Theor Appl Genet (2011) 123:95–108
DOI 10.1007/s00122-011-1569-9

ORIGINAL PAPER

Functional relationships of *phytoene synthase 1* alleles on chromosome 7A controlling flour colour variation in selected Australian wheat genotypes

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Received: 24 May 2010 / Accepted: 11 March 2011 / Published online: 27 March 2011

Abstract Flour colour measured as a Commission Internationale de l'Eclairage (CIE) *b** value is an important wheat quality attribute for a range of end-products, with genes and enzymes of the xanthophyll biosynthesis pathway providing potential sources of trait variation. In particular, the *phytoene synthase 1* (*Psy1*) gene has been associated with quantitative trait loci (QTL) for flour *b** colour variation. Several *Psy1* alleles on chromosome 7A (*Psy-A1*) have been described, along with proposed mechanisms for influencing flour *b** colour. This study sought to identify evolutionary relationships among known *Psy-A1* alleles, to establish which *Psy-A1* alleles are present in selected Australian wheat genotypes and establish their role in controlling variation for flour *b** colour via QTL analysis. Phylogenetic analyses showed seven of eight known *Psy-A1* alleles clustered with sequences from *T. urartu*, indicating the majority of alleles in Australian germplasm share a common evolutionary lineage. In this regard, *Psy-A1a*, *Psy-A1c*, *Psy-A1e* and *Psy-A1p* were common in Australian genotypes with flour *b** colour ranging from white to yellow. In contrast *Psy-A1s* was found to be related to *A. speltoides*, indicating a possible A–B genome translocation during wheat polyploidisation. A new allele *Psy-A1t* (similar to *Psy-A1s*) was discovered in genotypes with yellow flour, with QTL analyses indicating *Psy-A1t* strongly influences flour *b** colour in Australian germplasm. QTL LOD value maxima did not coincide with *Psy-A1* gene locus in two of three populations and, therefore, *Psy-A1a* and *Psy-A1p* may not be involved in flour colour. Instead two other QTL were identified, one proximal and one distal to *Psy-A1* in Australian wheat lines. Comparison of *Psy-A1t* and *Psy-A1p* predicted protein sequences suggests differences in putative sites for post-translational modification may influence enzyme activity and subsequent xanthophyll accumulation in the wheat endosperm. *Psy-A1a* and *Psy-A1p* were not involved in flour *b** colour variation, indicating other genes control variation on chromosome 7A in some wheat genotypes.

Communicated by G. Bryan.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-011-1569-9) contains supplementary material, which is available to authorized users.

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Introduction

Flour colour is a key quality trait for wheat, and plays a critical role in determining product end use and markets (Fu 2008; Mares and Campbell 2001). For example, many bread products and white salted noodles typically require a white or creamy flour type, whereas for yellow alkaline noodles a bright yellow colour is desired. In this regard, flour whiteness/yellowness is typically measured as a Commission Internationale de l'Eclairage (CIE) *b** value. There is generally a high correlation between *b** and the

yellow pigmentation of wheat flour, which is determined primarily by the presence of carotenoid/xanthophyll compounds (Fratianni et al. 2005; Mares and Campbell 2001; Miskelly 1984). The most abundant xanthophyll compound detected in the flour of mature wheat seed is lutein (Howitt et al. 2009; Howitt and Pogson 2006), which is formed by the addition of hydroxyl groups to α-carotene through carotenoid β-ring hydroxylase and carotenoid ε-ring hydroxylase activities (Botella-Pavía and Rodríguez-Concepción 2006).

Flour *b** colour (or xanthophyll content) in wheat is a highly heritable complex genetic trait, with different varieties often possessing significant quantitative trait loci (QTL) on different chromosomal groups (e.g. Mares and Campbell 2001; Patil et al. 2008). QTL on chromosome group 7 are most frequently reported to be involved in variation for flour colour. Specifically, major QTL have been detected on chromosomes 7A and 7B for both hexaploid common wheat (*Triticum aestivum* L.) (Howitt et al. 2009; Kuchel et al. 2006; Mares and Campbell 2001; Parker et al. 1998; Zhang et al. 2009) and tetraploid durum wheat (*Triticum turgidum* L. ssp. *durum*) (Elouafi et al. 2001; Patil et al. 2008; Pozniak et al. 2007). These loci have been shown to account for up to 60% of flour colour variation for *T. aestivum* mapping populations (Kuchel et al. 2006; Parker et al. 1998) and are important chromosomal regions for trait manipulation in wheat improvement.

The high correlation between xanthophyll accumulation and flour colour has resulted in intensified efforts to identify candidate genes from the xanthophyll pathway that are co-located with QTL for flour *b** on wheat chromosomes 7A and 7B. In this regard, several studies indicate the *phytoene synthase 1* (*Psy1*) gene contributes to flour *b** colour variation in a range of wheat genotypes. Phytoene synthase is the first committed enzyme of the carotenoid/xanthophyll pathway, producing phytoene via the condensation of two geranylgeranyl diphosphate molecules (Botella-Pavía and Rodríguez-Concepción 2006). Pozniak et al. (2007) were the first to co-locate *Psy1* with a QTL on 7B in a single durum wheat mapping population, and a homologous *Psy1* sequence was later identified and co-located with a QTL for flour *b** colour on chromosome 7A in three durum wheat mapping populations (Singh et al. 2009). In hexaploid wheat, He et al. (2008) identified *Psy1* on chromosome 7A co-segregating with white and yellow phenotypes, and then demonstrated a close association between *Psy-A1* allele type and yellow pigment content across a panel of 217 Chinese wheat and 342 CIMMYT Spring wheat accessions (He et al. 2009a). *Psy1* allelic variation has also been linked with flour *b** colour QTL on 7A in Australian hexaploid wheat cultivars (Howitt et al. 2009). To date, no other genes in the xanthophyll pathway have been mapped to group 7 chromosomes in wheat.

Psy-A1 alleles may have a functional role in controlling flour colour in Australian hexaploid wheats, given previous reports of *Psy1* co-locating with flour *b** colour QTL on chromosome 7A. In this regard, there are currently eight *Psy-A1* alleles described for *T. aestivum* (*Psy-A1a*, *Psy-A1b*, *Psy-A1c*, *Psy-A1e*, *Psy-A1p*, *Psy-A1q*, *Psy-A1r* and *Psy-A1s*), some of which have substantial sequence differences that may be linked to flour colour variation in different genotypes (He et al. 2008, 2009a; Howitt et al. 2009). For example, the alleles *Psy-A1b* and *Psy-A1e* have been linked to cultivars with white flour colour through QTL or association analyses (He et al. 2008; Howitt et al. 2009; Zhang and Dubcovsky 2008). These two alleles possess a 37 bp insertion in exon 2, which alters translation and probably results in non-functional gene products (Howitt et al. 2009). A simple co-dominant molecular marker (YP7A) allowing electrophoretic identification of germplasm possessing this insert was designed by He et al. (2008). In contrast, the *Psy-A1c* and *Psy-A1s* alleles have been tentatively associated with yellow flour types, attributed to the absence of a transposable element of around 700 bp in the fourth intron (He et al. 2009a; Howitt et al. 2009). A multiplex CAPS molecular marker system was developed by Howitt et al. (2009) that distinguishes four allele types associated with a range of flour colour types (*Psy-A1e*, *Psy-A1p*, *Psy-A1r*, and *Psy-A1s*). However, additional clarification regarding the involvement of various *Psy-A1* alleles in wheat flour colour variation is required, particularly for those proposed to be associated with yellow flour types.

The development of markers for genes such as *Psy-A1*, in conjunction with phylogenetic approaches, provides opportunities to study the origin and evolutionary relationships of alleles, their functional significance in trait variation and application to Australian wheat breeding. Therefore, the aim of this study was to clarify sequence relationships among available *Psy-A1* alleles in wheat through phylogenetic analysis and determine their functional association with flour *b** QTL in selected Australian hexaploid wheat genotypes. This information will provide a means to identify the value of using *Psy-A1* alleles for breeding wheat varieties with a range of flour *b** values.

Methods

Phylogenetic analyses

Geneious version 4.7.5 software (Drummond et al. 2007) was used for *Psy-A1* sequence analyses. *Psy1* sequences from wheat and related *Triticum* and *Aegilops* species were retrieved from the NCBI website (http://www.ncbi.nlm.nih.gov/) via BLASTn using *Psy-A1a* [EU096091] as the

query sequence. A total of 60 sequences were aligned using the 65% similarity cost matrix default setting, and a preliminary neighbour-joining phylogeny constructed with 100 bootstrap replications (data not shown). Phylogenetically uninformative sequences that were nearly identical in terms of sequence similarity and specific origin were removed from the dataset, with the exception of all *T. aestivum Psy-A1* alleles. A total of 42 *Psy1* DNA sequences were retained for further analysis, including *Thinopyrum ponticum* [EU096095] which was used as an outgroup sequence. Subsequent realignment produced a final ungapped dataset of 1,959 bp, comprising 830 bp of coding sequence and 1,129 bp of intron sequence (supplementary file). A maximum likelihood phylogeny was constructed with PHYML (Guindon and Gascuel 2003) using the general-time-reversible (GTR) substitution model with 1,000 bootstrap replications, estimated transition/transversion ratio and proportion of invariable sites. A neighbour-joining (NJ) phylogeny was also constructed using the Tamura–Nei genetic distance model.

Plant material

Hexaploid wheat genotypes were obtained from the seed collection at the Department of Agriculture and Food, Western Australia. The F_1-derived doubled haploid (DH) populations, Ajana/WAWHT2074 (179 individuals) and Carnamah/WAWHT2046 (121 individuals) were described previously by Francki et al. (2009) and Bariana et al. (2006), respectively. A third DH population derived from an F_1 cross between Ajana and WAWHT2046 (127 individuals) was developed using the wheat × maize system as described by Laurie and Bennett (1988).

DNA extraction and genotyping for *Psy-A1* alleles

DNA was extracted from wheat genotypes and individuals of mapping populations using standard phenol/chloroform techniques as described by Francki et al. (1997), quantified using a Nanodrop® ND-1000 v3.2 Spectrophotometer (Thermo Fisher Scientific Inc., DE, USA) and adjusted to a concentration of 25 ng/µl. All cultivars were screened with two separate primer pairs which amplify approximately 1,000 bp of sequence from the 5′ region of the *Psy1* gene on chromosome 7A. The primers PSY7A-1F/YP7AR were used to amplify all known *T. aestivum Psy-A1* alleles with the exception of *Psy-A1s*, which was amplified instead by the primers PSY7A-5F/PSY7A-5R. To further distinguish *Psy-A1p* and *Psy-A1q* alleles, primers PSY7A-7F and PSY7A-7R were designed to amplify a known SNP within exon 1. Primer sequences are provided in Table 1. PCRs consisting of 50 ng of genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM primer, 6% (v/v) dimethyl sulfoxide (DMSO) and 1.5 U Taq polymerase (Fisher Biotech, WA, Australia) were performed in 25 µl volumes. Thermocycling parameters were 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 60–62°C for 15 s and 72°C for 30 s, with a final extension of 72°C for 7 min. PCR products were electrophoresed in 50 µL aliquots on a 1.5% agarose gel pre-stained with GelGreen (Biotium, CA, USA) and visualised on a Dark Reader (Clare Chemical Research, CO, USA). PCR bands were gel-excised using GeneCatcher tips (The Gel Company, CA, USA) and purified using a QIAquick gel cleanup kit (QIAGEN, CA, USA). Products were sequenced using the Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, CA, USA) and analysed using Geneious (Drummond et al. 2007), with allele assignments based on DNA sequence.

Parents and individuals of each DH mapping population were genotyped for *Psy-A1* alleles via PCR. Two separate assays were performed for the Ajana/WAWHT2074 mapping population, the first using the primers PSY7A-1F/YP7AR to amplify the Ajana allele, and the second using the primers PSY7A-5F/PSY7A-5R to amplify the WAWHT2074 allele. The primers GAPDH-2F and GAPDH-1R (Table 1) were used as a positive control for both assays. To distinguish the parents and DH individuals of the Ajana/WAWHT2046 and Carnamah/WAWHT2046 mapping populations, a three primer PCR was developed whereby the primers PSY7A-1F and YP7AR were used as a positive control, with the Ajana and Carnamah haplotypes detected using the internal primer PSY7A-SNP2 (Table 1). PCR products were amplified and visualised as described above.

Trial designs

Phenotypic evaluation for flour *b** colour variation was designed and analysis using two-phase experiments as described by Smith et al. (2006) using grain samples taken from a field experiment (Phase I) and then processed further in a laboratory experiment (Phase II). Separate designs were generated for each phase, accounting for random allocation of genotypes in both field plots and grain samples for milling and *b** evaluation. A new class of designs (*p/q* designs) described by Cullis et al. (2006) used partial replication (percentage *p*) of genotypes in Phase I and a percentage, *q*, of the grain samples duplicated at Phase II. In this study, eight milling data sets from three DH populations (Ajana/WAWHT2074, Carnamah/WAWHT2046 and Ajana/WAWHT2046) were analysed from trials at three locations in Western Australia (Bullaring, Merredin and Wongan Hills) for the period 2002–2005. Details of the populations, trial locations, field and laboratory layouts for two-phase analysis of experiments are provided in Table 2. Apart from the DH lines, commercial cultivars and

Table 1 *Psy-A1* primer sequences used in the current study, with position based on *T. aestivum cv. Chinese Spring* [EF600063] or *cv. Schomburgk* [EU649795]

Primer	Sequence	Position	Reference
PSY7A-1F	GCTCACACGAGAGTGGTGAA	81–100	
YP7AR	TCATTCTCACTTCAGACCGTCA	993–972	Cenci et al. (2004)
YP7AF	GGACCTTGCTGATGACCGAG	800–819	Cenci et al. (2004)
PSY7A-5F	GCGGAGTGGTGACTGGTG	91–108[a]	
PSY7A-5R	GGCGGTCTGAACTCTGAAGTG	1,013–993[a]	
PSY7A-7F	GCCACCACTCCGGCCCATAC	25–44	
PSY7A-7R	TGCTGCTGTTGCTGCGAGGG	571–522	
PSY7A-LTF1	CAGTGCTCCCGCCTGCTACCCA	306–327	
PSY7A-LTR1	GGCGGAGCGAGGGGTGACTGAG	3,475–3,454	
M13F	CGCCAGGGTTTTCCCAGTCACGAC	–	
M13R	TCACACAGGAAACAGCTATGAC	–	
PSY7A-seq1	GGACCCTCCATTGTTGCTCCCC	894–915[a]	
PSY7A-seq2	CGCATCCCGTCGATCATGTCCTT	2,379–2,357[a]	
PSY7A-3R	ATGAAGTACCGCTCCACCAC	3,060–3,041	
PSY1-XR1	CTCCAAGGCGACAGCTGAGA	2,429–2,410	He et al. (2008)
Y3F	TATGGTGCAGGAGGACAGAC	1,442–1,461	Cenci et al. (2004)
PSY7A-8F	GCGGCCACCACTCCGGTC	16–33[a]	
PSY7A-8R	CCGCCTGCTTCACCACCACG	544–525[a]	
PSY7A-9F	CGCTCTGGCTCTCGGGCTG	2,527–2,545[a]	
PSY7A-9R	CGCGACACCGCCCTGTTTCT	3,392–3,373[a]	
PSY7A-SNP2	CCATGCACGACCAGACATA	796–778	
GAPDH-2F	CGAAGCCAGCAACCTATGAT	–	
GAPDH-1R	CAAAGTGGTCGTTCAGAGCA	–	

[a] Position based on *T. aestivum cv. Schomburgk*

Table 2 Summary of DH populations, environments and two-phase experiment for field design and laboratory analysis of grain samples for flour *b** colour evaluation

Populations	Site and year	Genotypes			Field layout columns/rows	Laboratory experiments # milling samples/# days
		Total (*n*)	DH population (*n*)	Cultivars and breeding lines (*n*)		
Ajana/WAWHT2074	Wongan Hills 2002	181	173	8	13 × 20	49 × 6
	Wongan Hills 2003	211	203	8	12 × 22	54 × 6
	Merriden 2003	211	202	9	12 × 22	54 × 6
Carnamah/WAWHT2046	Wongan Hills 2002	129	121	8	8 × 20	47 × 4
	Wongan Hills 2003	129	120	9	12 × 13	50 × 4
	Merriden 2003	129	120	9	12 × 13	50 × 4
Ajana/WAWHT2046	Wongan Hills 2005	157	127	30	12 × 16	60 × 4
	Bullaring 2005	157	127	30	12 × 16	53 × 6

advanced breeding lines were also included in the trials with different degrees of replication in both field and milling phases of each trial. The *p* and *q* values were in the range of 22–24 and 14–29%, respectively. Partially replicated designs for the field and laboratory experiments were designed using DIGGER software (Coombes 2002). Typically, the trials were laid out in rectangular arrays indexed by rows and columns. Each trial plot was of size 5 m × 1.25 m, reduced to 3 m × 1.25 m, prior to harvesting in order to reduce neighbouring effects. The design for the laboratory was a resolvable incomplete block design with milling days used as the block factor.

Flour *b** measurements

Grain samples (20 g) were conditioned to 13% moisture content and milled using a Quadramat Junior Mill with a 4XX screen with a 250 μm aperture. Flour samples were measured for colour using a Minolta CR-400 Chroma meter calibrated using a standard white tile and checked daily against a flour control sample. Flour colour was evaluated using International Commission on Illumination (CIE) *b** colour space parameters according to method 9-02 of the Royal Australian Chemical Institute's "Official Testing Methods of the Cereal Chemistry Division". (Reference Cereal Chemistry Division, RACI, Official Testing Methods, 4th Edition 2003.)

Statistical analysis

Linear mixed models (Smith et al. 2006) were used for the analysis of trials. In multi-phase quality trait experiments, possible trends associated with residuals for each of the phases including spatial trends in the field and temporal trends in the laboratory were modelled accordingly. Spatial variation was partitioned into three components (Gilmour et al. 1997) including global trend, local (natural) trend and extraneous variation. Global trends were reflected by non-stationary trends across the field. Local trend reflected mainly moisture and fertility trends in the soil. Extraneous variation was related to agronomic and/or management practices in the field, e.g. serpentine harvesting and sowing. Trials analyses of the Phase I followed the approach of Gilmour et al. (1997) and Stefanova et al. (2009) whereas Phase II included temporal variation and blocking structure (milling days) in the model. The covariance structure in the field was linked to field rows and columns (a separable autoregressive process of order 1, AR1 × AR1), whilst the covariance structure at laboratory level was linked to the milling order of the samples within a milling day. Therefore, the model used in the analysis was represented here as

$$y \sim 1 + type + genotype + millrep + frep + column.row + millday.millorder$$

where '*1*' represents an overall mean, *type* is a factor with number of levels corresponding to the parental genotypes, commercial varieties and DH (only one level assigned to all DH genotypes), *genotype* is a factor with number of levels equal to the number of genotypes in the trial, *millrep* and *frep* were factors reflecting the blocking structures associated with the two randomizations (randomization of genotypes to the field plots and randomization of field plots in the laboratory processing). Both factors were defined with two levels for the trials. Terms *column.row* and *millday.millorder* represent the field and laboratory residuals, respectively. The *1* and *type* corresponded to the fixed effects in the model whilst the remaining terms were fitted as random.

Random effects in the model were predicted using empirical best linear unbiased prediction (E-BLUP) and fixed effects were estimated using empirical best linear unbiased estimation (E-BLUE). Additionally, prediction error variance matrix for the genotype effects was obtained from the analysis and used to calculate generalized measure of broad-sense heritability using the average pairwise prediction error variance of genotype effects (Cullis et al. 2006) and calculated for each DH population in each trial. All analyses were conducted using ASREML (Gilmour et al. 2006) and samm (Butler et al. 2003) and adjusted mean values for flour *b** colour variation was used for QTL analysis. For the 30 Australian wheat genotypes, contrast analysis (ANOVA) of *b** values and *Psy-A1* allele type was performed using GenStat for Windows 12th Edition (Payne et al. 2009).

Genetic map construction and QTL analysis

A genetic map derived from the Ajana/WAWHT2074 DH population was described by Francki et al. (2009) and consisted of 575 markers (339 DArT and 234 SSR) covering 2,825 cM with an average of 6.6 cM distance between markers. The Carnamah/WAWHT2046 genetic map has been described previously (Bariana et al. 2006) but expanded to include 314 markers (59 stm, 157 SSR, 91 DArT and 7 other) covering 3,162 cM with an average of 10.1 cM distance between markers. The third genetic map was derived from the Ajana/WAWHT2046 DH population totalling 163 SSR and DArT markers similar to genetic maps described in Francki et al. (2009) and covering 2,616 cM with an average of 16.1 cM between markers. The maps were constructed using Mapmanager QTXb20 (Manly et al. 2001) using the "Distribute" command, linkage criterion of $P = 0.001$ and Kosambi function to calculate genetic distances from recombination fractions. The "Ripple" command was used to determine the best order of markers within linkage groups. Marker order was confirmed using RECORD (Ripples = 0, EQV threshold = 0) (Van Os et al. 2005).

The mean *b** values adjusted for field and laboratory spatial variation in the two-phase experimental design was used for composite interval mapping (CIM). CIM model one of QTL Cartographer version 2.5 (36) was used with conditional settings of 10 cM control intervals, five control markers (determined by QTL Cartographer to account for the genetic background variation), and forward regression (Zeng 1994). Experiment-wise critical thresholds for significance of potential QTL for each year were determined using CIM to conduct permutation tests as previously described (Churchill and Doerge 1994). Highly significant

($P = 0.01$) QTL thresholds were calculated from 1,000 permutations. Chromosome linkage and QTL traces were graphically represented using MapChart v2.1 (Voorrips 2002).

Psy-A1 allele sequencing and functional prediction

Long template PCR was performed on the cultivars Ajana and WAWHT2074 using the QIAGEN LongRange PCR kit with *Psy-A1* specific primers PSY7A-LTF1 and PSY7A-LTR1 (Table 1) to amplify approximately 3,000 bp of gene sequence. Approximately 25 µl of PCR product was separated on a 1.3% low melt agarose gel (Fisher Biotec, Australia) and DNA bands excised using GeneCatcher tips. Gene fragments were cloned directly from the gel slice following the technique described by Struhl (1987) using the pGEM®-T Easy Vector System II (Promega, WI, USA). Six DNA templates of cloned full length *Psy-A1* genes from each cultivar were purified from recombinant bacteria using Wizard® Plus SV Minipreps (Promega) and used for sequence analysis. The 5′ and 3′ ends of cloned fragments were sequenced using M13F/M13R primers. The remaining insert from Ajana was sequenced using the primers YP7AF, Y3F, PSY1-XR1 and PSY7A-3R; and from WAWHT2074 using primers PSY7A-seq1 and PSY7A-seq2 (Table 1). The respective PCR primer pairs PSY7A-8F/PSY7A-8R and PSY7A-9F/PSY7A-9R (Table 1) were designed to amplify and sequence the flanking 5′ and 3′ ends of the cloned DNA fragment from WAWHT2074.

Psy-A1 allele sequences from Ajana and WAWHT2074 were assembled and the translated amino acid sequences aligned using the PAM250 matrix in Geneious. Prediction of functional amino acid residues was performed using EMBOSS *sigcleave* (von Heijne 1986) to detect putative signal cleavage sequences, and ScanProsite was used for motif analysis via the ExPASy Proteomics Server (http://au.expasy.org/cgi-bin/prosite) (de Castro et al. 2006).

Results

Phylogenetic analysis of *Psy-A1* genes in Triticum species

Eight unique *T. aestivum Psy-A1* sequences and a number of similar gene sequences from related *Triticum* and *Aegilops* species were retrieved from Genbank. Phylogenetic analyses of *Triticum Psy-A1* sequences were investigated to identify genome origins of *Psy-A1* genes from progenitor species of hexaploid wheat. The resulting PHYML tree revealed the presence of several well-supported *Psy1* gene clusters (Fig. 1). Sub-tree I contains two

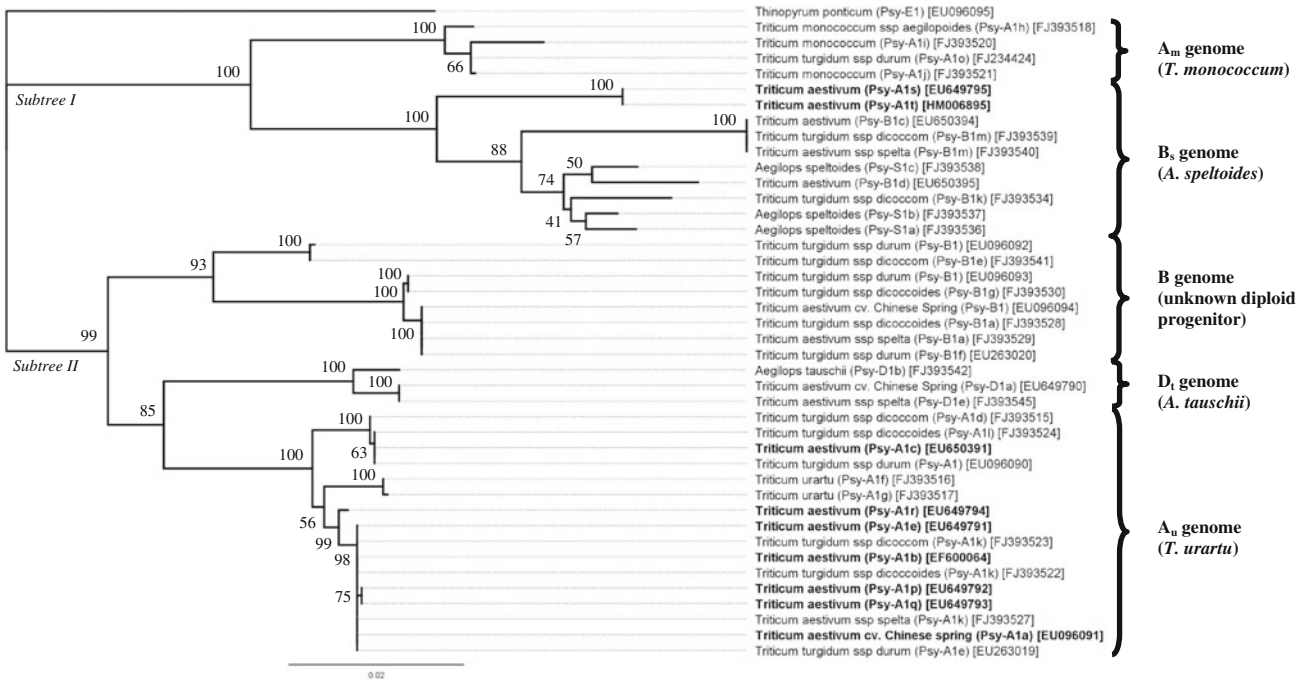

Fig. 1 Maximum likelihood phylogenetic tree of *Psy1* sequences from *T. aestivum* and related wheat progenitor species, outgrouped by *Thinopyrum ponticum* sequence [EU096095]. Genes are labelled by species name, with allele and GenBank accession number in *parentheses* and *brackets*, respectively, and *T. aestivum Psy-A1* alleles in *bold*. Bootstrap values are shown for major gene clusters, with number of substitutions/site indicated by *scale bar* below. The two major gene clusters are designated Subtree I and II, respectively

sequence clusters related to the wheat diploid species *T. monococcum* (A_m genome) and *A. speltoides* (B_s genome), whereas Subtree II is comprised of two sequence clusters related to the diploid species *T. urartu* (A_u genome) and *A. tauschii* (D genome) and a third cluster with a B genome but unknown diploid progenitor species. Nearly all *Psy-A1* alleles from *T. aestivum* grouped with sequences in the A_u cluster indicating their origin from *T. urartu*, however, the *Psy-A1s* and *Psy-A1t* alleles clustered with the B_s group of sequences. These results are supported by NJ analyses which show similar levels of support for the respective gene clusters (data not shown) with only minor sequence rearrangements within the B_s group. The clustering of both *Psy-A1s* and *Psy-A1t* with *A. speltoides* raises the question as to the relationship between the progenitor origin of this allele and its genetic location on the A genome.

Existing and novel *Psy-A1* alleles in selected Australian wheat genotypes

Psy-A1 allele type was characterised for a selection of 30 Australian hexaploid wheat cultivars or breeding lines, with average *b** values ranging from 7.27 for EGA Blanco (white in colour) to 13.67 for Schomburgk (yellow in colour) (Fig. 2). A total of 23 wheat genotypes had sequence identity to one of four known alleles with *Psy-A1e* having the lowest average *b** (8.12) followed by *Psy-A1p* (9.02), *Psy-A1a* (9.60), and *Psy-A1c* (10.5). The allele assignment for each wheat cultivar or breeding line and their corresponding *b** values are shown in Fig. 2.

Interestingly, partial *Psy-A1* gene sequences obtained for the cultivars and breeding lines Wyalkatchem, Cascades, WAWHT2074, Camm, Cadoux, BT-Schomburgk and Schomburgk were found to be highly similar to the previously described *Psy-A1s* allele. However, a single SNP within exon 1 for each of these wheat genotypes (corresponding to *Psy-A1s* sequence position 409 and resulting in a non-synonymous amino acid change at residue 62 from lysine to glutamate) has led to the identification of a novel allele, *Psy-A1t*. Australian wheat genotypes possessing *Psy-A1t* have relatively high average flour *b** values ranging from 9.83 to 13.67 (Fig. 2). Contrast analyses showed significant differences ($P < 0.05$) in *b** value between various *Psy-A1* alleles (Table 3), particularly those representing genotypes with extreme values for flour *b** (i.e. *Psy-A1e* and *Psy-A1t*).

Phenotypic and QTL analysis of flour colour in Australian hexaploid wheat genotypes

The putative function of three *Psy-A1* alleles in controlling *b** variation was evaluated by QTL analysis using bi-parental doubled haploid (DH) mapping populations. DH populations were selected for QTL analysis according to different *Psy-A1* alleles and *b** values of parental genotypes. WAWHT2046 (*Psy-A1a* allele), WAWHT2074 (*Psy-A1t* allele), Ajana and Carnamah (*Psy-A1p* allele) represented the most desirable colour ranges for Australian wheat genotypes with *b** values ranging from 8.01 (white) to 10.63 (yellow) as detailed in Fig. 2. Phenotypic variation for *b** value showed modal distribution (data not shown) for each DH population in three environments. A summary of the phenotyping data for each population at each site, including minimum, maxim and parental mean values is

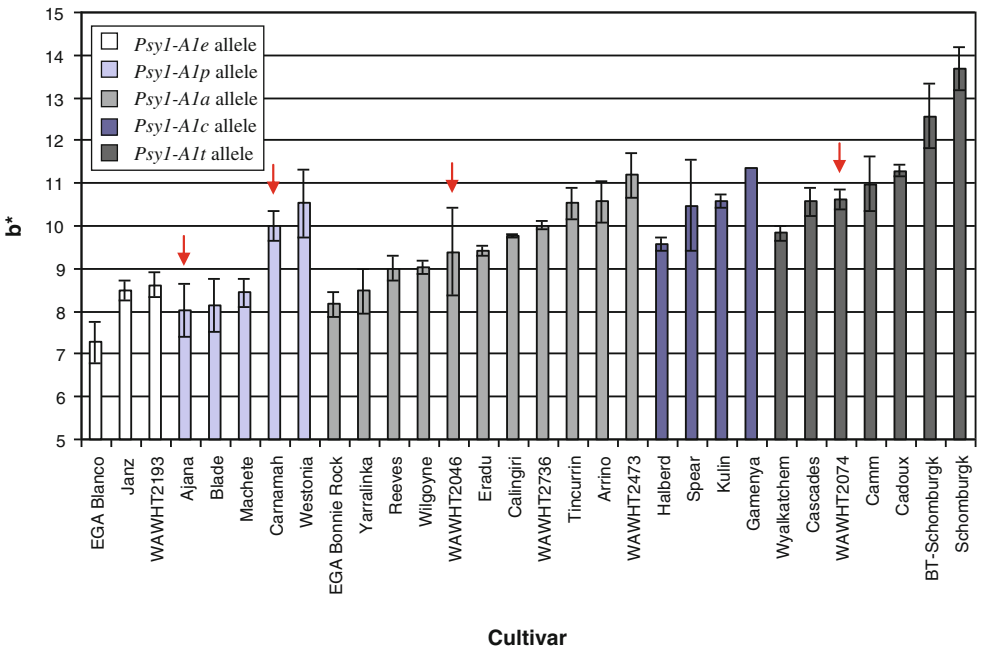

Fig. 2 Selected Australian wheat genotypes classified according to *Psy-A1* allele type and corresponding *b** values (±1 SD). *Arrows* indicate parental genotypes of doubled haploid populations used in subsequent mapping analyses

Table 3 Contrast analysis (ANOVA) probabilities for Australian wheat genotype *b** values grouped according to *Psy-A1* allele type, with significant values ($P < 0.05$) in bold type

	Psy-A1p	*Psy-A1a*	*Psy-A1c*	*Psy-A1t*
Psy-A1e	0.248	**0.039**	**0.006**	**<0.001**
Psy-A1p		0.313	**0.045**	**<0.001**
Psy-A1a			0.152	**0.002**
Psy-A1c				0.196

summarised in Table 4. Broad-sense heritability for each population at each site ranged from 0.798 to 0.958 (Table 4) indicating that 79.8–95.8% of the variation observed is due to between-line genotypic variation and suitable for subsequent QTL analysis. Pearson's correlation co-efficient of *b** values for each population between sites was high, ranging from 0.79 to 0.86 ($P < 0.001$). Analysis of *b** values identified a QTL on the distal end of chromosome 7A for each population grown at each site. The QTL on 7A identified in the Ajana/WAWHT2074 population accounted for 32–36% of the total variation whereas the phenotypic variation ranged from 15 to 24% in remaining populations at this locus (Table 5). Moreover, a QTL on chromosome 3A was consistently detected in the Ajana/WAWHT2074 and Ajana/WAWHT2046 populations for each environment. Additional QTL for individual populations and specific environments were also detected and are described in Table 5.

The position of *Psy-A1* alleles in QTL analyses provides evidence for those that may be functional in controlling flour *b** colour. *Psy-A1a, Psy-A1p* and *Psy-A1t* mapped to chromosome 7A in their respective populations (Fig. 3). *Psy-A1p* and *Psy-A1t* co-located with the maximum LOD peak of the QTL indicating that one or both alleles control 32–36% of the phenotypic variation for the Ajana/WAWHT2074 population (Table 5; Fig. 3). However, *Psy-A1a* and *Psy-A1p* did not have a significant association ($P = 0.01$) but rather flanked the QTL on chromosome 7A in the Carnamah/WAWHT2046 and Ajana/WAWHT2046 populations, indicating *Psy-A1* alleles are not involved in flour colour variation in these populations and other genes at this locus may control flour colour in some Australian wheat genotypes. Therefore, it is reasonable to assume the *Psy-A1t* allele contributes to controlling yellowness in flour colour in the Ajana/WAWHT2074 DH population (Fig. 3).

Sequence comparison of *Psy-A1p* and *Psy-A1t* alleles

Full sequence analysis of alleles from Ajana and WAWHT2074 parental genotypes allowed the prediction of functional protein domains that may be contributing to flour yellowness. The cloned partial gene sequence of 3,170 bp from Ajana was found to be identical to *Psy-A1p*, whereas the complete WAWHT2074 sequence of 3316 bp was a new allele *Psy-A1t* (Genbank accession number HM006895). *Psy-A1p* and *Psy-A1t* were found to be identical for 89% of nucleotide residues with 343 SNPs, and also contained 976 sequence gaps including a 680 bp indel within intron 4. The coding regions specifically contained 87 SNPs and 24 gaps, resulting in 16 translated amino acid changes and 8 gaps, respectively (Fig. 4). The majority of these changes were located in exon 1. Analysis of predicted amino acid residues for *Psy-A1p* and *Psy-A1t* using EMBOSS *sigcleave* and ScanProsite identified *Psy1* signature motifs in addition to putative signal cleavage regions and sites involved in phosphorylation, myristolation and glycosylation. There were no amino acid differences identified in the *Psy1* signature motifs at positions 259–274 and 295–320 (Fig. 4). However, *Psy-A1t* was found to possess a unique casein kinase II phosphorylation site (consensus pattern [ST]-x(2)-[DE], Pinna 1990) at alignment position 17–24, and protein kinase C phosphorylation site (consensus pattern [ST]-x-[RK], Kishimoto et al. 1985; Woodget et al. 1986) at position 132–134 (Fig. 4). In this regard, *Psy-A1t* and *Psy-A1s* are the only wheat *Psy1* sequences possessing these additional phosphorylation sites. A number of other amino acid differences were detected between the

Table 4 Summary of adjusted mean values for parental genotypes, minimum, maximum, mean values and broad sense heritability of DH populations for flour *b** colour at different sites

Populations	Site and year	Parental *b** mean	DH *b** range	DH *b** mean	H^2
Ajana/WAWHT2074	Wongan Hills 2002	Ajana = 8.60, WAWHT2074 = 11.78	7.89–13.23	10.77	0.958
	Wongan Hills 2003	Ajana = 8.42, WAWHT2074 = 11.16	7.51–11.31	10.8	0.907
	Merriden 2003	Ajana = 8.00, WAWHT2074 = 11.39	8.70–14.29	11.36	0.885
Carnamah/WAWHT2046	Wongan Hills 2002	Carnamah = 11.46, WAWHT2046 = 10.92	8.45–13.79	10.85	0.92
	Wongan Hills 2003	Carnamah = 11.81, WAWHT2046 = 10.60	8.13–13.63	10.99	0.911
	Merriden 2003	Carnamah = 11.70, WAWHT2046 = 11.15	8.79–14.34	11.36	0.916
Ajana/WAWHT2046	Wongan Hills 2005	Ajana = 8.07, WAWHT2046 = 8.66	6.79–18.08	11.50	0.798
	Bullaring 2005	Ajana = 9.58, WAWHT2046 = 9.95	7.28–16.96	10.62	0.818

Table 5 Summary of QTL analysis using composite interval mapping for flour *b** colour in three DH mapping populations

Population	Location	Chromosome	Marker interval	Distance (cM)	Composite interval mapping		Additive[c]	R^2
					LOD threshold[a]	Max. LOD[b]		
Ajana/WAWHT2074	Wongan Hills 2002	*QFc.daw-3A*	*wPt6376-wPt9215*	44.6	4.0	13.4 (*gwm666a*)	0.51	0.21
		QFc.daw-4D	*wmc89b-wmc457*	17.6	4.0	5.2 (*barc217*)	−0.26	0.04
		QFc.daw-5B	*wPt-8637-gwm213*	11.3	4.0	4.8 (*barc74*)	0.27	0.06
		QFc.daw-7A	*wPt4038-cfa2257a*	19.0	4.0	22.7 (*Psy-A1*)	0.61	0.32
	Wongan Hills 2003	*QFc.daw-2D*	*wmc25b-gwm484*	46.6	4.1	6.6 (*gwm484*)	−0.42	0.17
		QFc.daw-3A	*wPt6376-wmc388b*	34.1	4.1	6.4 (*gwm666a*)	0.33	0.10
		QFc.daw-4D	*wPt-0877-wmc48b*	65.7	4.1	5.3 (*Rht2*)	−0.32	0.10
		QFc.daw-7A	*wPt4038-cfa2257a*	19.0	4.1	19.26 (*Psy-A1*)	0.58	0.32
	Merredin 2003	*QFc.daw-3A*	*wPt6376-wmc388b*	34.1	4.1	9.6 (*gwm666a*)	0.50	0.16
		QFc.daw-4D	*wmc89b-wmc457*	17.6	4.1	5.0 (*wmc457*)	−0.29	0.06
		QFc.daw-7A	*wPt4038-cfa2257a*	19.0	4.1	24.1 (*Psy-A1*)	0.70	0.36
Carnamah/WAWHT2046	Wongan Hills 2002	*QFc.daw-7A*	*stm545tgag-wPt5533*	29.2	6.0	7.4 (*wPt-4220*)	0.51	0.20
		QFc.daw-7B	*stm521acat-barc0056*	7.8	6.0	6.9 (*gwm046*)	−0.45	0.15
	Wongan Hills 2003	*QFc.daw-7A*	*wPt-4553-wPt-4220*	31.0	4.2	7.5 (*stm545tgag*)	0.45	0.24
	Merriden 2003	*QFc.daw-7A*	*stm545tgag-wPt5533*	29.2	4.3	5.5 (*wPt-4220*)	0.43	0.15
		QFc.daw-7B	*wPt-4902-wPt0504*	11.5	4.3	4.7 (*wPt0504*)	−0.37	0.11
Ajana/WAWHT2046	Wongan Hills 2005	*QFc.daw-3A*	*gwm0674-gwm0499*	7.1	3.7	4.5 (*gwm0674*)	0.24	0.12
		QFc.daw-7A	*wPt-6495-cfa2257*	70.6	3.7	5.3 (*cfa2240*)	0.32	0.21
	Bullaring 2005	*QFc.daw-3A*	*gwm0674-gwm0499*	7.1	4.0	5.0 (*gwm0674*)	0.32	0.14
		QFc.daw-7A	*wPt-6495-cfa2257*	70.6	4.0	5.4 (*cfa2240*)	0.38	0.21

[a] LOD threshold values calculated using 1,000 permutations and significant at $P = 0.01$

[b] Maximun LOD score for QTL with corresponding marker in parentheses

[c] Positive and negative values indicate that paternal allele increases and decreases for each trait, respectively

translated allele sequences, however, their functional significance is unclear (Fig. 4).

Discussion

This study identified phylogenetic relationships among *Psy-A1* alleles and between other *Psy1* genes, providing insights into the mechanisms of gene and genome inheritance and evolution in modern day Australian hexaploid wheat cultivars. Moreover, this study provided information on the possible functional role of *Psy-A1* alleles in controlling flour *b** colour and how this information can be used for wheat improvement.

Phylogenetic analyses provided further insights into the evolution of modern polyploid wheats. Observations of two major gene clusters for both the A and B genomes concurs with previous phylogenetic investigations for *Psy1* sequence relationships by Wang et al. (2009), who identified multiple lineages within the A, B and D genomes of wheat. These results are supported by increasing evidence that hexaploidisation in wheat has occurred at least twice, and has involved two distinct tetraploid wheat lineages (Caldwell et al. 2004; Giles and Brown 2006; Gu et al. 2004; Haudry et al. 2007; Ozkan et al. 2005). The process of recurrent polyploidization combined with allele sorting could explain conflicting results observed for other genetic studies. For example, analyses of both repetitive sequences (Dvorak et al. 1993) and *Acc-1* and *Pgk-1* plastid genes (Huang et al. 2002) suggested *T. urartu* was the A genome donor of polyploid wheats. In contrast, examination of ITS sequences indicated *T. monococcum* was the A genome donor of tetraploid wheats (Zhang et al. 2002). In this regard, understanding the evolutionary history of wheat is important for making inferences regarding gene sequence relationships and their functional link to traits of interest.

The maximum likelihood phylogeny described here has strong bootstrap support for all major nodes, confirmed by neighbour joining methods. There are two main points of difference however, between this study and that of Wang et al. (2009). First, the durum wheat *Psy-A1o* allele (not examined by Wang et al. 2009) was found to cluster with sequences from the A genome donor, *T. monococcum* (A_m). Therefore, it can be concluded that *T. urartu* is not the sole

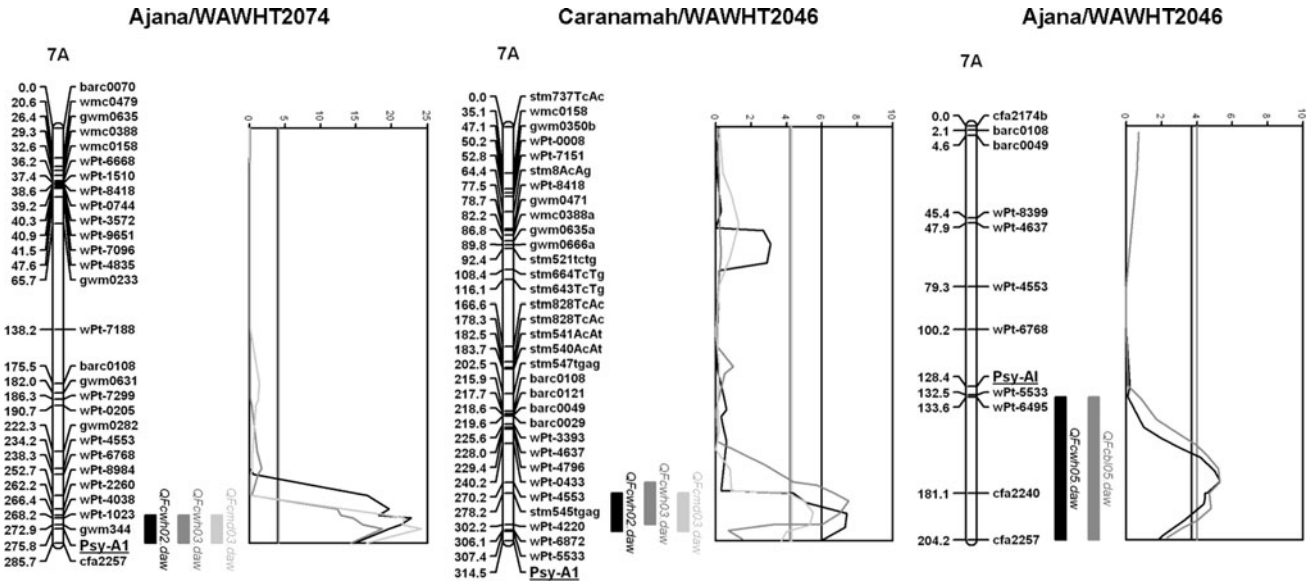

Fig. 3 Molecular marker map of chromosome 7A and alignment of QTL traces for flour *b** colour (*QFc.daw*) in three DH populations grown in several environments. The position of markers and genetic distance (in) between markers is shown to the *right* and *left* of the chromosome map, respectively. The *shaded bars* represent the position of the QTL interval for flour *b** colour for each environment. QTL traces representing each environment is shown with *shaded lines*. The *y* axis in QTL traces show LOD scores and *vertical lines* show threshold LOD scores (calculated by permutation analysis using 1,000 reiterations) for detection of significant ($P = 0.01$) QTL for each environment

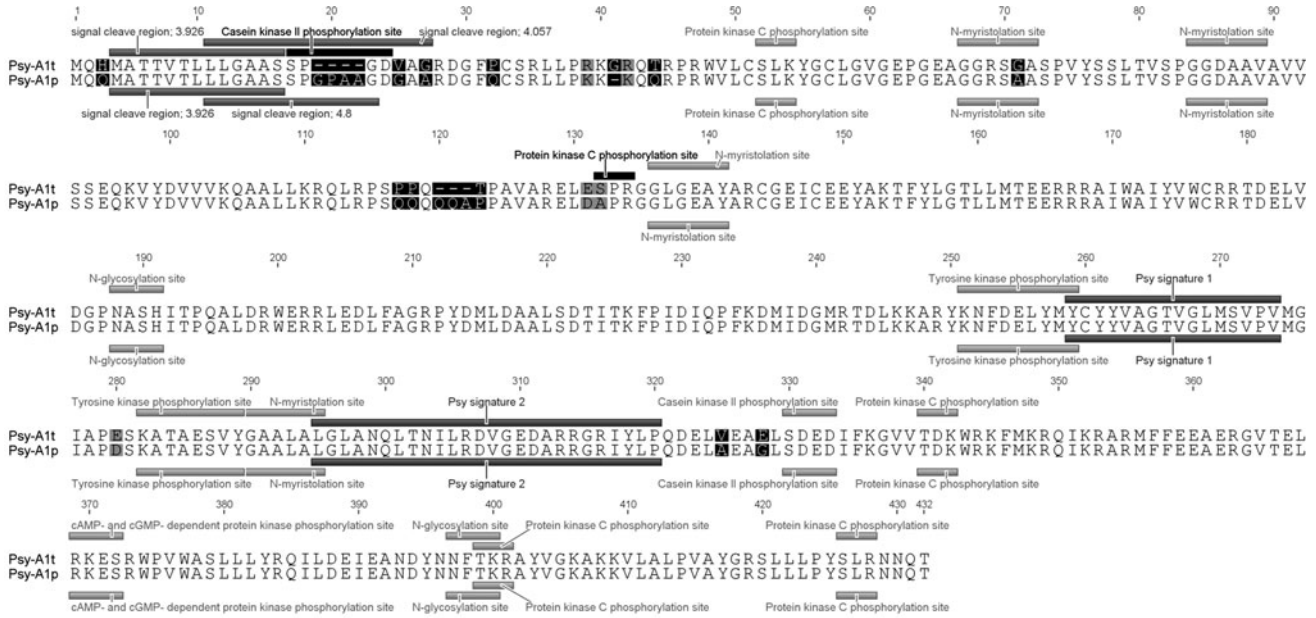

Fig. 4 Amino acid sequence comparison for alleles *Psy-A1t* (GenBank accession no. HM006895) and *Psy-A1p* (Genbank accession no. EU649792). Identical residues are *unshaded*, with similar and dissimilar residues highlighted *grey* and *black*, respectively. Predicted ScanProsite and EMBOSS motifs are indicated *above* and *below* the respective sequences, with homologous regions in *light grey*, signal cleavage regions/scores and Psy (squalene and phytoene synthases) signature sequences in *dark grey*, and unique motifs in *black*

A genome progenitor of polyploid wheats. This is supported by a previous phylogenetic study of genes for polyphenol oxidase (*Ppo1*) which produced two distinct A genome sequence clusters for polyploid wheat, also linked to *T. urartu* and *T. monococcum* (He et al. 2009b). Second, while all other *Psy-A1* alleles reported for *T. aestivum* are probably derived from *T. urartu*, the alleles *Psy-A1s* (also not included in the study by Wang et al. 2009) and *Psy-A1t*

formed a more distant cluster with B genome donor *A. speltoides* (B_s). In this regard, mapping of *Psy-A1t* in this study confirms the genetic location of this allele on chromosome 7A rather than 7B. As such, the phylogenetic position of *Psy-A1s* in relation to B_s sequences may be a consequence of ancestral allele sorting. Alternatively, *Psy-A1s* could be derived from the B_s genome through an ancestral inter-genomic translocation event. It is possible that sporadic genomic and gene rearrangements such as this may occur frequently in allopolyploids, and could be key drivers of genetic variability (Feldman and Levy 2009; Liu et al. 2009). This phenomenon is not uncommon since translocation events in wheat from the B genome to the A genome during polyploidisation has been reported previously (Belyayev et al. 2000) which may explain the close evolutionary relationship of the *Psy-A1s* allele to the B genome progenitor species *A. speltoides*. It is interesting to note that *Psy-A1t* is common in wheat genotypes with higher flour *b** values, which indicates this allele provides significant genetic variability for flour yellowness. *Psy-A1t* probably originated from an earlier parental ancestor and was retained during the subsequent domestication of Australian wheat breeding lines.

Detailed characterisation of the *Psy-A1* alleles present in various Australian wheat genotypes has provided further information on the association of different alleles with flour colour types. These alleles defined a range of flour *b** colour values, including varieties producing extremes in phenotype from white flour (represented by *Psy-A1e*) to yellow flour (represented by *Psy-A1t*). *Psy-A1* allele typing of wheat cultivars enables selection individuals from populations with a predicted window of *b** values during marker-assisted breeding, irrespective of functional significance. However, the broad phenotypic variation observed for wheat genotypes with the same allele indicates that possible promoter region sequence variation for *Psy-A1* alleles, or the influence of genetic factors other than *Psy-A1*, needs further consideration for more accurate prediction of flour *b** colour when applying marker-assisted breeding.

This study used genetic mapping and QTL analysis to putatively assign *Psy-A1* allele function in controlling flour yellowness in Australian hexaploid wheat genotypes. It was expected that the selected *Psy-A1a*, *Psy-A1p* and *Psy-A1t* alleles would co-locate with the QTL for flour *b** variation. Surprisingly, only the allele combination of *Psy-A1t* and *Psy-A1p* mapped within the QTL on chromosome 7A. As *Psy-A1p* and *Psy-A1a* mapped outside the QTL (in two other mapping populations) this indicates that variation only at *Psy-A1t* has a significant effect on flour colour. These findings confirm a strong link between genetic diversity from the B_s progenitor genome and yellow flour colour in modern day wheat. It is interesting to note that previous studies have confirmed the involvement of specific *Psy-A1* alleles in relation to white flour colour types, with the mapping of *Psy-A1a* with *Psy-A1b* (He et al. 2008) and *Psy-A1*e with *Psy-A1p* (Howitt et al. 2009). For these studies, *Psy-A1b* and *Psy-A1*e are assumed to be the alleles dominating trait variation based on differences in gene transcription (see Howitt et al. 2009). However, involvement (or lack thereof) of the alleles *Psy-A1a* and *Psy-A1p* in flour colour variation has not been confirmed until now. The current study also demonstrates that although *Psy-A1* has been associated with flour *b** QTL in this and a number of other studies (He et al. 2008; Howitt et al. 2009; Singh et al. 2009; Zhang et al. 2009), it cannot be assumed that allelic variation for this gene is a common mechanism for variation in endosperm carotenoid content in wheat, as proposed by Howitt et al. (2009).

The locations of *Psy-A1* loci and genetic distance to common markers (such as *cfa2257*) reflects differences in recombination rates across populations but useful to assess the relative position of QTL. The QTL proximal to *Psy-A1* in the Carnamah/WAWHT2046 population appears to be a similar QTL reported on chromosome 7A in Singh et al. (2009). However, the scarcity of similar markers mapped in populations across studies precluded finer comparison of these regions. An alternative QTL in this study was located distal to the *Psy-A1* locus in the Ajana/WAWHT2046 population on chromosome 7A and not previously reported in other studies. The use of a common parent in two populations indicates that WAWHT2046 has two possible chromosomal regions, both distal and proximal to *Psy-A1*, controlling increases in flour *b** colour. Similar studies in durum wheat by Zhang and Dubcovsky (2008) and Singh et al. (2009) support the hypothesis that QTL and genes on group 7 chromosomes other than those associated with *Psy-A1* alleles contribute to flour *b** variation and yellowness. Since only one QTL on 7A was detected in the Carnamah/WAWHT2046 and Ajana/WAWHT2046 population in all environments, it appears that other genetic factors influence the causal effects of only one QTL on phenotype. Further studies are warranted to elucidate other factors influencing QTL expression.

Analysis of *Psy-A1* alleles indicated that alternative splicing (Howitt et al. 2009) affected carotenoid accumulation in some Australian genotypes. This study investigated *Psy1* gene expression in developing grain (early, mid and late stages of grain maturity) from parents of mapping populations but did not identify any significant differences in transcript size or abundance (data not shown). The co-location of *Psy-A1t* with QTL for flour *b** variation warranted sequencing of exon and intron regions of this allele. The translated protein sequence was compared with *Psy-A1p* to ascertain potential differences in protein function. Interestingly, no difference in the predicted amino acid

sequence of the phytoene synthase signature motif was detected, indicating that the catalytic domain responsible for the conversion of farnesyl pyrophosphate to phytoene (Römer et al. 1993) is conserved between alleles. However, differences in amino acid residues were observed in regions of the translated protein that may be important for enzyme function. Major differences were observed in predicted amino acid regions for post-translational modification involving phosphorylation. Protein kinases can have a strong impact on protein activity (Stone and Walker 1995) with multi-functional roles in activating proteins in various metabolic and physiological pathways associated with disease resistance (Ivanov et al. 2003), DNA repair (Krohn et al. 2003), osmotic stress (Riera et al. 2004), circadian rhythms (Daniel et al. 2004), auxin signalling (Moreno-Romero and MartÃnez 2008), transcription (Klimczak et al. 1995) and translation (Dennis et al. 2009). However, the precise role of post-translational modification on the function of enzymes involved in the xanthophyll cycle (including phytoene synthase) in plants or other organisms is unknown. Nevertheless, recent evidence has shown phosphorylation is important for maintaining the xanthophyll cycle (Takahashi et al. 2006), and changes in sites for post translational modification could alter enzyme function. Variation in genes encoding proteins that modify enzymes or products of the xanthophyll pathway may also represent alternative targets for flour *b** QTL on chromosome 7A and other regions of the wheat genome. Further studies are warranted to authenticate the biochemical significance of sequence differences between *Psy-A1t* and *Psy-A1p* at predicted sites of post-translational modification that may affect enzyme function and subsequent flour *b** variation.

Psy-A1 alleles differ in their functional relationships controlling flour *b** colour variation but, nevertheless, are useful markers in commercial variety development. It is unlikely that selection for particular *Psy-A1* alleles would provide accurate prediction of *b** variation. Instead, genotyping for *Psy-A1* alleles would enrich individuals from breeding populations with broader *b** values. For example, *Psy-A1e*, *Psy-A1c* and *Psy-A1t* alleles would identify lines with broader *b** ranges of 7–9, 9–11 and 10–14, respectively. Selection of individuals with accurate *b** values would require the identification of other candidate genes and specific allelic combinations for QTL on 7A and other chromosomes.

Acknowledgments The authors would like to thank Ms K. Shaw for technical assistance, Ms F. Brigg for helpful comments regarding sequencing of GC rich templates, and Mr D. Diepeveen for statistical assistance. This work was supported by the Grains Research Development Corporation through project CWQ0009 and CWQ0013 and Value Added Wheat Cooperative Research Centre through project 4.3.9.

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