenoids from developing endosperm and pericarp tissues of UC1113 and UC1041 (low GYPC

Table 1. Carotenoid concentrations in endosperm of developing tetraploid and hexaploid wheat grains. Data shown are the average of three biological replicates ($n = 3$) with SE. Each biological replicate contains tissues dissected and pooled from multiple grains.

Genotype	Stage	Carotenoid concentration		
		Violaxanthin	Lutein	Total
		————— μg carotenoid pigment g tissue ⁻¹ —————		
UC1113	3	1.2 ± 0.14	3.12 ± 0.2	4.33 ± 0.28
	4	0.75 ± 0.05*†	2.27 ± 0.28*	3.03 ± 0.23*
	5	0.63 ± 0.05*	2.32 ± 0.01*	2.94 ± 0.05*
Kofa	3	1.22 ± 0.25	3.79 ± 0.01	5.01 ± 0.26
	4	1.12 ± 0.06	4.05 ± 0.22	5.18 ± 0.28
	5	0.57 ± 0.02*	3.47 ± 0.16	4.04 ± 0.18*
UC1041	3	0.61 ± 0.16	0.73 ± 0.05	1.35 ± 0.22
	4	0.24 ± 0.04*	0.55 ± 0.05*	0.79 ± 0.08*
	5	0.06 ± 0.01**	0.42 ± 0.01**	0.48 ± 0.01**
Agatha	3	1.09 ± 0.08	4.89 ± 0.37	5.98 ± 0.45
	4	0.56 ± 0.2*	4.16 ± 0.07	4.71 ± 0.27*
	5	0.42 ± 0.03**	3.15 ± 0.21**	3.57 ± 0.24**
Agatha 28-4	3	0.65 ± 0.11	1.95 ± 0.17	2.61 ± 0.28
	4	0.51 ± 0.06	1.87 ± 0.01	2.38 ± 0.05
	5	0.08 ± 0.01**	0.91 ± 0.05**	0.99 ± 0.04*

† Asterisks indicate statistically significant differences in carotenoid concentrations among the three developmental stages (relative to Stage 3) of each wheat genotype (* $P < 0.05$ and ** $P < 0.01$).

tetraploid and hexaploid wheat genotypes, respectively), Kofa and Agatha (high GYPC tetraploid and hexaploid wheat genotypes, respectively), and the Agatha 28-4 mutant were analyzed (Table 1, 2).

In endosperm, only violaxanthin and lutein accumulated at Stages 3 through 5, with lutein being more abundant than violaxanthin in all wheat genotypes (Table 1). Both individual and total carotenoids decreased progressively during endosperm development. At Stage 5, higher levels of total carotenoids were observed in the endosperm tissue of Agatha and Kofa than UC1113, UC1041, and Agatha 28-4, which is consistent with the previously reported GYPC in mature grains of these wheat genotypes (Zhang and Dubcovsky, 2008; Zhang et al., 2005) (Table 1). Although the Agatha 28-4 mutant showed a 72% reduced carotenoid concentration compared with Agatha, total carotenoids in Agatha 28-4 are still twice that in UC1041 at Stage 5 of grain endosperm (Table 1).

In pericarp, the order of carotenoid abundance was lutein, violaxanthin, β -carotene, and neoxanthin for tetraploid wheat genotypes at Stages 3 through 5 (Table 2). While the order of carotenoid abundance was lutein, β -carotene, violaxanthin, and neoxanthin for hexaploid wheat genotypes at Stage 3, there were relatively more violaxanthin than β -carotene at Stages 4 and 5 of the hexaploid wheat pericarp tissue (with the exception of comparable violaxanthin and β -carotene in UC1041 at Stage 4 and more neoxanthin than β -carotene in UC1041 at Stage 5) (Table 2). In contrast to endosperm, total carotenoids and lutein

Table 2. Carotenoid concentrations in pericarp of developing tetraploid and hexaploid wheat grains. Data shown are the average of three biological replicates ($n = 3$) with SE. Each biological replicate contains tissues dissected and pooled from multiple grains.

		Carotenoid concentration				
Genotype	Stage	Neoxanthin	Violaxanthin	Lutein	β-carotene	Total
μg carotenoid pigment g tissue ⁻¹						
UC1113	3	2.78 ± 0.36	5.21 ± 0.53	8.63 ± 1.35	3.51 ± 0.62	20.13 ± 2.84
	4	1.72 ± 0.19**†	3.35 ± 0.58*	5.68 ± 0.64*	1.88 ± 0.3*	12.63 ± 1.7*
	5	1.67 ± 0.15**	3.39 ± 0.37**	5.58 ± 0.95*	1.95 ± 0.19*	12.59 ± 1.63*
Kofa	3	3.27 ± 0.27	6.07 ± 0.19	10.84 ± 0.98	4.43 ± 0.36	24.61 ± 1.51
	4	2.64 ± 0.14*	5.18 ± 0.5*	8.77 ± 0.67*	3.04 ± 0.22**	19.63 ± 1.54*
	5	1.54 ± 0.05***	3.03 ± 0.14***	5.65 ± 0.35***	1.57 ± 0.09***	11.79 ± 0.63***
UC1041	3	3.48 ± 0.46	5.86 ± 0.44	11.46 ± 0.91	6.57 ± 0.56	27.38 ± 1.73
	4	4 ± 0.23	5.03 ± 0.26	10 ± 0.42	5.64 ± 0.5	24.67 ± 1.41
	5	0.84 ± 0.02**	1.43 ± 0.19***	1.61 ± 0.08***	0.44 ± 0.03***	4.32 ± 0.29***
Agatha	3	5.56 ± 0.04	7.5 ± 0.4	20.6 ± 0.14	9.47 ± 0.11	43.14 ± 0.61
	4	3.12 ± 0.15***	5.58 ± 0.36**	11.38 ± 1.13***	4.82 ± 0.43***	24.91 ± 1.89***
	5	1.22 ± 0.02***	2.65 ± 0.26***	4.8 ± 0.54***	2.18 ± 0.22***	10.85 ± 0.98***
Agatha 28-4	3	5.5 ± 0.2	7.64 ± 0.17	18.18 ± 0.8	8.42 ± 0.38	39.75 ± 1.2
	4	3.25 ± 0.43**	6.22 ± 0.65*	11.31 ± 2.23**	5.04 ± 0.82**	25.83 ± 4.12**
	5	1.46 ± 0.24***	3 ± 0.59***	4.72 ± 1.16***	2.15 ± 0.56***	11.33 ± 2.55***

$\dagger$ Asterisks indicate statistically significant differences in carotenoid concentrations among the three developmental stages (relative to Stage 3) of each wheat genotype (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$).

in pericarp did not correlate with the previously reported GYPC in the wheat genotypes analyzed (GYPC measures the yellow pigment color of wheat flour). Additionally, total carotenoids in Agatha 28-4 were not significantly different from Agatha in pericarp (Table 2). Furthermore, the total carotenoid concentration in Agatha and Agatha 28-4 was nearly twice the concentration of the other wheat genotypes at Stage 3 and UC1041 had the lowest total carotenoids of all wheat genotypes at Stage 5 (Table 2).

Characterization of Wheat PSY1 Homoeolog Enzyme Activities

To compare the enzymatic activities of various wheat PSY1 homoeologs, substrate conversion by the representative tetraploid and hexaploid wheat PSY-A1 (UC1041), the normal tetraploid wheat PSY-B1 (UC1113), the conversion tetraploid wheat PSY-B1 (Kofa), the hexaploid wheat PSY-B1 (UC1041), the hexaploid wheat PSY-D1 (UC1041), as well as PSY-E1 from Agatha, and the Agatha 28-4 mutant was determined using an *E. coli* system that has been engineered for carotenoid accumulation (Fig. 2). PSY-A1, normal tetraploid wheat PSY-B1, hexaploid wheat PSY-B1, and Agatha PSY-E1 exhibited similar capacity for turning GGPP into downstream products (Fig. 2). However, the conversion tetraploid wheat PSY-B1 showed a 22% reduction in enzymatic activities compared with the normal tetraploid wheat PSY-B1. Interestingly, PSY-D1 was ~60% more efficient in product formation than the other wild-type wheat PSY1 homoeologs (Fig. 2). PSY-E1 from Agatha 28-4 had 75% reduced activities relative to PSY-E1 from Agatha, suggesting that the white endosperm phenotype of Agatha 28-4 is at least partially a result of its reduced PSY-E1 enzyme activity (Fig. 2).

Transcript Levels of PSY1 Homoeologs in Endosperm and Pericarp of Developing Wheat Grains

To examine whether transcript levels of PSY1 homoeologs could be contributing factors for differential carotenoid accumulation in wheat grains, PSY1 homoeolog-specific expression in endosperm and pericarp tissues of various tetraploid and hexaploid wheat genotypes was analyzed by real-time qPCR (Fig. 3). Measurement of transcript abundance using the absolute quantification method allowed for direct comparison of PSY1 homoeolog (Fig. 3) and gene expression (Fig. 4) from wheat cultivars and breeding lines with diverse genetic background.

In endosperm, transcript levels of PSY-A1 and PSY-B1 homoeologs were always higher in tetraploid wheat than that of hexaploid wheat genotypes (Fig. 3A–C). For the two tetraploid wheat genotypes, there were more PSY-A1 transcripts in Kofa than UC1113 at Stages 3 and 4. On the other hand, transcript levels of the conversion (Kofa) and the normal (UC1113) PSY-B1 alleles were similar at Stages 3 and 5, while there were more PSY-B1 transcripts of the conversion allele than the normal allele at Stage 4 (Fig. 3A–C). Among hexaploid wheat genotypes, all three PSY1 homoeologs were expressed at very low levels or undetectable by real-time qPCR in UC1041. While Agatha and Agatha 28-4 had significantly higher PSY-E1 expression than the corresponding A- and B-genome PSY1 homoeologs at Stages 3 and 4, PSY-E1 expression largely decreased at Stage 5 of the endosperm tissue as compared to Stages 3 and 4 (Fig. 3A–C).

In pericarp, PSY-B1 was the dominantly expressed PSY1 homoeolog in tetraploid wheat genotypes (Fig. 3D–F). While PSY-A1 transcript levels were similar

Fig. 2. Characterization of homoeologous wheat PSY1 activities in carotenoid-accumulating *E. coli* cells. High-performance liquid chromatography elution profiles of carotenoid extracts from *E. coli* cells expressing (A) pAC-BETA, (B) pAC-85b + pDEST17-*GUS*, (C) pAC-85b + pDEST17-*PSY-A1* (UC1041), (D) pAC-85b + pDEST17-*PSY-B1* (UC1113), (E) pAC-85b + pDEST17-*PSY-B1* (Kofa), (F) pAC-85b + pDEST17-*PSY-B1* (UC1041), (G) pAC-85b + pDEST17-*PSY-D1* (UC1041), (H) pAC-85b + pDEST17-*PSY-E1* (Agatha), and (I) pAC-85b + pDEST17-*PSY-E1* (Agatha 28-4) are shown. PSY1 activities were determined based on β -carotene (β -car) produced in *E. coli* cells coexpressing pAC-85b and different wheat *PSY1* homoeologs (J). Different letters indicate significant differences ($P < 0.05$) in PSY1 homoeolog activities.

Fig. 3. Transcript levels of different *PSY1* homoeologs in endosperm and pericarp of developing tetraploid and hexaploid wheat grains. Different letters indicate significant differences ($P < 0.05$) in transcript levels of *PSY1* homoeologs in the same tissue and at the same developmental stage for each genome. Data shown are the average of three biological replicates ($n = 3$) with SE. Each biological replicate contains tissues dissected and pooled from multiple grains. Technical replications of each biological replicate were averaged and used as subsamples.

between Kofa and UC1113 at each stage of pericarp development, *PSY-B1* expression was significantly lower in Kofa than UC1113 at Stage 3 and became comparable at Stages 4

and 5 (Fig. 3D–F). In hexaploid wheat, *PSY-A1* transcript accumulation exhibited a decreasing trend in Agatha and Agatha 28-4 during pericarp development; in contrast, it

Fig. 4. Transcript levels of *PSY1* genes in various tetraploid and hexaploid wheat tissues. Transcripts of *PSY1* homoeologs from different genomes were added together to obtain the transcript levels of *PSY1* genes. Different letters indicate significant differences ($P < 0.05$) in transcript levels of *PSY1* genes in the same tissue (and also at the same developmental stage for grain tissues). Data shown are the average of three biological replicates ($n = 3$) with SE. Each biological replicate contains tissues dissected and pooled from multiple grains. Technical replications of each biological replicate were averaged and used as subsamples.

peaked at Stage 5 in UC1041 (Fig. 3D–F). *PSY-B1* transcript levels showed a gradual reduction in all three hexaploid wheat genotypes. Steady or decreased *PSY-E1* transcript levels were observed in Agatha and Agatha 28-4, respectively, during pericarp development (Fig. 3D–F).

To assess the contribution of *PSY1* gene expression to carotenoid accumulation, transcripts of different *PSY1* homoeologs in each wheat genotype were added together and compared (Fig. 4). In endosperm, Kofa *PSY1* expression was among the highest, and UC1041 *PSY1* expression was among the lowest for all wheat genotypes at different developmental stages (Fig. 4A). In addition, *PSY1* expression was reduced for Kofa, UC1041, and Agatha 28-4, but not for UC1113 and Agatha, at Stage 5 of grain endosperm (Fig. 4A). In pericarp, the most notable difference was lower *PSY1* expression levels in Kofa at Stage 3 and in Agatha 28-4 at Stage 5 than the other wheat genotypes; *PSY1* expression was mostly similar for all wheat genotypes at Stage 4 (Fig. 4B).

Overall, there were significant correlations between *PSY1* transcript levels and carotenoid concentrations in endosperm and pericarp of tetraploid and hexaploid wheat grains (Supplemental Fig. S2, S3). However, when transcripts of *PSY-A1* and *PSY-B1* homoeologs were compared with carotenoid concentrations in wheat grain tissues, only *PSY-A1* transcripts were significantly correlated with violaxanthin and total carotenoids and *PSY-B1* transcripts with violaxanthin in endosperm of tetraploid and hexaploid wheat grains (Supplemental Fig. S2). Transcripts of the A- and B-genome *PSY1* homoeologs did not exhibit significant correlations with carotenoid concentrations in pericarp of the wheat grains analyzed in this study (Supplemental Fig. S3).

Transcript Levels of *PSY1* Homoeologs in Vegetative Tissues

To investigate the impact of *PSY1* homoeolog transcript accumulation on carotenoid concentrations in vegetative tissues, *PSY1* homoeolog expression was also analyzed in leaves, stems, and roots of different wheat genotypes (Fig. 5). In leaves, the normal (UC1113) and the conversion (Kofa) tetraploid wheat *PSY-B1* homoeologs had two- to fivefold higher expression than *PSY-A1* in UC1113 and Kofa. While expression of *PSY-A1* and *PSY-B1* homoeologs was not significantly different, *PSY-D1* transcripts accumulated at much lower levels than the A- and B-genome homoeologs in UC1041 (Fig. 5A). Higher expression of *PSY-A1* and *PSY-B1* homoeologs was observed in Agatha than Agatha 28-4 (Fig. 5A). Interestingly, when *PSY1* gene expression in leaves was compared, *PSY1* transcripts in Kofa were two- to fourfold lower than the other wheat genotypes. In addition, *PSY1* expression in Agatha was significantly lower than those in UC1113 and UC1041 (Fig. 4C).

In stems, the differential expression patterns of *PSY1* homoeologs in UC1113 and Agatha 28-4 generally resembled those in leaves (Fig. 5B). On the other hand, *PSY-A1* and *PSY-B1* transcripts accumulated to a comparable extent in Kofa. Unlike Agatha 28-4, where *PSY-E1* and *PSY-A1* expression was similar and higher than *PSY-B1*, *PSY-E1* was the highest expressed *PSY1* homoeolog in Agatha (Fig. 5B). As was noted in leaves, Kofa *PSY1* was also the lowest expressed *PSY1* gene in stems among the five wheat genotypes (Fig. 4C).

In roots, *PSY1* homoeolog expression was much lower than in leaves and stems for all wheat genotypes (Fig. 5). Notably, *PSY-B1* was the only *PSY1* homoeolog with above-baseline transcript accumulation in tetraploid and hexaploid wheat (Fig. 5C; note the scale of the y-axis). At the gene level, *PSY1* was lowly expressed in all wheat genotypes (Fig. 4C).

Comparison of Promoter Sequences of Wheat *PSY-A1* Homoeologs

While *PSY-A1* proteins from UC1113, Kofa, and UC1041 differ in a single conserved amino acid and likely have similar enzyme activities, *PSY-A1* homoeologs in the three wheat genotypes exhibited distinct expression profiles in vegetative tissues as well as endosperm and pericarp of developing grains (Fig. 3, 5). To explore the molecular control mechanisms underlying *PSY-A1* expression in different wheat genotypes, tissues, and during grain development, promoter sequences (900 bp upstream of the translational start) of *PSY-A1* homoeologs from UC1113, Kofa, and UC1041 were obtained and compared (Fig. 6). The multiple-sequence alignment revealed highly conserved *PSY-A1* promoter sequences (98.5% identity) among the three wheat genotypes that vary at only four locations (Fig. 6). The *PSY-A1* promoter sequences were also searched against previously identified *cis*-regulatory motifs in plants and the differing promoter sequences did not match to any known *cis*-motifs (Fig. 6).

As was shown previously for *PSY* promoters from other plant species (Li et al., 2009; Qin et al., 2011; Welsch et al., 2008), several putative light response elements (LREs) are present in wheat *PSY-A1* promoters (Fig. 6). In addition, sequences that match to the response elements to three plant hormones, including abscisic acid, methyl jasmonate, and gibberellin, were also found, suggesting that wheat *PSY-A1* expression may respond to hormonal signals. However, the ethylene response RAP2.2 motif previously discovered in *Arabidopsis* is absent in the wheat *PSY-A1* promoters (Welsch et al., 2007) (Fig. 6).

Fig. 5. Transcript levels of different *PSY1* homoeologs in leaf, stem, and root of tetraploid and hexaploid wheat genotypes. Different letters indicate significant differences ($P < 0.05$) in transcript levels of *PSY1* homoeologs in the same tissue for each genome. Data shown are the average of three biological replicates ($n = 3$) with SE. Each biological replicate contains tissues dissected and pooled from multiple grains. Technical replications of each biological replicate were averaged and used as subsamples.

UC1113_PSY-A1	TCCATGCAAATGCAAGCAAGATGGTTCTGTCTACTAGAACAAAGGTATGGTAATGCAAACGTGATTTTTTTGGATGCTACCA	LRE
Kofa_PSY-A1	TCCATGCAAATGCAAGCAAGATGGTTCTGTCTACTAGAACAAAGGTATGGTAATGCAAACGTGATTTTTTTGGATGCTACCA	
UC1041_PSY-A1	TCCATGCAAATGCAAGCAAGATGGTTCTGTCTACTAGAACAAAGGTATGGTAATGCAAACGTGATTTTTTTGGATGCTACCA	
UC1113_PSY-A1	CCCACACATGTTTAAACCACCGGCGCCAGGAATGCATGACGCCATGTACCGGGCGCGAGGCGCCACGTAGGAGCGCCCTG	LRE LRE LRE MeJA response ABRE
Kofa_PSY-A1	CCCACACATGTTTAAACCACCGGCGCCAGGAATGCATGACGCCATGTACCGGGCGCGAGGCGCCACGTAGGAGCGCCCTG	
UC1041_PSY-A1	CCCACACATGTTTAAACCACCGGCGCCAGGAATGCATGACGCCATGTACCGGGCGCGAGGCGCCACGTAGGAGCGCCCTG	
UC1113_PSY-A1	GCCGGATTTCGTCCATCCATCCCTTTCCAGGTGTCACTACTGAGCGGCGCCCAAGGCACG	LRE
Kofa_PSY-A1	GCCGGATTTCGTCCATCCATCCCTTTCCAGGTGTCACTACTGAGCGGCGCCCAAGGCACG	
UC1041_PSY-A1	GCCGGATTTCGTCCATCCATCCCTTTCCAGGTGTCACTACTGAGCGGCGCCCAAGGCACG	
UC1113_PSY-A1	ATAACCCATATCCCCTCATCTCACCAGCGCGGCCATTGTCTCCTCCACGTCTCCCTCT	LRE LRE
Kofa_PSY-A1	ATAACCCATATCCCCTCATCTCACCAGCGCGGCCATTGTCTCCTCCACGTCTCCCTCT	
UC1041_PSY-A1	ATAACCCATATCCCCTCATCTCACCAGCGCGGCCATTGTCTCCTCCACGTCTCCCTCT	
UC1113_PSY-A1	TCTCTCTCTCTCTCTCTCTCAAAGGCAGAGGAAGCAAGAGGACACTGGACGCAGAGAGAGGGAGACACTGGACTTTGTCC	LRE
Kofa_PSY-A1	TCTCTCTCTCTCTCTCTCTCAAAGGCAGAGGAAGCAAGAGGACACTGGACGCAGAGAGAGGGAGACACTGGACTTTGTCC	
UC1041_PSY-A1	TCTCTCTCTCTCTCTCTCTCAAAGGCAGAGGAAGCAAGAGGACACTGGACGCAGAGAGAGGGAGACACTGGACTTTGTCC	
UC1113_PSY-A1	TGCGATGGAACCTTGGCAGCAGCATATTACATACGCGCGCTCACTCTCAGCGGGATCTGGCCGTACGTGTGTATCGGCT	ABRE
Kofa_PSY-A1	TGCGATGGAACCTTGGCAGCAGCATATTACATACGCGCGCTCACTCTCAGCGGGATCTGGCCGTACGTGTGTATCGGCT	
UC1041_PSY-A1	TGCGATGGAACCTTGGCAGCAGCATATTACATACGCGCGCTCACTCTCAGCGGGATCTGGCCGTACGTGTGTATCGGCT	
UC1113_PSY-A1	GCTTGTGTTGACGGCGATTGCGCGAGGTTAAGGCGGCATATGCCGTGAGCTTGTAGAGGGATCGAGCTTGACGCGGGCGT	MeJA response LRE
Kofa_PSY-A1	GCTTGTGTTGACGGCGATTGCGCGAGGTTAAGGCGGCATATGCCGTGAGCTTGTAGAGGGATCGAGCTTGACGCGGGCGT	
UC1041_PSY-A1	GCTTGTGTTGACGGCGATTGCGCGAGGTTAAGGCGGCATATGCCGTGAGCTTGTAGAGGGATCGAGCTTGACGCGGGCGT	
UC1113_PSY-A1	GCTGATAAGAGGTGAGTGGCCTAAGCCGTTCTCTCGTGCATAAAGGCCTTTTGCCCAAGCACAAAGAGAATTGTCGCCATTAT	LRE P Box LRE
Kofa_PSY-A1	GCTGATAAGAGGTGAGTGGCCTAAGCCGTTCTCTCGTGCATAAAGGCCTTTTGCCCAAGCACAAAGAGAATTGTCGCCATTAT	
UC1041_PSY-A1	GCTGATAAGAGGTGAGTGGCCTAAGCCGTTCTCTCGTGCATAAAGGCCTTTTGCCCAAGCACAAAGAGAATTGTCGCCATTAT	
UC1113_PSY-A1	ATCTCACTCACTGTGTAGTATCTCTATGCAGGGGTGCATGGCAAACCTAGTGGTCGGTAGTGCAGGCCACCACTCCGGCCCA	
Kofa_PSY-A1	ATCTCACTCACTGTGTAGTATCTCTATGCAGGGGTGCATGGCAAACCTAGTGGTCGGTAGTGCAGGCCACCACTCCGGCCCA	
UC1041_PSY-A1	ATCTCACTCACTGTGTAGTATCTCTATGCAGGGGTGCATGGCAAACCTAGTGGTCGGTAGTGCAGGCCACCACTCCGGCCCA	
UC1113_PSY-A1	GACAAAGCGTGTGCGTGTCCAAGAAGCGCCTTTCCCCCGGCTCACACGAGAGTGGTGAATCCATCCACGGTGCCTGCCTC	LRE
Kofa_PSY-A1	GACAAAGCGTGTGCGTGTCCAAGAAGCGCCTTTCCCCCGGCTCACACGAGAGTGGTGAATCCATCCACGGTGCCTGCCTC	
UC1041_PSY-A1	GACAAAGCGTGTGCGTGTCCAAGAAGCGCCTTTCCCCCGGCTCACACGAGAGTGGTGAATCCATCCACGGTGCCTGCCTC	
UC1113_PSY-A1	AATTCTCTCCCTTGACAGGGGAGAGCAGAGCTCCGTTTCTCTCCTCGGCGCGGCGGAGACGAGCTCCGGCGGTTTGGGGTTG	LRE ABRE
Kofa_PSY-A1	AATTCTCTCCCTTGACAGGGGAGAGCAGAGCTCCGTTTCTCTCCTCGGCGCGGCGGAGACGAGCTCCGGCGGTTTGGGGTTG	
UC1041_PSY-A1	AATTCTCTCCCTTGACAGGGGAGAGCAGAGCTCCGTTTCTCTCCTCGGCGCGGCGGAGACGAGCTCCGGCGGTTTGGGGTTG	
UC1113_PSY-A1	GCGTTGCGCGCATGCAGCAG	ATG
Kofa_PSY-A1	GCGTTGCGCGCATGCAGCAG	
UC1041_PSY-A1	GCGTTGCGCGCATGCAGCAG	

Fig. 6. Analysis of *PSY-A1* promoters from UC1113, Kofa, and UC1041. The putative light response element (LRE), gibberellin response element (P Box), MeJA response element (MeJA response), and abscisic acid response element (ABRE) predicted by sequence analysis are shown. Different sequences among the three *PSY-A1* promoters are indicated by boxes.

DISCUSSION

The functional characterization and gene expression analysis collectively indicate that carotenoid accumulation is better correlated with transcript levels of *PSY1* homoeologs than their enzyme activities in endosperm of wheat genotypes with varied GYPC. Consistent with our findings in wheat, a previous study in maize (*Zea mays* L.) also revealed correlations between *PSY1* transcript levels and total carotenoids in mature maize kernels (Fu et al., 2013). In addition, maize genotypes with low levels of *crtRB1* (encoding β -carotene hydroxylase) and *LCY ϵ* (encoding lycopene ϵ -cyclase) transcripts, from natural genetic variations, were shown to correlate with high levels of β -carotene accumulation in mature maize kernels (Harjes et al., 2008; Yan et al., 2010). These results in wheat and maize collectively suggest that transcript levels of carotenoid metabolic genes (and homoeologs in polyploids) may be indicative of the amount of carotenoid product accumulating in endosperm of cereal grains.

The enzyme activity assays also provide new insights into the structure–function relationship of wheat *PSY1* enzymes. Although amino acid substitutions, particularly Phe at position 324 to Val, from the normal tetraploid wheat *PSY-B1* to the hexaploid wheat *PSY-B1* were suggested to impact enzyme activities (Zhang and Dubcovsky, 2008), assays in the *E. coli* system indicated that these amino acid changes had little effect on substrate conversion and the Phe–Val residue is not essential for *PSY-B1* enzyme activities (Fig. 2). On the other hand, the enzyme assays revealed the critical role of Pro at position 422 in enzyme function; the single amino acid mutation from Pro in Agatha to Leu in Agatha 28-4 led to 80% reduction in *PSY-E1* activities (Fig. 2). Since *PSY-E1* transcript levels were also reduced in Agatha 28-4 as compared with Agatha (Fig. 3), this amino acid change may also affect the stability of *PSY-E1* transcripts. Given that only the *PSY-E1* gene was sequenced in Agatha and the EMS-mutagenized Agatha 28-4 mutant, it cannot be ruled out that mutations in genes other than *PSY-E1* could also contribute to the white endosperm phenotype of Agatha 28-4. However, since Agatha is derived from backcrosses and can be considered near isogenic to Thatcher (Sharma and Knott, 1966), one may argue that the potential unidentified mutations is closely linked to *PSY-E1*.

While Agatha 28-4 and Agatha had similar carotenoid concentrations in the pericarp tissue at Stages 3 through 5, total carotenoids were largely reduced in endosperm of Agatha 28-4 relative to Agatha, indicating that the single amino acid mutation in *PSY-E1* and potentially unidentified mutations in Agatha 28-4 have more influence on carotenoid accumulation in endosperm than pericarp of wheat grains (Table 1, 2). One possibility is that the reduced *PSY-E1* activity in pericarp of Agatha 28-4 could be compensated for by *PSY-A1* and *PSY-B1* activities in this tissue, whereas *PSY-E1* may be the dominant activity

in grain endosperm and a reduction in *PSY-E1* activities will lead to a decrease in total carotenoid accumulation in endosperm (Fig. 3; Table 1, 2).

Since the Kofa conversion *PSY-B1* showed a 22% reduced activity compared with the normal tetraploid wheat *PSY-B1* when assayed in *E. coli*, it suggests that the high GYPC QTL linked to Kofa *PSY-B1* is not a result of increased enzyme activities of the conversion *PSY-B1* allele (Fig. 2). Other factors, including, but not limited to, transcriptional control, may account for the difference in GYPC between Kofa and the low GYPC tetraploid wheat varieties. Similarly, *PSY1* homoeologs of UC1041 (low GYPC hexaploid wheat) had comparable or even higher *PSY* activities than those of tetraploid wheat genotypes with high GYPC (Fig. 2), suggesting the control of carotenoid accumulation by factors other than *PSY* enzyme activities in hexaploid wheat.

As implicated by the expression data, the wheat breeding process has preferentially selected *PSY1* homoeologs with little endosperm expression in low GYPC wheat genotypes (Fig. 3). Aside from genotypic variation, the presence of putative LREs and hormonal response elements in *PSY-A1* promoters suggests that *PSY-A1* expression and carotenoid accumulation in wheat may be controlled by abiotic and biotic signals from the environment (Fig. 6). However, since these predictions are based on sequence analysis of short motifs, light, and hormonal regulation of wheat *PSY-A1* homoeolog expression will need to be experimentally verified in the future.

The differences in *PSY-A1* promoter sequences of UC1113, Kofa, and UC1041 have also uncovered potential new *cis*-regulatory motifs, though further experimentation is also required to test this hypothesis (Fig. 6). It was recently shown that the ORANGE (OR) protein, a previously identified regulator of chromoplast biogenesis, could interact with *PSY* and modulate *PSY* protein abundance and enzymatic activities in *Arabidopsis* (Zhou et al., 2015). It will be interesting to investigate whether *PSY1* could also be posttranscriptionally regulated by OR in wheat.

Given the overall correlation between *PSY1* homoeolog transcript levels in grain endosperm and carotenoid concentrations, wheat breeders interested in increasing carotenoid levels in the endosperm can select lines with high transcript levels for particular *PSY1* homoeologs (e.g., highly expressed *PSY-A1* homoeologs for high carotenoid accumulation in tetraploid and hexaploid wheat grains; highly expressed *PSY-E1* homoeolog for high carotenoid accumulation in hexaploid wheat grains) and then combine them in a single wheat genotype. Future dissection of the transcriptional and posttranscriptional control mechanisms of wheat *PSY1* homoeologs will advance our understanding of the molecular and genetic basis of endosperm carotenoid accumulation and the GYPC trait in tetraploid and hexaploid wheat.

Conflict of Interest

The authors declare that there is no conflict of interest.

Supplemental Information Available

Supplemental information is available with the online version of this manuscript.

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