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ORIGINAL PAPER

Identification of QTL and association of a phytoene synthase gene with endosperm colour in durum wheat

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Abstract The yellow colour 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. We hypothesized that variation in the genes coding for phytoene synthase (*Psy*), a critical enzyme in carotenoid biosynthesis, may partially explain the phenotypic variation in endosperm colour observed among durum cultivars. Using rice sequence information, primers were designed to PCR clone and sequence the *Psy* genes from Kofa (high colour) and W9262-260D3 (medium colour) durum cultivars. Sequencing confirmed the presence of four *Psy* genes in each parent, corresponding to a two member gene family designated as *Psy1-1, Psy1-2* and *Psy2-1* and *Psy2-2*. A genetic map was constructed using 155 F_1-derived doubled haploid lines from the cross W9262-260D3/Kofa with 194 simple sequence repeat and DArT® markers. Using *Psy1-1* and *Psy2-1* allele-specific markers and chromosome mapping, the *Psy1* and *Psy2* genes were located to the group 7 and 5 chromosomes, respectively. Four quantitative trait loci (QTL) underlying phenotypic variation in endosperm colour were identified on chromosomes 2A, 4B, 6B, and 7B. The *Psy1-1* locus co-segregated with the 7B QTL, demonstrating an association of this gene with phenotypic variation for endosperm colour. This work is the first report of mapping *Psy* genes and supports the role of *Psy1-1* in elevated levels of endosperm colour in durum wheat. This gene is a target for the further development of a molecular marker to enhance selection for endosperm colour in durum wheat breeding programs.

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Introduction

One of the primary quality traits targeted by durum wheat (*Triticum turgidum* L. var *durum*) breeding programs is the bright yellow colour of semolina and pasta products as this trait becomes increasingly important in global markets (Dexter and Marchylo 2000). 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 (Manna et al. 1998; Borrelli et al. 1999).

Numerous studies have been conducted on the genetics of yellow colour in wheat grain. In durum, this trait is largely controlled by additive gene effects and is highly heritable (Johnston et al. 1983; Elouafi et al. 2001; Clarke et al. 2006). Using classical quantitative genetic analyses, the minimum number of factors controlling endosperm colour was estimated to be three in one of the environment durum-cross combinations and a median of 6–7 over all the environment-cross

combinations (Clarke et al. 2006). Quantitative trait loci (QTL) for endosperm colour have been mapped to at least seven chromosomes. Major genes exist on the group 2 chromosomes (Joppa and Williams 1988) with minor effect QTL being reported on chromosomes 4A and 5A (Hessler et al. 2002) and on 3BS (Mares and Campbell 2001). However, the group 7 chromosomes appear to contain genes most critical to yellow colour. Elouafi et al. (2001) identified QTL on the group 7 chromosomes which together explained 62% of the phenotypic variation observed for that trait. The 7B QTL alone explained 53% of the phenotypic variation. In hexaploid wheat, QTL for flour colour and xanthophyll content have been reported on chromosome 7A (Parker et al. 1998; Mares and Campbell 2001) and 7B (Kuchel et al. 2006).

In durum, the 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, the biosynthesis of carotenoids has been characterized extensively, and begins with the formation of phytoene via the condensation of two geranylgeranyl pyrophosphate (GGPP) molecules by phytoene synthase (*Psy*) (Cunningham and Gantt 1998). Phytoene then undergoes four sequential desaturation steps catalysed by phytoene desaturase (*Pds*) (Bartley et al. 1999) and ζ-carotene desaturase (*Zds*) (Albrecht et al. 1995) to produce tetracis prolycopene. Carotenoid isomerase (*CRTiso*) catalyses the cis to trans conversion of tetracis prolycopene to all trans lycopene (Matthews et al. 2003). Lycopene cyclization occurs through the action of two enzymes, lycopene-β-cyclase (*Lcy-b*) and lycopene-ε-cyclase (*Lcy-e*) to produce β-, ε- or α-carotene. Subsequent modifications transform these compounds into lutein and/or other xanthophylls (Fraser and Bramley 2004). The majority of the genes coding for critical enzymes involved in plant carotenoid biosynthesis have been cloned (Fraser and Bramley 2004).

Locating genes controlling colour expression in durum grain would facilitate breeding efforts to select genotypes with elevated colour by directly selecting for desirable alleles at critical loci. Using a PCR based approach, Cenci et al. (2004) identified and mapped clones from a durum wheat BAC library thought to contain genes coding for critical enzymes involved in the carotenoid biosynthetic pathway. In their work, putative *Psy*, *Pds*, and *Zds* clones were localized to the group 5, 4, and 2 chromosomes, respectively. However, because no gene sequences or associations with phenotypic differences were presented, it is difficult to ascertain any genetic association with expression of yellow colour in the grain. Interestingly, none of the BAC clones identified map to chromosomes 7A or 7B, the chromosomes known to contain major QTL for yellow colour (Parker et al. 1998; Elouafi et al. 2001; Mares and Campbell 2001).

Phytoene synthase appears to be the rate-limiting step in the carotenoid biosynthethic pathway (Lindgren et al. 2003). Recently, two functional *Psy* genes (*Psy1* and *Psy2*) were identified in rice (*Oryza sativa* L.), maize (*Zea mays* L.) and common wheat (*Triticum aestivum* L.) (Gallagher et al. 2004). However, in maize, association and QTL mapping studies have shown that only *Psy1* is associated with elevated levels of endosperm carotenoids (Palaisa et al. 2003; Wong et al. 2004). Furthermore, only *Psy1* expression is correlated with carotenoid accumulation during grain fill (Gallagher et al. 2004). In rice, *Psy1* maps to chromosome 6 which shares extensive synteny to wheat group 7 chromosomes (Francki et al. 2004; La Rota and Sorrells 2004; Van Deynze et al. 1995). Thus, we hypothesize that *Psy1* loci reside on homeologous group 7 chromosomes in durum wheat, and may be the gene or genes associated with the 7A and 7B QTL identified by others. To confirm our hypothesis, we have developed a genetic map using a doubled haploid (DH) population that was previously characterized for endosperm colour in six environments (Clarke et al. 2006). The objectives of this study were to map the orthologues of the rice *Psy1* and *Psy2* genes to determine if either of these genes is associated with variation in endosperm colour in our mapping population.

Materials and methods

Plant material and trait evaluation

The mapping population consisted of 155 F_1-derived doubled haploid lines from the cross W9262-260D3 × Kofa. W9262-260D3 is a line selected from the cross Kyle*2/'Biodur' made in the Agriculture and Agri-Food Canada-Swift Current breeding program. Kofa is a US semidwarf cultivar developed by WestBred, LLC. This population has been characterized extensively for endosperm colour (Clarke et al. 2006). Briefly, the 155 DH lines were grown together with Kofa and W9262-260D3 and 15 locally adapted check cultivars using an alpha lattice design with two replications at Swift Current, Indian Head, and Regina, Canada in 2001 and 2002. The yellow colour data used for QTL mapping is that reported by Clarke et al. (2006) where endosperm colour was measured using near infrared reflectance (NIR) spectrophotometry with a NIRSystem 6,500 A 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.

In addition to the mapping population, Kofa, hexaploid wheat (*Triticum aestivum* L.) cultivar Chinese Spring and Chinese Spring nullisomic–tetrasomic lines (Sears 1966) N7AT7D (nullisomic for 7A, tetrasomic for 7D), N7BT7D (nullisomic for 7B, tetrasomic for 7D), N5AT5D (nullisomic for 5A, tetrasomic for 5D) and N5BT5D (nullisomic for 5B, tetrasomic for 5D) were used to assign polymerase chain reaction (PCR) fragments to chromosomes.

Phytoene synthase gene cloning

A PCR based approach was utilized to clone *Psy* genes from Kofa and W9262-260D3. Forward primer PsyF3 (5′-TCT GCG AGG AGT ATG GCC AAG-3′) and reverse primer PsyR2 (5′-CCC TCT TGG TGA AGT TGT TGT-3′) were designed based on rice *Psy1* (AY445521.1) and *Psy2* (AY024351.1) sequences to simultaneously amplify durum *Psy1* and *Psy2* fragments. Polymerase chain reactions were performed in 25 μl reactions consisting of 200 μg 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 *Pfu* DNA polymerase, an enzyme with proof-reading ability (Stratagene, La Jolla, CA, USA). The PCR cycling conditions were as follows: 95°C for 5 min, 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min 20 sec, final extension at 72°C for 10 min. Using these PCR conditions, two amplicons approximately 2,450 and 2,110 bp in length were consistently amplified from both Kofa and W9262-260D3. Following electrophoresis, the two amplicons were independently gel purified and cloned using the Zero Blunt Topo Cloning Kit (Invitrogen, Burlington, ON, Canada) following the instructions of the manufacturer. Isolated plasmid DNA from 20 clones for the 2,450 bp fragment and 20 clones for the 2,110 bp fragment for each parent were sequenced at the McGill University and Quebec Genome Innovation Centre, Montreal, Quebec, Canada. For each of the parents, sequences were aligned to verify single-nucleotide differences and to exclude sequences of PCR induced chimeric DNA molecules (Cronn et al. 2002) and consensus sequences generated and aligned with the reported rice *Psy1* and *Psy2* cDNA sequences. The multiple 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 multiple sequence alignments (Felsenstein 1985) where a consensus tree indicating the proportion of bootstrapped trees showing that same clade is presented. Based on sequence similarity, parental consensus sequences were assigned to one of four allelic groups. Two of the four allelic groups most similar to the reported rice *Psy1* sequences were designated as *Psy1-1* and *Psy1-2*. The remaining two allelic groups most similar to the reported *Psy2* sequences were designated as *Psy2-1* and *Psy2-2*. All nucleotide sequences presented have been submitted to GenBank (http://www.ncbi.nlm.nih.gov).

Genetic linkage map and mapping of *Psy* genes

A total of 810 microsatellite primer pairs (368 WMC, 192 GWM, 65 GDM, 17 INRA, 168 BARC) were used to amplify DNA of the parents and evaluated on agarose gels for polymorphism. The PCR reactions consisted of 50 mM KCl, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM microsatellite primers, 1.75 U of Taq DNA polymerase and 50 μg of genomic DNA. Temperature cycling was 94°C for 3 min, followed by 44 cycles of 94°C for 1 min. Annealing was allowed for 1 min (temperatures were dependent on the individual microsatellite primer), and 1 min at 72°C for extension, followed by a final extension at 72°C for 10 min before cooling to 4°C. Primer sequences and annealing temperature were obtained from Röder et al. (1998) for GWM primers, Pestsova et al. (2000) for GDM primers and the GrainGenes database (http://www.wheat.pw.usda.gov) for BARC, WMC (Wheat Microsatellite Consortium), and INRA (Institut National de la Recherche Agronomique) primers. Eighty-two of the primer sets were polymorphic between the two parents and were subsequently screened on the DH mapping population. Amplification products were resolved by electrophoresis in mixed 2% Metaphor and 1% agarose LE gels at 4 V/cm in 0.5× TBE buffer and stained with ethidium bromide (0.5 μg/ml). The DNA banding patterns were visualized with UV light and recorded by a Kodak EDAS-290 digital camera imaging system.

A further 158 microsatellite primer pairs were tested for polymorphism between parents by capillary electrophoresis (CE) using an ABI3100-Avant. The forward microsatellite primer of each pair was modified by incorporating the M13 sequence to the 5′ end during synthesis (Schuelke 2000). Reactions were

performed in 0.2 ml strip tubes containing 10 µl of a reaction mixture consisting of 50 mM of KCl, 10 mM of Tris–HCl, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.02 pmol of M13 sequence-modified forward microsatellite primer, 0.2 pmol of reverse microsatellite primer, 0.18 pmol of Universal dye-labeled M13 primer, 0.5 U of *Taq* DNA polymerase and 24 µg of genomic DNA. The Universal M13 primer was labeled with either FAM, VIC, NED or PET fluorescent dyes. The PCR conditions were as described above for the GWM, WMC, BARC, and INRA markers. Amplification products (0.5 µl) were combined with 9.0 µl HiDi formamide (ABI, Foster City, CA), 0.45 µl deionized water and 0.05 µl LIZ size standard. Samples were run on a 36 cm capillary and processed with Applied Biosystems Data Collection Software version 2.0. Electropherograms were analysed with GeneMapper version 3.0. Forty-seven primer pairs generated polymorphism using CE.

DArT® markers (Wenzl et al. 2004; Xia et al. 2005; Wittenberg et al. 2005) were generated by Triticarte Pty Ltd (Canberra, Australia; http://www.triticarte.com.au), a high-throughput, low cost whole-genome profiling service laboratory. Approximately 5,300 random clones isolated from 85 genetically diverse durum wheat cultivars were produced following *PstI/TaqI* digestion and size purification and blotted on a microarray (Eric Huttner, personal communication). For the DArT assay, DNA from a random sample of 94 lines from the mapping population were subject to the same *PstI/TaqI* digestion and size purification and probed against the durum DArT array. Individual genotypes were scored for the presence or absence of hybridization based on fluorescence signal intensities. One hundred and twenty four loci were identified as being polymorphic between the parents and were scored in the DH mapping population as being present or absent. Each DArT clone (marker) was given a preliminary name, which will be revised in the future using a more applicable naming system.

To map the *Psy* genes, CAPS markers were developed for *Psy1-1* and *Psy2-1*. For *Psy1-1*, PCR of 300 µg of genomic DNA from Kofa, W9262-260D3 and the 155 DH lines was performed using forward primer Psy1F5 (5′-GCG AGG AGT ATG CCA AGA CCT-3′) and reverse primer Psy1R5 (5′-AAG GCC GAC AAA CGA AAC AAT-3′). This primer pair amplified 1,125 bp only from the two *Psy1* loci. Reaction conditions for these primers were identical to that for primers PsyF3/PsyR2, except that *Taq* DNA polymerase was used. Following PCR, 12 µl aliquots were transferred into a 96-well microtiter plate and were subjected to restriction digestion with 0.5 U of *Eco*RI at 37°C for 1 h. Following digestion, fragments were separated on a 1% agarose gel and DH lines scored for the presence of the 677/448 bp fragments indicative of the Kofa *Psy1-1* allele. To map the *Psy2-1* locus, PCR was performed on the parents and DH population using forward primer Psy2-1F (5′-TTC TTG GAT GGA CGG AGG TTA-3′) and PsyR2. This forward primer was designed specifically to amplify 1,585 and 1,605 bp of the Kofa and W9262-260D3 *Psy2-1* alleles, respectively. PCR were performed using 300 µg of genomic DNA in 25 µl reactions using the same reaction conditions as described for PsyF3/PsyR2, except that no DMSO was used. Following PCR, 12 µl of each reaction was digested with 0.5 U of *Bsr*I (5′-actggn|n-3′) and fragments separated on a 1.5% (w/v) agarose gel. The DH lines were scored for the presence of either two fragments 927/656 bp in length (indicative of the Kofa *Psy2-1* allele) or three fragments 927/360/317 bp length (indicative of the W9262-260D3 allele). The *Psy1* and *Psy2-2* loci were assigned to chromosomes using Chinese Spring nullisomic–tetrasomic substitution lines. For *Psy1*, PCR of 100 µg of genomic DNA was performed using primers *Psy1* specific primers Psy1F5/Psy1R5 as described above. Following amplification, PCR amplicons were digested with *Hpa*II in a 15 µl reaction following instructions of the manufacturer. For *Psy2-2*, PCR of 100 µg genomic DNA using primers Psy2-2F (5′-TTC AGT TAG TCG TCC ACG TCG-3′) and PsyR2 was performed using conditions identical to Psy1F5/Psy1R5. This primer pair was designed specifically to amplify *Psy2-2*, and the absence of amplification in substitution lines was used to assign that locus to a wheat chromosome.

Map construction and QTL analysis

A genetic linkage map of the W9262-260D3/Kofa population was constructed using the Haldane mapping function within the software JoinMap 3.0 (van Ooijen and Voorrips 2004) at a minimum LOD score of 3.0. A total of 109 simple sequence repeat (SSR) and 125 DArT® markers were successfully scored on the DH population, but significant segregation distortion was observed for eight SSR and seven DArT markers which were removed prior to map construction. Linkage groups were assigned chromosome names by comparing markers on the generated map to previously published durum maps (Korzun et al. 1999; Elouafi et al. 2001; Nachit et al. 2001; Elouafi and Nachit 2004) and the hexaploid wheat SSR consensus map (Somers et al. 2004).

Least square means of endosperm colour from six environments from previous work (Clarke et al. 2006)

were used in QTL analysis. Single marker analysis was performed (Lynch and Walsh 1998; Knapp 2001) with PROC MIXED (Littell et al. 1996) on the least square means, as was simple interval mapping (SIM) with MQTL (Tinker and Mather 1995) to identify markers most significantly associated with variation in yellow endosperm colour. To enhance the power of QTL detection (Jansen and Stam 1994), the analyses was repeated using those markers identified by SIM as being significantly associated with yellow colour as co-factors for QTL in a multiple locus model (MLM) in MapQTL Version 5.0 (van Ooijen 2004). For each environment, the significance threshold ($P < 0.01$) of the LOD score was determined as described previously (van Ooijen et al. 1999). Single factor ANOVA was used to assess marker association with phenotypic variance for those markers not assigned to linkage groups. For each QTL, the average QTL effect (one half the difference between parental marker class means) was estimated by MapQTL.

Results

Isolation, sequence analysis and mapping of *Psy* genes

The PCR-based cloning approach produced four partial *Psy* sequences from each of the parents of our mapping population, and based on homology, each could be assigned to one of four groups (Fig. 1). Two of the four sequence groups displayed greater homology to the rice *Psy1* gene and the remaining two sequence groups being most similar to the rice *Psy2* sequence (Fig. 1). The greatest variation between Kofa and W9262-260D3 was observed at *Psy1-1* followed by the *Psy2-1* locus (Fig. 1). The sizes of the partial *Psy2-1* sequences were variable between the two parents; 2,124 bp from W9262-260D3 and 2,103 bp from Kofa. The reduced size of the Kofa allele was due to a 21 bp deletion in a region corresponding to the fourth exon of the rice *Psy2* sequence (Fig. 2a). Although alleles of *Psy1-1* were similar in size between Kofa (2,439 bp) and W9262-260D3 (2,436 bp), a 17 bp deletion was identified that resulted in an *Eco*RI digestion site unique to the Kofa *Psy1-1* allele (Fig. 2b). A 25 bp deletion was also observed in the W9262-260D3 *Psy1-1* allele (Fig. 2c). Based on alignments with the rice *Psy1* cDNA sequence, both of the Kofa and W9262-260D3 *Psy1-1* deletions are present in putative intron regions. Kofa and W9262-260D3 sequences of *Psy1-2* were identical along the 2,444 bp sequenced (Fig. 1), and are thus the same gene. Because the 2,114 bp *Psy2-2* sequences of Kofa and W9262-260D3 were identical (Fig. 1), they were also assumed to be allelic.

A total of 101 microsatellite and 114 DArT® markers were assigned to 22 linkage groups, with the number of loci per group ranging from 4 to 23. Four SSR markers and four DArT® markers could not be assigned to any linkage group. The final map for QTL analysis consisted of 194 markers spanning greater than 1,400 cM. All chromosomes were represented, with chromosomes 6B and 7B having the greatest coverage. The *Psy1-1* and *Psy2-1* allele-specific CAPS markers (Fig. 3) developed from the *Eco*RI restriction site unique to the Kofa *Psy1-1* allele (Fig. 2b) and a polymorphic *Bsr*I restriction digestion site unique to the W9262-260D3 *Psy2-1* allele (Fig. 2d) are shown in Fig. 3. Application of the CAPS markers on the DH mapping population and subsequent linkage analysis indicated that *Psy1-1* mapped to chromosome 7B and the *Psy2-1* locus to chromosome 5B (Fig. 4).

Chromosome mapping of the *Psy1-2* and *Psy2-2* loci was performed using nullisomic–tetrasomic substitution lines given the lack of sequence variation between the two parents from which to develop polymorphic markers for mapping. Following amplification and

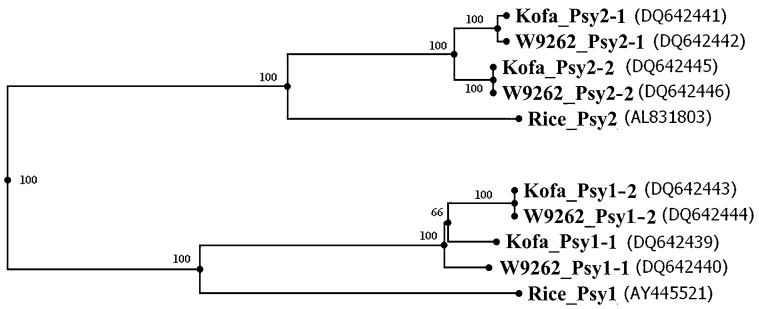

Fig. 1 Similarity dendrogram of partial *Psy* consensus sequences from Kofa and W9262-260D3 and the rice *Psy1* and *Psy2* cDNA sequences. Bootstrap values are shown at selected nodes as percentages of 1,000 replicates. All *Psy* gene sequences were deposited in GenBank with accession numbers presented

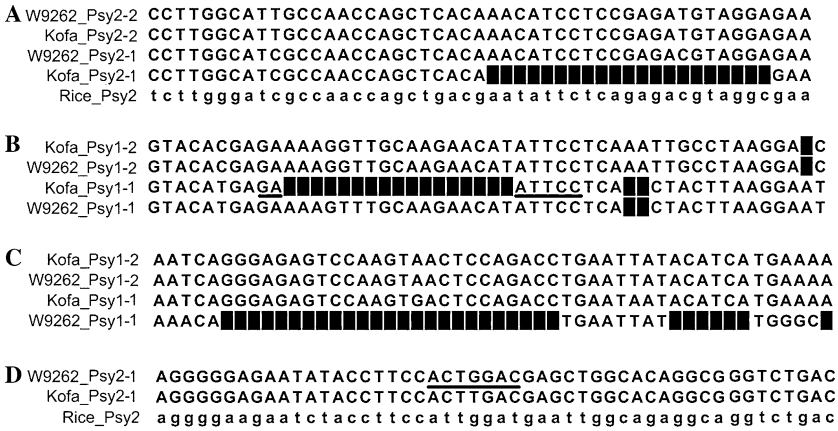

Fig. 2 Partial sequence alignments of the durum *Psy* genes illustrating **a** the presence of a 21 bp deletion in the Kofa *Psy2-1* allele in a region corresponding to the fourth exon of the rice *Psy2* sequence, **b** a 17 bp deletion in the *Psy1-1* allele from Kofa that results in the creation of an *Eco*RI digestion site (*underlined sequence*), **c** the presence of deletions in the W9262-260D3 *Psy1-1* allele; and **d** a *Bsr*I restriction digestion site (*underlined sequence*) unique to the W9262-260D2 *Psy2-1* allele in a region corresponding to the fifth exon of the rice *Psy2* sequence. The polymorphic *Eco*RI (**b**) and *Bsr*I (**d**) digestion sites were utilized to develop CAPS markers for genetic mapping of the *Psy1-1* and *Psy2-1* loci (see Materials and methods for details)

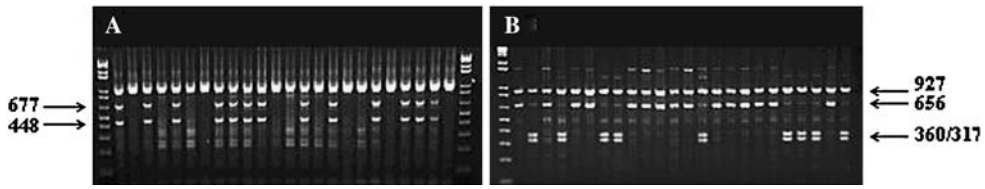

Fig. 3 An allele specific CAPS marker for *Psy1-1* (**a**) and *Psy2-1* (**b**). In both gels, *lane 1* is the DNA ladder, *lane 2* is Kofa; *Lane 3* is W9262-260D3. *Lanes 4–23* are a random set of DH lines from the W9262-260D3/Kofa mapping population. For *Psy1-1* (**a**), the Kofa allele contains an *Eco*RI restriction site that cleaves the amplified product into two fragments 677 and 448 bp in length. For *Psy2-1* (**b**), the Kofa allele possesses only a single *Bsr*I digestion resulting in the production of two fragments 927/656 bp in length, whereas digestion of the W9262-260D3 allele results in three fragments 927/360/317 bp in length due to the presence of two *Bsr*I digestion sites

digestion of the *Psy1* loci with *Hpa*II, the expected 857 and 760 bp fragments derived from *Psy1-1* and *Psy1-2*, respectively, were present in Kofa and Chinese Spring (Fig. 5). The absence of the 760 bp fragment in N7AT7D (Fig. 5) confirmed that *Psy1-2* is located on chromosome 7A. The 857 bp fragment from *Psy1-1* was absent in N7BT7D (Fig. 5), confirming genetic mapping experiments that *Psy1-1* is located on chromosome 7B. Amplification of *Psy2-2* using primers Psy2-2F/PsyR2 produced the expected 1,675 bp fragment from Kofa, Chinese Spring, and N5BT5D, but not in N5AT5D (Fig. 5b) indicating that *Psy2-2* is located on chromosome 5A.

QTL analysis and association of *Psy1-1* with endosperm colour

The W9262-260D3/Kofa population was chosen for QTL analysis and mapping of *Psy* genes because transgressive segregation for endosperm colour was previously reported in all six testing environments (Clarke et al. 2006), with the average difference between the high and low DH transgressive lines being 3.6 mg kg^{-1} (range 3.3–3.8 mg kg^{-1}) (Table 1). Both single factor ANOVA and simple interval mapping identified *Psy1-1* as being significantly associated with variation in colour (Fig. 4). Three other markers, *Xgwm193, Xgwm495* and *Xgwm425* were also identified and all four markers selected as cofactors in a multiple locus QTL analysis. Using the multiple QTL model, four QTL were identified in the W9262-260D3/Kofa mapping population on chromosomes 2A, 4B, 6B, and 7B (Table 2). The significance of each QTL varied among the environments, but all were significant in at least four of the six environments (Table 2). Using MLM analysis, the QTL on chromosome 7B spanned 4.7 cM and was highly significant in 2001 (Fig. 4), but not significant in two environments in 2002 (Table 2). The *Psy1-1* locus explained the majority of variation at the 7B QTL with an average effect ranging from 0.20 to

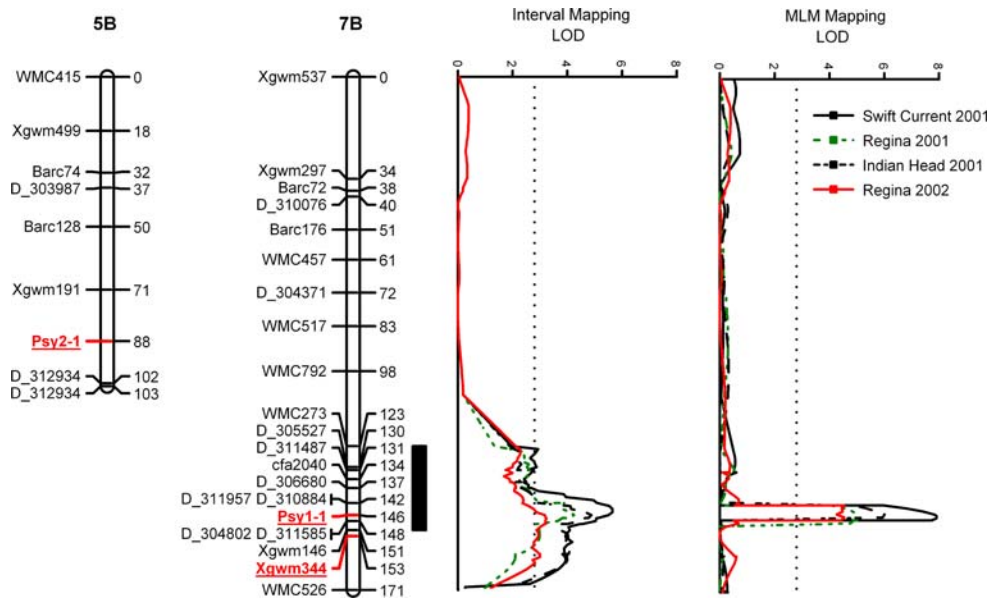

Fig. 4 Genetic linkage map of chromosomes 5B and 7B illustrating the map location of *Psy1-1* and *Psy2-1*. DArT® markers are labeled as clone ID numbers preceded with the letter D. Genetic distance of markers are indicated in cM to the right of the chromosome bar. QTL analyses of 7B by interval and MLM mapping are presented as LOD. The black bar represents the location of a previously reported QTL for yellow flour colour (Kuchel et al. 2006) and *Xgwm344*, a marker previously identified to be tightly linked to butanol-extracted yellow pigments in durum wheat (Elouafi et al. 2001)

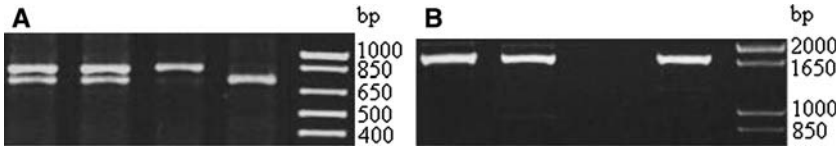

Fig. 5 Chromosome mapping of (**a**) *Psy1* loci and *Psy2-2* (**b**) using Chinese Spring nullisomic–tetrasomic substitution lines. Amplification of the two *Psy1* loci with Psy1F5/Psy1R5 and subsequent digestion with *Hpa*II resulted in the expected 857 and 760 bp fragments for *Psy1-1* and *Psy1-2*, respectively, in Kofa (*Lane A1*) and Chinese Spring (*Lane A2*). The 760 bp fragment was absent in N7AT7D (*Lane A3*) as was the 857 bp fragment in N7BT7D (*Lane A4*). Primers specific for *Psy2-2* successfully amplified the expected 1,675 bp fragment from Kofa (*lane B1*), Chinese Spring (*lane B2*), and N5BT5D (*lane B4*), but not from N5AT5D (*Lane B3*), indicating that *Psy2-2* is located on chromosome 5A

0.23 mg kg^{-1} (Table 2). The yellow colour QTL marked by *Xgwm193* on chromosome 6B was highly significant in three of the environments common to the 7B QTL, but not at Regina (2002) where the 7B locus was significant (Table 2). The average effect of the 6B QTL ranged from 0.15 to 0.21 mg kg^{-1} (Table 2). A QTL localized to chromosome 2A spanning 13.1 cM was significant at Swift Current (2001 and 2002), Regina (2002), and Indian Head (2002), with an average effect ranging from 0.15 to 0.21 mg kg^{-1} (Table 2). The QTL on 4B marked by *Xgwm495* was significant in the same four environments as the QTL on 2B, but was most pronounced at Indian Head 2002 with a LOD score of 4.0 (Table 2). The average effect at this locus ranged from 0.14 to 0.20 mg kg^{-1}. Unlike *Psy1-1*, *Psy2-1* was not associated with variation for endosperm colour (Table 2). The eight markers that could not be assigned to any linkage group were not associated with variation in yellow colour, regardless of environment (data not shown).

Both parents contributed positive alleles (elevated colour) to the DH progeny with Kofa contributing the positive alleles at the 7B and 4B QTL and W9262-260D3 at the QTL on 6B and 2A (Table 2). Doubled haploid lines with the positive alleles at marker loci *Psy1-1, Xgwm193*, *Xgwm425* and *Xgwm495* had on average 1.4 mg kg^{-1} higher colour expression (range of 1.3–1.6 mg kg^{-1}) than those DH lines possessing the negative alleles (Table 3). Similarly, those DH lines with the favorable alleles at each of the four QTL had

Table 1 Least square means of endosperm colour for the DH mapping population, Kofa, W9262-260D3 and the high and low transgressive genotypes (modified from Clarke et al. 2006)

	Swift Current 2001	Swift Current 2002	Regina 2001	Regina 2002	Indian Head 2001	Indian Head 2002
High transgressive	9.3	9.4	8.9	9.4	8.3	9.5
Kofa	7.8	8.1	8.0	7.7	7.1	7.9
W9262-260D3	7.5	7.3	7.3	7.2	6.5	7.6
Low transgressive	5.5	6.1	5.1	5.9	4.8	6.0
$LSD_{0.05}$	0.7	0.8	0.7	0.7	0.7	0.8

Values are yellow colour measurements predicted using NIRS calibrated yearly with butanol extracted yellow pigments (mg kg^{-1}) using AACC method 14–50

significantly greater colour than W9262-260D3 (Table 3), the parent with lower colour. At Swift Current (2001), the average endosperm colour of DH lines with all four positive QTL alleles had significantly higher yellow colour than Kofa, and at the remaining five environments, similar colour to Kofa (Table 3). Regardless of environment, lines with colour significantly greater than Kofa were noted to possess the four positive QTL alleles (Table 3). The DH lines carrying the alleles for reduced colour at all four QTL had significantly lower yellow colour than Kofa, and colour numerically lower than W9262-260D3 (Table 3).

Discussion

Four QTL for endosperm colour were identified in the Kofa/W9262-260D3 mapping population, and each varied from non-significant to highly significant depending on the environment. Only at Swift Current (2001) were all four QTL identified as being significant. Although endosperm colour is highly heritable (Elouafi et al. 2001; Clarke et al. 2006), our results demonstrated that colour genes are responsive to environmental cues, confirming earlier reports of QTL × environment interactions for grain pigment (Elouafi et al. 2001). Our results suggest that some environmental conditions trigger more loci to contribute greater expression of pigment variation, such as at Swift Current 2001, whereas other conditions trigger fewer loci to contribute expression of pigment variation such as at Regina 2001. Despite some environments generating fewer significant loci, the range in pigment was as great in these environments as the Swift Current 2001 environment (Table 1) where all four QTL were significant (Table 2). If multiple environments had not been evaluated, any combination of the loci may not have been represented. Perhaps more loci contributing to pigment expression can be discovered if the W9262-260D3/Kofa population were tested in more and varied environments.

Given the importance of *Psy* in carotenoid biosynthesis, we set out to determine if *Psy* gene(s) were associated with variation in endosperm colour in durum wheat. Duplicate *Psy* genes designated as *Psy1* and *Psy2* encoding unique, functional enzymes have been reported in other grass species (Gallagher et al. 2004). Because durum wheat is a tetraploid species, two loci, one in the A genome and one in the B genome, would be expected for each of the *Psy* genes. We successfully isolated four partial *Psy* sequences corresponding to a two member gene family from both Kofa and W9262-260D3. Between parents, allelic variation was greatest at *Psy1-1*, followed by the *Psy2-1* locus (Fig. 1). For *Psy1-1*, the most notable differences between Kofa and W9262-260D3 were allele specific deletions within putative intron regions (Fig. 2b, c). We did, however, predict the *Psy1-1* protein sequences using rice cDNA to define intron boundaries, and noted three amino acid differences between the Kofa and W9262-260D3 (data not shown). Since functional studies were not conducted, we are uncertain if these specific genetic changes are the causal factor(s) influencing variation in endosperm colour. In maize, insertions in the 5′ regulatory region of *Psy1* were found to be most associated with elevated yellow endosperm, likely due to increased expression of the gene product in the endosperm (Palaisa et al. 2003). More work is needed to sequence the complete *Psy1-1* sequences, including 5′ untranscribed regions, to determine if this is also the case in durum. Alternatively, *Psy1-1* maybe tightly linked to other gene(s) that influence the expression of yellow endosperm colour.

The Kofa *Psy2-1* allele, possessed a 21 bp deletion in a region corresponding to the fourth exon of the rice *Psy2* gene (Fig. 2a), which results in the loss of seven amino acids in the translated protein sequence. Despite this large deletion, nucleotide differences at the *Psy2-1* locus were not associated with phenotypic variation in yellow colour (Table 2). Others have noted that unlike *Psy1*, *Psy2* is not associated with carotenoid

Table 2 QTL loci associated with variation for yellow endosperm colour in the W9262-260D3/Kofa doubled haploid population

Chromosome	Marker	Positive Allele [a]	QTL Interval (cM)	Swift Current 2001		Swift Current 2002		Regina 2001		Regina 2002		Indian Head 2001		Indian Head 2002	
				LOD	Effect	LOD	Effect	LOD	Effect	LOD	Effect	LOD	Effect	LOD	Effect
2A	*Xgwm425*	W	6.1	3.5**	0.16 ± 0.05	2.6*	0.15 ± 0.04	0.6^{ns}	–	4.8**	0.21 ± 0.04	0.8^{ns}	–	3.0**	0.17 ± 0.05
4B	*Xgwm495*	K	13.1	3.3**	0.14 ± 0.05	3.6**	0.19 ± 0.05	0.7^{ns}	–	2.6*	0.15 ± 0.05	0.3^{ns}	–	4.0**	0.20 ± 0.05
6B	*Xgwm193*	W	13.2	6.4**	0.21 ± 0.04	3.3**	0.15 ± 0.04	4.2**	0.19 ± 0.05	2.5^{ns}	–	4.7**	0.18 ± 0.04	0.8^{ns}	–
7B	*Psy1-1*	K	5.1	8.0**	0.23 ± 0.05	2.3^{ns}	–	5.0**	0.20 ± 0.06	4.5**	0.21 ± 0.06	6.0**	0.20 ± 0.05	2.2^{ns}	–
5B	*Psy2-1*	–	–	0.5^{ns}	–	0.8*	ns	0.3^{ns}	–	0.5^{ns}	–	0.5^{ns}	–	0.2^{ns}	–

QTL effects (mg kg^{-1}) ± standard deviation explained by markers closest to the centre of the QTL are presented

[a] Parent contributing the positive allele (elevated endosperm colour) at each QTL where W = W9262-260D3 and K = Kofa

**LOD score significant at the 1% level, *LOD significant at the 5% level, *ns* not significant at the 5% significance level

accumulation as that gene is down regulated during grain fill (Gallagher et al. 2004). Because carotenoids are involved in protection of the photosynthetic apparatus, it has been suggested that *Psy* gene duplication may provide plants with a mechanism to modify gene expression in the seed (i.e., regulate *Psy1* without altering *Psy2* expression) thereby avoiding deleterious effects on photosynthetic organs from over or under expression appropriate to the seed (Gallagher et al. 2004). Furthermore, the duplicate *Psy* genes may code for enzymes that localize to different plastid membranes given the variant peptide N and C terminal sequences predicted based on their gene sequences (Gallagher et al. 2004).

Genetic mapping revealed *Psy1-1* located to chromosome 7B (Fig. 4), which was confirmed using chromosome mapping (Fig. 5). Genetic mapping also confirmed that *Psy2-1* resided on chromosome 5B. Because Kofa and W9262-260D3 partial sequences were identical at both the *Psy1-2* and *Psy2-2* loci, we could not design polymorphic markers for mapping. However, it is logical to assume that they are homeologous to *Psy1-1* and *Psy2-1*, respectively. To test this hypothesis, we assigned these loci to chromosomes using available wheat substitution lines and confirmed that *Psy1-2* mapped to 7A and *Psy2-2* to 5A. Obviously, further sequencing is required to identify sequence variation from which to develop polymorphic markers for genetic mapping of *Psy1-2* and *Psy2-2*, but since no QTL for endosperm colour were identified on chromosomes 7A and 5A in our mapping population, we did not pursue detailed genetic mapping of these loci.

Using both interval and MLM mapping, the *Psy1-1* locus explained the majority of variation at the 7B QTL (Fig. 4), confirming our hypothesis that this gene is associated with variation in endosperm colour. Although *Psy1-1* was not significant at Swift Current (2002) and Indian Head (2002), the LOD scores were ≥2.2 at those sites (Table 2), suggestive that *Psy1-1* did have a small effect, albeit to a lesser extent than at other environments, on expression of endosperm colour. In durum, Elouafi et al. (2001) reported a QTL for butanol-extracted yellow pigments on 7B flanked by *Xgwm344*. Although *Xgwm344* was not polymorphic in our population, that marker maps 1.5 cM from *Xgwm146* on the hexaploid wheat consensus map (Fig. 4; Somers et al. 2004). In our study, *Xgwm146* flanked the 7B QTL, mapping approximately 5 cM from *Psy1-1* (Fig. 4). Similar to this study, Kuchel et al. (2006) identified a QTL on 7B associated with variation in yellow flour colour in a hexaploid wheat mapping population

Table 3 Least square means of endosperm colour (NIR predicted yellow pigment concentration, mg kg^{-1}) of Kofa, W9262-D063 for genotypes homozygous for alleles with a positive effect (*WWKK*) and negative effect (*kkww*) on endosperm colour at the four QTL identified in this study are presented in bold

	Swift Current 2001[b]	Swift Current 2002	Regina 2001	Regina 2002	Indian Head 2001	Indian Head 2002
Kofa	7.8a	8.1a	8.0a	7.7ab	7.1ab	7.9a
W9262-D063	7.5a	7.3b	7.3ab	7.2bc	6.5bc	7.6ab
WWKK[a]	**8.6b**	**8.2a**	**8.1a**	**8.4a**	**7.6a**	**8.5a**
MIN	*8.1*	*7.5*	*7.4*	*7.4*	*7.1*	*7.5*
MAX	*9.3*	*9.4*	*8.9*	*9.4*	*8.3*	*9.1*
kkww[a]	**6.9a**	**6.7b**	**6.7b**	**6.9c**	**6.3c**	**7.2b**
MIN	*5.5*	*6.1*	*5.1*	*6.1*	*4.8*	*6.0*
MAX	*7.7*	*7.7*	*7.1*	*7.4*	*7.1*	*7.3*
Difference	**1.6**	**1.5**	**1.4**	**1.5**	**1.3**	**1.3**
SED	**0.21**	**0.17**	**0.19**	**0.22**	**0.17**	**0.20**

The LS means of genotypes with minimum and maximum colour in each marker class are presented in italics. The standard error of the difference (SED) between the two marker classes is presented

[a] Genotype where W (w) indicates the W9262-260D3 and K (k) indicates Kofa alleles at the four QTL ordered as follows: *Xgwm193* (6B), *Xgwm425* (2A), *Xgwm495* (4B) and *Psy1-1* (7B)

[b] Means in columns followed by the same letter are not significantly different at the 5% significance level

spanning the interval *Xgwm273-Xgwm146* (Fig. 4). Taken together, the 7B QTL identified in previous reports is the same QTL identified in this study. Given the importance of *Psy* in carotenoid biosynthesis (Fraser and Bramley 2004) and its association with elevated pigment in other species (Gallagher et al. 2004), *Psy1-1* is a strong candidate gene for this QTL. Since *Psy1-2* is homeologous to *Psy1-1*, it may be associated with the yellow colour QTL identified on chromosome 7A in other durum wheat mapping populations (Elouafi et al. 2001; Cervigni et al. 2005), further supporting the role of *Psy1* in expression of endosperm colour.

The W9262-260D3/Kofa DH population has proven to be useful for detecting QTL for yellow colour at a variety of loci whereas to date, the majority of the QTL detected have been reported separately in different durum and hexaploid wheat mapping populations. The W9262-260D3/Kofa population will thus be useful for mapping putative candidate genes thought to be associated with the other yellow colour QTL identified in this study. Joppa et al. (1988) concluded that there were major genes for yellow colour on the group 2 chromosomes, which is in agreement with the QTL identified on 2A in this study. Recently, Cenci et al. (2004) mapped two putative homeologous ζ-carotene desaturase (*Zds*) clones to group 2 chromosomes in durum wheat. Because the gene coding for this enzyme has been cloned (Matthews et al. 2003) and its importance to the accumulation of carotenoids in maize seeds verified (Wong et al. 2004), we are pursuing this gene(s) as a possible candidate for the QTL localized to 2A in our mapping population.

Hessler et al. (2002) reported a QTL for yellow colour on chromosome 4A, and the QTL on 4B identified in the W9262-260D3/Kofa population at *Xgwm495* may be homeologous to that identified in their study. Putative genes coding for phytoene desaturase (*Pds*), an enzyme important to the biosynthesis of carotenoids, have been mapped to the group 4 chromosomes in durum (Cenci et al. 2004), and may be associated with the 4B QTL identified in the W9262-260D3/Kofa population. Alternatively, Hessler et al. (2002) mapped a lypoxygenase gene, *Lxp-B1* on chromosome 4B near *Xgwm165*. Because *Xgwm165* maps only 4 cM from *Xgwm495* on the wheat SSR consensus map (Somers et al. 2004) it is possible that the 4B QTL identified in this study is associated with the *Lxp-B1* locus. This latter hypothesis is supported by the fact that *Lxp-B1* has recently been mapped to 4B in a population derived from a cross involving Kofa, with the Kofa allele contributing to reduced lipoxygenase activity (Cervigni et al. 2005). Kofa contributed the positive allele for yellow colour at the 4B QTL in the W9262-260D3/Kofa population (Table 2), and given the negative effects of lipoxygenase on yellow colour (Borrelli et al. 1999; Manna et al. 1998), reduced lipoxygenase activity could result in increased colour. Although pigment loss due to lipoxygenase activity is most pronounced following pasta manufacturing (Borelli et al. 1999), we cannot rule out the possibility that it is a factor influencing yellow colour in our mapping population.

The QTL identified on chromosome 6B in this study has yet to be reported in the literature, and maybe a novel QTL for endosperm yellow colour. Alternatively,

previous work has shown that kernel weight and endosperm colour are negatively correlated in some populations (Alvarez et al. 1999; Clarke et al. 2006) and may be due to pigment dilution by increased starch content in larger seeds (Hessler et al. 2002). Elouafi and Nachit (2004) identified a QTL on chromosome 6B marked by *Xgwm582*, which explained 20% of the phenotypic variation in kernel weight. In that study, *Xgwm582* mapped approximately 30 cM away from *Xgwm193*, the marker most significantly associated with pigment in our study. We are currently conducting studies to determine if the 6B QTL is responsible for elevated yellow colour, or if it is associated with seed size and a corresponding pleiotropic effect.

In this study, we chose to measure total yellow colour using NIR prediction, as numerous studies have shown that with appropriate calibration, this technique is a precise tool for yellow colour measurement (McCaig et al. 1992; Edwards et al. 1996; Brenna and Bernardo 2004). Furthermore, NIR prediction has proven useful for prediction of individual carotenoid pigments in maize semolina (Brenna and Berardo 2004). We do, however, acknowledge that a proportion of phenotypic variation maybe associated with prediction error, potentially biasing our estimates of QTL effects. It is also possible that predicting colour using NIR could have resulted in the identification of QTL that are not associated with colour *per se*, but with other factors impacting on light reflectance. Indeed Mares and Campbell (2001) reported QTL associated with variation in instrumental measure of flour yellowness that were not associated with variation in direct chemical measures of xanthophylls. However, several lines of evidence support our use of NIR for colour evaluation and QTL mapping including the high r^2 of prediction ($r^2 = 0.94$–0.98) for our NIR calibration and the fact that the majority of the colour QTL detected in this study have been reported separately in other durum and hexaploid wheat mapping populations. Most notably, the 7B QTL for flour colour identified by Kuchel et al. (2006) was found to span the interval *Xgwm273-Xgwm146*, the same interval identified in this study (Fig. 4). The association of *Psy1-1*, a gene important in the biosynthesis of carotenoids, with variation in NIR predicted colour further supports that the QTL identified in this study are associated with endosperm colour, at least for the 7B QTL.

Bidirectional transgressive segregation for yellow colour was observed in the W9262-260D3/Kofa mapping population consistently across multiple environments (Clarke et al. 2006; Table 1), and indicates that neither Kofa nor W9262-260D3 carries all of the desirable alleles for elevated endosperm colour. This was confirmed by the QTL analysis showing QTL contributions to colour from both parents (Table 2). The markers associated with the QTL identified in this study will be useful for increasing yellow colour by combining the positive alleles at all four loci. Regardless of environment, the mean of the DH lines with all four positive alleles had higher yellow colour than W9262-260D3 (Table 3), the lower pigment parent. Although the mean colour of these DH lines was similar to the high pigment parent Kofa (Table 3), transgressive segregants with colour significantly greater than Kofa were identified in all environments (Table 3). Although there was overlap in expression of yellow colour between DH lines with the four positive and negative QTL alleles (Table 3), selection with these markers could aid in the identification of lines with a minimum acceptable level of yellow colour, similar to Kofa.

When selecting markers for use in marker assisted breeding, it would be most efficient to choose markers that are significant over a wide range of germplasm and environmental conditions. The 7B QTL localized in this study has consistently been reported in a number of tetraploid and hexaploid wheat mapping populations. The strong association of *Psy1-1* with the 7B QTL suggests that this gene maybe a useful target for marker assisted selection for yellow colour in a range of wheat germplasm. In this study, we developed a CAPS marker for *Psy1-1*, which is specific for the Kofa allele, but we have not ascertained if this marker would be useful in other crosses. However, we are currently sequencing *Psy1* alleles from a set of 96 genetically diverse durum wheat cultivars with a large range in phenotypic expression of pigment and performing associative genetic analyses (Thornsberry et al. 2001) to identify those alleles that have the greatest effect on increasing yellow colour. This strategy will allow the identification and development of a molecular marker for molecular-based selection of the most desirable *Psy1-1* allele(s) and will represent the first step toward implementation of allele-specific marker assisted selection for elevated endosperm colour.

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