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ORIGINAL PAPER

Allelic variation at *Psy1-A1* and association with yellow pigment in durum wheat grain

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Abstract The yellow pigment (YP) of durum wheat (*Triticum turgidum* L. var *durum*) semolina is due in part to the presence of carotenoid pigments found in the endosperm and is an important end-use quality trait. Phytoene synthase (*Psy*) is considered a rate-limiting enzyme in the carotenoid biosynthetic pathway and in this study, three alleles of *Psy1-A1* were sequenced from four durum wheat cultivars and a co-dominant marker was developed for genetic mapping. *Psy1-A1* mapped to chromosome 7AL near *Xwmc809* in three durum mapping populations and was significantly associated with a pigment quantitative trait loci (QTL) identified on that chromosome. A second QTL localized 25 cM proximal to *Psy1-A1* in two populations, and the interaction between the two QTL was not significant. Consistent with QTL mapping data, the *Psy1-A1o* allele was associated with elevated pigment in a validation population comprising 93 diverse cultivars and breeding lines. These results confirm an earlier hypothesis that *Psy1*, and at least one additional gene in the distal region of 7AL, are associated with grain YP differences in durum wheat. The functional co-dominant marker developed in this study differentiates the *Psy1-A1* alleles reported here and could be used as a target to enhance YP selection in durum wheat breeding programs.

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Introduction

Elevated yellow pigment (YP) concentration is a desirable end-use quality trait in durum wheat (*Triticum turgidum* L. var *durum*) and is the target of durum breeding programs worldwide (Troccoli et al. 2000). In contrast, low YP is a breeding target in hexaploid wheat (*Triticum aestivum* L.) where bright white flour is preferred. In durum, the degree of yellowness is influenced by several factors, including the presence of carotenoid pigments (Hentschel et al. 2002; Panfili et al. 2004), semolina extraction rate (Matsuo and Dexter 1980), processing conditions (Borrelli et al. 1999), and oxidative degradation by lipoxygenases (Borrelli et al. 1999).

The inheritance of YP is complex and is controlled largely by additive gene action (Johnston et al. 1983; Elouafi et al. 2001; Mares and Campbell 2001) and is highly heritable (Parker et al. 1998; Elouafi et al. 2001; Clarke et al. 2006; Patil et al. 2008). Several QTL for YP have been identified in both durum and hexaploid wheat on chromosomes 1A (Patil et al. 2008), 1B (Cervigni et al. 2005; He et al. 2008), 3A (Parker et al. 1998), 3B (Mares and Campbell 2001; Patil et al. 2008), 4A and 5A (Hessler et al. 2002), 2A, 4B and 6B (Pozniak et al. 2007), 5B (Patil et al. 2008), and 6A (Cervigni et al. 2005). However, the majority of mapping studies are in agreement that the group 7 chromosomes largely influence the expression of grain pigment in wheat and durum (Parker et al. 1998; Elouafi et al. 2001; Cervigni et al. 2005; Atienza et al. 2007; Pozniak et al. 2007; He et al. 2008; Patil et al. 2008; Zhang and Dubcovsky 2008). Elouafi et al. (2001)

identified two minor QTL located on chromosome 7A and a third major QTL on chromosome 7BL, believed to be homoeologous to one of the QTL on chromosome 7AL. The 7BL QTL has, since, been validated in two other durum wheat populations (Pozniak et al. 2007; Zhang and Dubcovsky 2008) and is the same QTL identified in hexaploid wheat (Kuchel et al. 2006). A QTL for yellow pigment in durum was identified by Patil et al. (2008) on chromosome 7A that accounted for up to 55% of the variation for the trait. Mares and Campbell (2001) identified QTL on 7A in two hexaploid wheat populations that have a large effect on flour yellowness, and correspond to regions associated with xanthophyll concentration.

Identification of genes controlling expression of YP in durum grain would facilitate breeding efforts to select genotypes with elevated pigment by direct selection for desirable alleles at critical loci. In durum, yellow colour of semolina is largely due to the presence of carotenoids, mainly the xanthophyll lutein, with small traces of zeaxanthin (Hentschel et al. 2002; Panfili et al. 2004). In other plants, biosynthesis of carotenoids has been characterized extensively and phytoene synthase (*Psy*) is considered a rate-limiting step in the carotenoid biosynthetic pathway (Burkhardt et al. 1997; Hirschberg, 2001; Lindgren et al. 2003). Three functional *Psy* genes (*Psy1*, *Psy2*, *Psy3*) have been identified in the *Poacea* (Gallagher et al. 2004; Li et al. 2008), but only *Psy1* expression is correlated with carotenoid accumulation during grain fill (Gallagher et al. 2004; Li et al. 2008). To date, four *Psy* genes have been identified in durum, forming two paralogous series on the group 5 (*Psy2*) and group 7 (*Psy1*) chromosomes (Pozniak et al. 2007). One of these, *Psy1-B1*, co-segregates with a QTL for YP on 7BL (Pozniak et al. 2007; Zhang and Dubcovsky 2008). In hexaploid wheat, *Psy1-A1* has been localized to the distal end of 7AL and was associated with variation for flour yellowness in one mapping population (He et al. 2008). In contrast, Zhang and Dubcovsky (2008) reported a QTL for YP on the distal end of 7AL, but the QTL was proximal to the physical position of *Psy1-A1*. Given the presence of two QTL on chromosome 7AL (Elouafi et al. 2001), we hypothesize that the variation at *Psy1-A1* is associated with the most distal of the QTL on that chromosome. Here, we report the characterization of three *Psy1-A1* alleles and examine the association of molecular variation at *Psy1-A1* with phenotypic variation for YP content in three durum wheat mapping populations.

Materials and methods

Mapping populations and validation set

Three mapping populations were used in this study. The first, designated as D03.77, was derived from the cross Commander/DT733 and consisted of 110 $F_{2:7}$ recombinant inbred lines (RILs) developed using single seed descent. DT733 was developed at the Semiarid Prairie Agricultural Research Centre, Agriculture and Agri-Food Canada from the cross DT663/W9262-260D1. W9262-260D1 is a sister line of W9262-260D3, which is a parent of a mapping population previously used for localizing YP QTL (Pozniak et al. 2007). A second population designated as A0022& is a doubled haploid (DH) population produced from the F_1 hybrid of Strongfield/Blackbird using the maize pollen method (Knox et al. 2000) and consisted of 89 DH lines. Blackbird is a selection out of *T. carthlicum* accession REB68421 obtained from Dr. Maxime Trottet of INRA, Centre de Recherches de Rennes, in France. A genetic map for the Strongfield/Blackbird population has been reported previously (Somers et al. 2006) and was used in this study. A third population designated as D05.58 was developed from the cross Strongfield/Commander, developed at the Crop Development Centre, University of Saskatchewan, Saskatoon, Canada. The population consisted of 106 $F_{2:7}$ RILs developed using single seed descent. Strongfield (Clarke et al. 2005a) has lower grain YP than Commander (Clarke et al. 2005b). A validation population consisting of a global collection of 93 genetically diverse inbred breeding lines and cultivars was used to determine the frequency of *Psy1-A1* alleles identified in this study. This population has been described previously (Reimer et al. 2008) and is presented as supplemental information (Table S1).

Cloning of Psy1-A1 alleles

Three primer sets (Table 1) were developed based on the *Psy1-A1* sequence from hexaploid wheat (GenBank accession no.: EF600063) to obtain the durum *Psy1-A1* sequences reported here, including portions of the 5′ and 3′ untranslated regions (UTR). For each primer set, polymerase chain reactions (PCR) were performed in 25-μL reactions consisting of 200 ng of genomic DNA, 1× PCR buffer, 400 μM of each dNTP, 0.5 μM of each primer, 5% (v/v) dimethyl sulfoxide (DMSO) and 2.5 Units (U) of *Taq* DNA polymerase. The PCR cycling conditions were 95°C for 5 min, 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min 20 s, and a final extension at 72°C for 10 min. Following electrophoresis, amplicons of the approximate size were cloned using the TOPO TA Cloning Kit (Invitrogen, Burlington, Ontario, Canada) following the manufacturer's instructions. The plasmid DNA from clones carrying the inserted PCR product were sequenced at the Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada. Allele sequences reported here have been designated based on the recommended rules for gene symbolization in wheat and sequence designations have

Table 1 Primer sets used for cloning of *Psy1-A1* from durum wheat and a *Psy1-A1* co-dominant marker (Psy1-A1_STS) for differentiating the three identified alleles of *Psy1-A1* in durum wheat

Name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)	Approx. sizes (bp)
Psy1-5′UTR	CCCACTCCGGCCCATACAAA	CTGCCATGCACGACCAGACA	700
Psy1_FR	AGGAGTACGCCAAGACCT	AAGGCCGACAAACGAAACAAT	1,135
Psy1-3′UTR	TCCCAACGCGTCGCACATCA	GGCCAACTAACTGGATGCAGAG	1,860/1,700
Psy1-A1_STS	GTGGATATTCCCTGTCAGCATC	GCCTCCTCGAAGAACATCCTC	*Psy1-A1l* = 1,089
			Psy1-A1o = 897
			Psy1-A1a = 1,776

UTR untranslated region

been confirmed with the curator of the *Psy1* wheat sequences (Xia Xianchun, personal communication).

All primers were assayed on the durum wheat cultivar Langdon and the Langdon-Chinese Spring disomic substitution lines LDN(7D)7A (missing chromosome 7A, contains Chinese Spring chromosome 7D) and LDN(7D)7B (missing chromosome 7B, contains Chinese Spring chromosome 7D) to localize amplicons to wheat chromosomes. Fragments from the Psy1-3′UTR primer set were digested with *PvuII* (New England Biolabs, Ipswich, MA, USA) following the instructions of the manufacturer to detect A and B genome polymorphisms. Genomic sequences obtained from the three primer pairs were contiged using Vector NTI Contig Express and intron/exon boundaries predicted using GeneScan (http://genes.mit.edu/GENSCAN.html). The genomic and deduced amino acid sequences were aligned using Vector NTI (Invitrogen, Burlington, Canada). Genomic sequence alignments were used to construct a sequence similarity dendrogram using the neighbour-joining method (Saitou and Nei 1987) function within the program ClustalX (Version 1.81). Those portions of the multiple sequence alignment with gaps were not used in the neighbour-joining method when constructing the dendrogram. Robustness of the dendrogram was determined by performing bootstrap analysis on 1,000 random samples taken from the sequence alignments. A consensus tree indicating the proportion of bootstrapped trees showing that same clade is presented. All nucleotide sequences presented have been submitted to GenBank (http://www.ncbi.nlm.nih.gov) with accession numbers presented.

Field trials and yellow pigment analysis

The D03.77 population was assessed in replicated trials in 2006 and 2007, near Saskatoon, Saskatchewan, Canada. In 2006, the RILs were grown in a randomized complete block, two-replicate test and plots consisted of a single 2 m row spaced 20 cm apart. In 2007, the experiment was planted in a two-replicate, alpha-lattice design. Plots consisted of five 2.5 m row plots spaced 20 cm apart. The A0022& population was grown in 2005 and 2006 at Regina and Swift Current, Saskatchewan, Canada in an alpha-lattice design with two replicates. The D05.58 population was grown as a two-replicate test using an alpha-lattice design at Saskatoon in 2008. Plots consisted of a single 3.7 m row spaced 20 cm apart. The validation set was grown in two-replicate trials in 2005 and 2006 at Saskatoon and Swift Current, Saskatchewan as described previously (Reimer et al. 2008). Briefly, the 93 accessions were grown at Swift Current and Saskatoon, in 2005 and 2006 in a two-replicate test using an alpha-lattice design.

For the D03.77, D05.58 and validation populations, total yellow pigment was assessed on whole grain meal from individual plots using AACC Method 14-50 (AACC 2000). Briefly, grain was ground in a UDY Cyclone Sample Mill (UDY Corporation, Fort Collins, Colorado) fitted with a 1 mm screen and water-saturated butyl alcohol (40 mL) was added to 8 g of whole meal (13.5% moisture basis), shaken and extracted for 16 h. Extract was then filtered through Whatman No. 1 filter paper, and absorbance measured at 435 nm using a Microplate Reader (BioRad, CA, USA). Three individual absorbance measurements per extracted sample were recorded and values were averaged and converted to yellow pigment concentration (mg kg^{-1}) using the extinction coefficient for β-carotene (AACC 2000). For the A0022& population, YP was assessed using near infrared reflectance (NIR) spectrophotometry with a NIR System 6500A instrument (McCaig et al. 1992). The NIR was calibrated each year against standard samples characterized by total solvent-extractable pigments (mg kg^{-1}) based on AACC approved method 14-50 (AACC 2000), with an r^2 of 0.94. For each population, YP data was analyzed separately for each environment using the PROC MIXED procedure of SAS® (Littell et al. 1996) with lines considered as fixed effects and replications considered as random. Broad-sense heritability was estimated as the proportion of genetic variance (σ_g^2) to

phenotypic variance, such that $h^2 = \sigma_g^2/(\sigma_g^2 + \sigma_{residual}^2/$ no. replications). Parental data was removed for heritability estimation.

Marker development and genetic mapping

Genetic maps for the distal region of chromosome 7AL were constructed by screening wheat microsatellite markers known to map in this region (Somers et al. 2004; He et al. 2008; Zhang and Dubcovsky 2008) on the parents of the D03.77, A0022& and D05.58 populations to identify polymorphic markers. Polymorphic markers were assayed on lines of each population using an ABI 3130 capillary electrophoresis instrument (Applied Biosystems, Foster City, CA). The forward primer of each microsatellite marker was M13-tailed (Schuelke 2000) and PCR reactions were performed as described previously (Pozniak et al. 2007). In addition, a co-dominant marker designated as Psy1-A1_*STS* was developed to distinguish the *Psy1-A1* alleles identified in this study and was evaluated on the mapping populations. PCR of genomic DNA using primers for Psy1-A1_*STS* (Table 1) was performed using 100 ng of DNA, 1× PCR buffer (Invitrogen, Burlington, Canada), 1 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.4 μM of each primer. PCR cycling conditions were an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C (30 s), 56°C (30 s) and 72°C (1 min). A final extension at 72°C for 10 min was used to complete the PCR. The sizes of amplicons expected for each allele are presented in Table 1. A second primer set known to differentiate *Psy1-A1* alleles (Zhang and Dubcovsky 2008) was also evaluated in the mapping and validation populations to confirm correct scoring of the *Psy1-A1a* allele. The D03.77 and D05.58 populations were segregating at *Psy1-B1*, and a co-dominant marker differentiating the alleles at this locus has been described (Zhang and Dubcovsky 2008) and scored on lines from these two populations.

Genetic linkage maps were constructed using the Haldane mapping function with the software JoinMap 3.0 (van Ooijen and Voorips 2004) at a minimum LOD score of 3.0. To test the association of polymorphic markers with variation in YP, single marker analysis (Lynch and Walsh 1998; Knapp 2001) was conducted on least square (LS) means using the PROC MIXED procedure of SAS (Littell et al. 1996). Markers were considered fixed and the marker effect was estimated as one-half the difference between parental marker class means. The interaction between *Psy1-A1* and *Psy1-B1* was assessed on LS means using a two locus model with the two loci and their interaction considered as fixed effects.

Results

Characterization of *Psy1-A1* from durum wheat

Three primer sets were used to amplify overlapping, contiguous fragments to generate full-length genomic sequences of *Psy1-A1* from durum wheat. Because two homeologous genes of *Psy1* exist in tetraploid durum, it was necessary to ensure that only the *Psy1-A1* sequence was contiged to complete the full-length sequence of *Psy1-A1*. Using the Langdon-Chinese Spring D-genome substitution lines, it was possible to differentiate A and B genome derived *Psy1* sequences (Fig. 1). The Psy1-5′UTR primers amplified a 580 bp fragment from Langdon and LDN(7D)7B, but the amplicon was absent in LDN(7D)7A, indicating that this primer set only amplified *Psy1* sequence from 7A. The two remaining primers cross-amplified fragments from both of the group 7 chromosomes (Fig. 1), but it was possible to determine which fragments localized to 7A (fragments missing in LDN(7D)7A; Fig. 1). Only sequences from fragments assigned to chromosome 7A were contiged to obtain the *Psy1-A1* full-length sequence.

The genomic sequences of *Psy1-A1* from Commander were 3,093 bp in length and smaller than the 3,266 bp sequences obtained from Strongfield and DT733. The *Psy1-A1* sequence from Blackbird was 3,949 bp in length and is identical to the *Psy1-A1a* allele reported previously (EF600063; He et al. 2008). Alignment of Strongfield and DT733 sequences (3,269 bp) indicated that these two lines possessed the *Psy1-A1l* allele reported previously (GenBank accession no. EU096090; Pozniak et al. 2007; Zhang

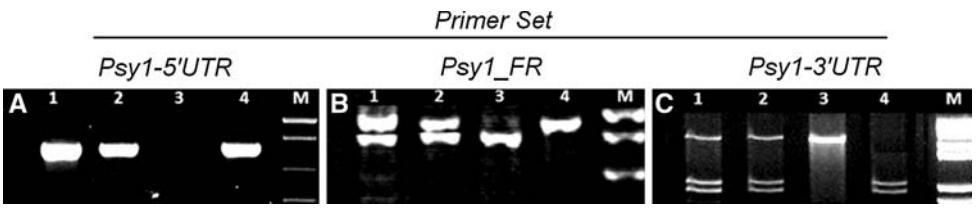

Fig. 1 Chromosomal localization of PCR amplicons derived from the three primer sets (see Table 1) used to clone *Psy1-A1*. Fragments were amplified from Strongfield (*Lane 1*), Landgon (*Lane 2*) and LDN(7D)7A (*Lane 3*) and LDN(7D)7B (*Lane 4*) substitution lines. Amplicons form Psy1-3′UTR (C) were digested with *PvuII* to detect the homeologous polymorphisms

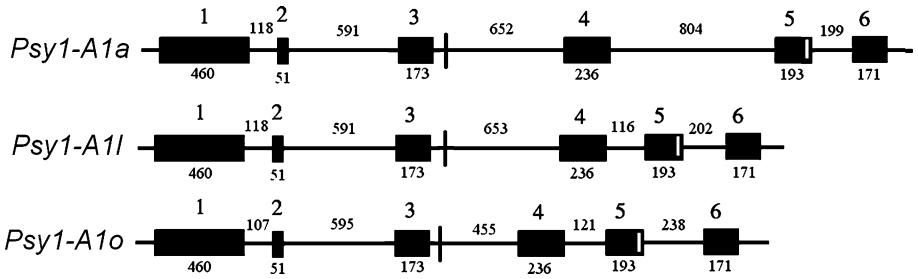

Fig. 2 Intron–exon structure of *Psy1-A1a* (EF600063), *Psy1-A1 l* (EU096090) and *Psy1-A1o* (FJ234424). *Solid black boxes* represent the six identified exons, and *lines* represent the spanning introns. Numerical values below the *boxes* and above the *lines* are sizes (bp) of the exons and introns. Corresponding exons are *numbered* above the *box*. The *vertical bars* are the approximate binding sites for the two Psy1-A1-STS primers

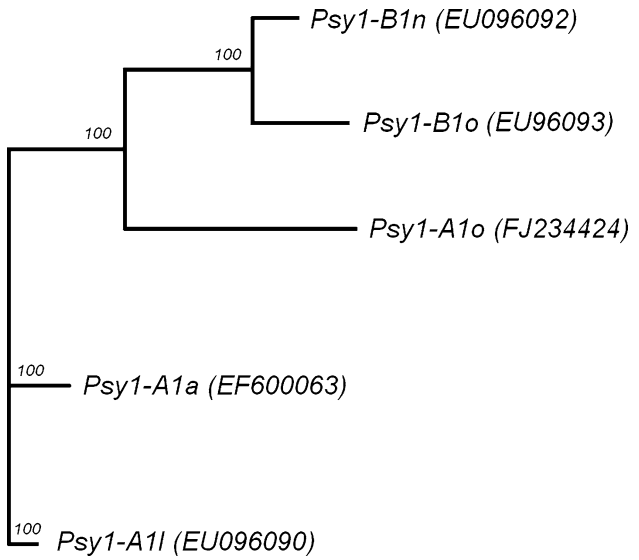

Fig. 3 Similarity dendrogram of *Psy1* sequences. Bootstrap values are shown at selected nodes as percentages of 1,000 replicates

and Dubcovsky 2008), The *Psy1-A1* sequence derived from Commander (FJ234424) is novel, and could be easily differentiated by a 198 bp insertion/deletion (INDEL) in the third intron (Fig. 2). Based on the recommended rules for gene symbolization in wheat, this allele was designated as *Psy1-A1o*. Based on nucleotide sequence, *Psy1-A1o* was more similar to the two reported *Psy1-B1* alleles (Zhang and Dubcovsky 2008; Pozniak et al. 2007) than the other *Psy1-A1* sequences presented here (Fig. 3).

Based on predicted intron/exon boundaries, all three alleles contained six exons, but intron length was variable (Fig. 2). The length of the predicted cDNAs were identical for all three alleles, containing an open reading frame of 1,284 bp and a deduced protein of 428 amino acid residues (Fig. 4). Alignment of *Psy1-A1* amino acid (aa) sequences revealed six residue differences among the three durum alleles (Fig. 4). Compared to the other two *Psy1-A1* aa sequences, *Psy1-A1o* was unique at positions 112 and from 116-118 (Fig. 4). These differences are in a non-conserved region coding for the chloroplast transit peptide of *Psy1*. The *Psy1-A1a* and *Psy1-A1l* alleles contained a E-D[276] substitution and a T[283] was observed in the deduced aa sequence of *Psy1-A1l*, whereas all other compared sequences possessed an S[283].

Marker development and genetic mapping

A co-dominant marker designated as Psy1-A1_STS (Table 1) was developed to differentiate the alleles of *Psy1-A1a*, *Psy1-A1l*, and *Psy1-A1o* by designing primers flanking the INDELs in introns 3 and 4 (Fig. 2). In agreement with the sequence data, Strongfield and DT733 carried the 1,089 bp fragment amplified by Psy1-A1_STS indicative of *Psy1-A1l* (Fig. 5). Langdon also carried the *Psy1-A1l* allele, but the 1,089 bp fragment was absent in LDN(7D)7A (Fig. 5). Commander produced the expected 897 bp fragment indicative of *Psy1-A1o*, but a second band similar in size to *Psy1-A1l* was also present (Fig. 5). To rule out possible heterogeneity at *Psy1-A1*, the 1,089 bp fragment was sequenced from Commander and was identical to the *Psy1-B1n* allele located on 7BL (EU096092) identified in previous studies (Pozniak et al. 2007; Zhang and Dubcovsky 2008). Reimer et al. (2008) confirmed that Commander carried the *Psy1-B1n* allele. AC Pathfinder is a parent of Commander (Clarke et al. 2005b) and lacks the *Psy1-B1n* allele (Reimer et al. 2008) and when evaluated with the *Psy1-A1* co-dominant marker, only the 897 bp fragment indicative of *Psy1-A1o* was present (Fig. 5). Consistent with sequence data, PCR of Blackbird produced the expected 1,776 bp fragment indicative of *Psy1-A1a*. A second primer set known to differentiate *Psy1-A1a* and *Psy1-A1l* was evaluated and confirmed the presence of the *Psy1-A1a* in Blackbird (data not shown).

A genetic map of 7AL was constructed in the three mapping populations and marker order was generally conserved among populations (Fig. 6). In the D03.77 population, *Psy1-A1* was flanked by *Xcfa2257* and

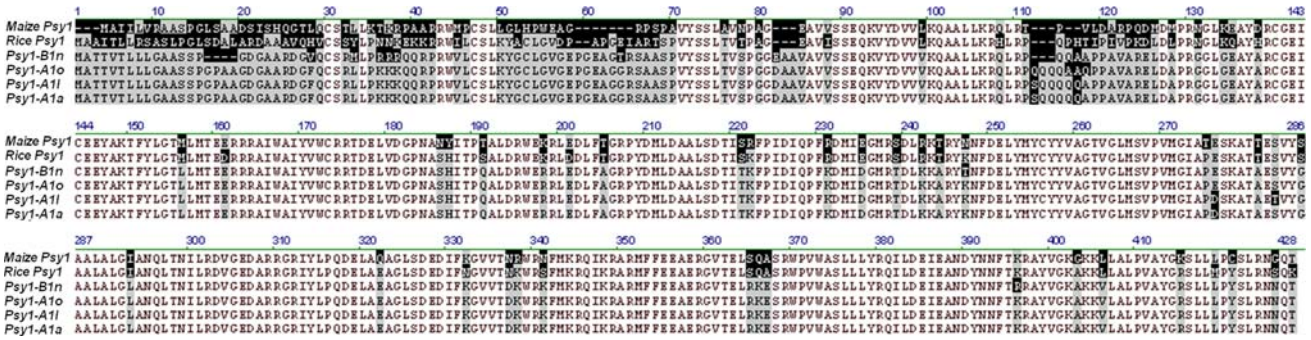

Fig. 4 Alignment of deduced amino acid sequences from three *Psy1-A1* alleles. Reported *Psy1* sequences from rice (AY445521), maize (UC32636) and wheat (*Psy1-B1n* EU096092) are presented for comparison

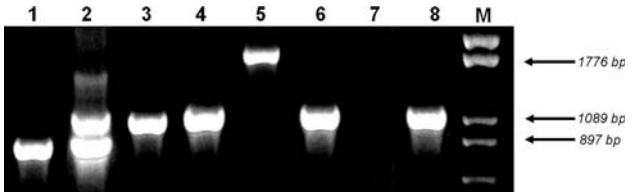

Fig. 5 Amplicon sizes of the *Psy1-A1*_STS co-dominant marker differentiating three alleles of Psy1-A1 are 1,089 bp (*Psy1-A1l*), 897 (*Psy1-A1o*), and 1,776 bp (*Psy1-A1a*). Genotypes are AC Pathfinder (*Lane 1*), Commander (*Lane 2*), Strongfield (*Lane 3*); DT733 (*Lane 4*), Blackbird (*Lane 5*); Langdon (*Lane 6*), LDN(7D)7A (*Lane 7*), LDN(7D)7B (*Lane 8*). Commander possessed both the 1,089 and 897 bp fragments, but sequencing results confirmed that the 1,089 bp amplicon is the *Psy1-B1*n allele located on 7BL (Pozniak et al. 2007). The 1,095 bp fragment was absent in AC Pathfinder, a parent of Commander, which does not carry *Psy1-B1n*

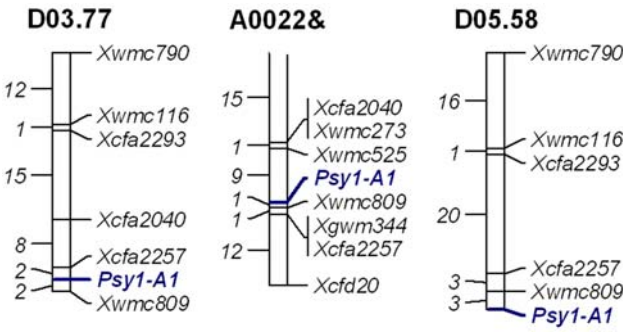

Fig. 6 Genetic maps of the distal region of chromosome 7AL in the three durum wheat mapping populations. Genetic distance between loci is presented in cM to the *left* of each linkage group

Xwmc809. *Psy1-A1* also mapped proximal to *Xwmc809* in the A0022& population. In the D05.58 population, *Psy1-A1* mapped 3 cM distal to *Xwmc809* (Fig. 6).

Phenotypic Data and QTL Analysis

Yellow pigment concentration was variable in all populations and in all environments (Table 2). In the D03.77 population, average YP was higher in 2006 than 2007, but the range in pigment was similar for the two environments (Table 2). In the A0022& population, the range in YP was higher in 2006 compared to 2005. In a single year of testing, the range in YP in the D05.58 population was similar to that observed in the D03.77 population. In the D03.77 population, heritability estimates for YP were 0.78 in 2006 and 0.91 in 2007. Heritability estimates in D05.58 (0.91 in 2008) and A0022& were high (Table 2) and are consistent with previous reports that YP is moderately to highly heritable (Clarke et al. 2006; Reimer et al. 2008).

Single marker analysis revealed a QTL centered at *Psy1-A1* which was significant in the D03.77 and A0022& populations in all of the environments evaluated (Table 3). In the A0022& population, the *Psy1-A1l* allele contributed by Strongfield elevated pigment, and the effect was similar among environments (Table 3). In the D03.77 population, lines carrying *Psy1-A1o* had higher pigment than lines carrying *Psy1-A1l* (Table 3). In a single year of testing, *Psy1-A1* was not significantly ($P > 0.05$) associated with variation in YP in the D05.58 population. A second YP QTL on 7A located approximately 23 cM proximal to *Psy1-A1* at *Xwmc790-Xwmc116-Xcfa2293* was identified in both the D03.77 and D05.58 populations. In the D03.77 population, *Xcfa2040* separated the two QTL and was not significantly ($P > 0.05$) associated with variation in YP in either year (Table 3). The average effects of the second QTL were similar in both populations (Table 3). Commander contributed the allele for elevated YP at the second QTL and the interaction between the QTL was not statistically significant ($P = 0.92$).

Commander, one of the parents of the D03.77 and D05.58 populations, carries the *Psy1-B1n* allele (Reimer et al. 2008; Fig. 5), and this allele has been associated with elevated pigment in other populations (Pozniak et al. 2007; Zhang et al. 2008). In this study, *Psy1-B1* was also significantly associated with YP variation in D05.58 (2008) and D03.77 (2007) (Table 3). The effect of *Psy1-B1* was greater in the D05.58 population than in D03.77 (Table 3).

Table 2 Summary of yellow pigment concentration (mg kg^{-1}), genetic variance ((σ_g^2), (mg kg^{-1})^2) and heritability (h^2) estimates for three durum mapping populations segregating at *Psy1-A1*

Population parameters	D03.77		D05.58	A0022&			
	Saskatoon 2006	Saskatoon 2007	Saskatoon 2008	Regina 2005	Swift Current 2005	Regina 2006	Swift Current 2006
Minimum	7.90	6.30	5.59	4.60	4.64	4.22	4.27
Maximum	11.21	9.51	9.14	8.38	8.54	8.72	8.93
SED	0.39	0.21	0.28	0.29	0.33	0.21	0.28
Range	3.31	3.21	3.55	3.78	3.90	4.50	4.66
Pop. average	9.15	8.13	7.37	6.52	6.40	6.54	6.69
σ_g^2	0.27	0.26	0.52	0.51	0.56	0.87	0.89
h^2	0.78	0.91	0.91	0.92	0.90	0.96	0.94

SED standard error of a difference between the minimum and maximum

The interaction between the two markers was not significant in any population, including the D03.77 population grown at Saskatoon (2007), the only environment where both genes were significantly associated with YP (Table 3). In this population, the independent effects of *Psy1-B1* and *Psy1-A1* were similar.

Allelic diversity at *Psy1-A1* in durum wheat

Analysis of the Psy1-A1_STS marker in a diverse set of 93 durum wheat cultivars and breeding lines revealed that the majority (83%) carried *Psy1-A1l*, with near equal frequencies of *Psy1-A1o* and *Psy1-A1a* (Table 4; Table S1). Field evaluation of this validation population over four environments identified large phenotypic variation in YP concentration, with YP values ranging from <4 to >11 mg kg^{-1} within environments (Table 4). Single marker analysis of Psy1-A1_STS data in this population revealed that *Psy1-A1* was significantly associated with variation in YP ($F = 27.9$; $P = 0.007$). Over all environments, the YP of lines carrying *Psy1-A1o* was on average 2.2 mg kg^{-1} higher than lines carrying *Psy1-A1l*, consistent with the genetic mapping data in the D03.77 population that this allele is associated with elevated pigment. Over all four environments, the average YP of lines carrying *Psy1-A1a* was at least 2.4 mg kg^{-1} (2.04–2.85 mg kg^{-1}) lower than lines carrying *Psy1-A1l* and 4.6 mg kg^{-1} (3.82–5.37 mg kg^{-1}) lower than lines with the *Psy1-A1o* allele (Table 4).

Discussion

The association of *Psy1-B1* with variation in yellow pigment has been demonstrated in durum wheat (Pozniak et al. 2007; Zhang and Dubcovsky 2008) but the role of *Psy1-A1* has not been clarified, given the lack of polymorphisms in studied populations. In this study, we identified molecular variation at *Psy1-A1* in three durum wheat mapping populations and localized the gene to a 4–8 cM interval on the distal end of 7AL. Like other *Psy1* genes, the three *Psy1-A1* alleles identified had six exons, with the majority of molecular variation occurring in intronic regions. The *Psy1-A1o* allele has yet to be reported in the literature and was easily differentiated from the other alleles by a 198 bp INDEL in the third intron. This allele was identified from Commander, and three of the four amino acids which differentiated *Psy1-A1o* from the remaining durum alleles are all found in the chloroplast targeting sequence. It is possible that these amino acid differences could confer the increased grain YP phenotype associated with this allele, perhaps due to enhanced transport of the enzyme to the chloroplasts. Although sequence information encoded by transit peptides is not fully understood, amino acid substitutions in transit peptides can alter targeting of functional proteins to chloroplasts (Lee et al. 2006), and thus enzyme activity.

In two of the three mapping populations used here, *Psy1-A1* was significantly associated with phenotypic variation in grain YP in all environments evaluated, and supports the role of this gene in expression of YP in wheat and durum grain (He et al. 2008; Zhang and Dubcovsky 2008). Although *Psy1-A1* was not significant in the D05.58 population, only a single environment was evaluated (Table 3). The effects of *Psy1-B1* are environmentally dependent (Pozniak et al. 2007), so perhaps the effects of *Psy1-A1* could have been detected if additional environments had been sampled.

In the mapping and validation populations, the average effects of the three *Psy1-A1* alleles were *Psy1-A1o* > *Psy1-A1l* > *Psy1-A1a*. Similar to hexaploid wheat, the *Psy1-A1a* allele identified from Blackbird was associated with a large reduction in pigment in the A0022& population. This allele was less frequent in durum germplasm (Table 4), likely the

Table 3 Single marker analysis for variation in yellow pigment concentration (mg kg^{-1}) in three durum wheat mapping populations

Marker	D03.77				D05.58		A0022&							
	Saskatoon 2006		Saskatoon 2007		Saskatoon 2008		Regina 2005		SC 2005		Regina 2006		SC 2006	
	F	Effect	*F*	Effect	*F*	Effect	*F*	Effect	*F*	Effect	*F*	Effect	*F*	Effect
Xwmc790	14.5**	0.15	12.6**	0.16	3.9*	0.13	–[a]	–	–	–	–	–	–	–
Xwmc116	14.4**	0.13	10.1**	0.17	9.1**	0.30	–	–	–	–	–	–	–	–
Xcfa2293	9.8**	0.12	16.5**	0.21	4.9*	0.24	–	–	–	–	–	–	–	–
Xcfa2040	2.1	ns	1.7	ns	–	–	13.6**	0.29	5.6**	0.19	6.2**	0.25	12.6**	0.35
Xwmc273	–[a]		–	–	–	–	15.6**	0.30	6.2**	0.21	8.2**	0.29	11.6**	0.35
Xwmc525	–	–	–	–	–	–	12.9**	0.28	5.0**	0.19	6.1**	0.25	11.5**	0.34
Xgwm344	–	–	–	–	–	–	28.2**	0.38	7.7**	0.23	21.1**	0.44	23.2**	0.45
Xcfd20	–	–	–	–	–	–	29.6**	0.37	8.5**	0.23	22.0**	0.43	22.3**	0.43
Xcfa2257	8.5**	0.11	11.2**	0.17	0.3	ns	31.5**	0.39	9.5**	0.24	23.5**	0.44	26.6**	0.47
Psy1-A1	**10.6****	**0.15**	**16.2****	**0.21**	**0.5**	**ns**	**34.8****	**0.41**	**11.5****	**0.27**	**24.5****	**0.46**	**32.7****	**0.52**
Xwmc809	5.1**	0.12	8.3**	0.15	0.1	ns	31.4**	0.4	8.5**	0.24	20.5**	0.42	22.5**	0.45
Psy1-B1[b]	0.3	ns	8.2**	0.19	17.9**	0.38	–	–	–	–	–	–	–	–
*A1*B1*[c]	0.1	ns	0.6	ns	0.8	ns	–	–	–	–	–	–	–	–

The distance between markers is presented in Fig. 6. Data for *Psy1-A1* are highlighted in bold. The *F* values for each marker are presented along with the marker effect (one-half the difference between parental marker class means). Commander contributed the allele for elevated YP at *Psy1-A1* and *Psy1-B1* in the D03.77 and D05.58 populations, whereas Strongfield contributed the allele for higher pigment in A0022&

** *F* value significant at $P < 0.001$

* *F* value significant at $P < 0.05$

ns *F* value not significant ($P > 0.05$)

[a] Markers were not polymorphic

[b] *Psy1-B1* is homeologous to *Psy1-A1* and is located on the distal region of 7BL (Pozniak et al. 2007)

[c] *F* test for interaction between *Psy1-A1* and *Psy1-B1*

result of breeders eliminating this allele through pigment selection given its negative effect on YP. However, the amino acid sequences of *Psy1-A1a* and the two remaining *Psy1-A1* alleles were similar, despite the presence of the 676-bp INDEL in the fourth intron. The strong negative effect of *Psy1-A1a* on expression of YP, despite similar amino acid sequences, could suggest that this insertion is linked to mutations in a regulatory region(s) of the gene that alter its expression. Alternatively, we cannot rule out the hypothesis that this insertion is linked to other gene(s) influencing grain YP.

The D03.77 and D05.58 populations segregated at the *Psy1-B1* locus, and allowed the opportunity to assess the combined effects of *Psy1-A1*/*Psy1-B1*. *Psy1-B1* was significant in both populations, but the statistical interaction between *Psy1-B1* and *Psy1-A1* was not significant (Table 3). This implies no additive effects in combining the two *Psy1* alleles and in populations segregating for both loci, selection for one of the alleles should be sufficient to elevate YP. The effect of these genes is likely to improve substrate conversion to phytoene, the precursor to lutein (Hentschel et al. 2002; Panfili et al. 2004). The lack of additivity suggests that enzymes downstream from *Psy* may be limiting the full expression of YP. However, both *Psy1* genes were only significant in one population at one environment and testing in additional populations and environments is still required to clarify the combined effects of these genes.

Zhang et al. (2008) hypothesized that allelic differences at *Psy1*, and at least one additional gene in the distal region of homeologous group 7L, are associated with differences in YP content. Our results support this hypothesis because two linked QTL for YP were identified on 7AL, one centered at *Psy1-A1*, and a second more than 25 cM proximal to *Psy1-A1*, near *Xwmc116*. The QTL at *Xwmc116* was also identified by Zhang et al. (2008). In the D03.77 population, the two QTL were separated by *Xcfa2040*, which was not significantly associated with YP in this population. It is possible that the additional gene(s) associated with *Xwmc116* could either be enzymes involved in synthesis of xanthophylls or other yellow pigments or gene(s) coding for transcription factors affecting the regulation of one or more carotenoid biosynthetic genes (Corona et al. 1996). Zeta-carotene desaturase and phytoene desaturase have been previously mapped to the group 2 and group 4 chromosomes, respectively (Cenci et al. 2004) and can be ruled out as candidates. Lycopene-β-cyclase is involved in converting lycopene to lutein (Cunningham et al. 1996) and

Table 4 Frequency of *Psy1-A1* alleles in a global collection of 93 durum wheat cultivars and breeding lines

Allele	Freq.	2005		2006	
	(%)	SC	SK	SC	SK
Psy1-A1l	83	7.91 ± 0.18	7.17 ± 0.14	8.56 ± 0.20	7.60 ± 0.16
Psy1-A1o	10	9.96 ± 0.52	8.95 ± 0.40	11.08 ± 0.58	10.03 ± 0.48
Psy1-A1a	7	5.46 ± 0.59	5.13 ± 0.45	5.71 ± 0.65	5.28 ± 0.54
Pop. average		7.94	7.18	8.59	7.67
Minimum		4.15	4.30	4.01	3.84
Maximum		12.54	11.03	13.56	11.96
LSD(0.05)		0.71	0.74	0.50	0.53

The average yellow pigment concentration ± SD (mg kg^{-1}) of lines carrying each allele is presented. A summary of the variation in yellow pigment concentration for the validation population is presented

can also be eliminated because we have localized that gene to the group 6 chromosomes (unpublished results). Recently a third paralogue of the *Psy* gene family has been identified in maize (Li et al. 2008), suggesting widespread duplication of this gene in the *Poaceae*. Based on wheat–rice synteny (La Rota and Sorrells 2004), it is doubtful that *Psy3* resides on chromosome 7AL, but given the apparent widespread duplication of this gene perhaps another, yet unidentified *Psy* paralog is located at or near *Xwmc116*.

In this and in a previous study (Pozniak et al. 2007), we revealed an association of homeologous loci of *Psy1* with elevated YP in durum grain. However, we are still not certain that these genes directly influence pigment expression in durum grain because they could be tightly linked to casual factors. Additional fine mapping and function characterization is required to confirm their causal effects. We have developed near-isogenic lines carrying all possible combinations of *Psy1-A1* (*l* and *o* alleles)/*Psy1-B1* (*n* and *o* alleles). These lines will be used to study the expression of these genes in relation to YP, and are being used to develop the necessary populations for high-resolution mapping of this region in durum. In the meantime, the co-dominant marker developed to differentiate *Psy1-A1* alleles is useful to select for *Psy1-A1o* to increase grain pigment in durum through allele-specific marker-assisted selection. In addition, the marker developed here cross-amplifies *Psy1-B1n* and could be used to identify genotypes in populations also segregating for that allele.

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