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Development of a robust marker for *Psy-1* homoeologs and its application in improvement of yellow pigment content in durum wheat

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Abstract Phytoene synthase-1 (*Psy-1*) homoeologs are associated with yellow pigment content (YPC) in endosperm of durum and bread wheat. In the present study, microsatellite variation in promoter region of *Psy-A1* was identified in durum wheat and marker Psy-1SSR, targeting the microsatellite variation was developed which amplifies variation in *Psy-A1* and *Psy-B1* loci simultaneously. Psy-A1SSR was mapped within *QYp.macs-7A*, a major QTL for YPC identified earlier in PDW 233/Bhalegaon 4 population. Marker Psy-A1SSR was further validated in two different RIL populations and a set of 222 tetraploid wheat accessions including less cultivated tetraploid wheat species. Eight alleles of Psy-A1SSR were identified in 222 wheat accessions, while seven alleles were observed for Psy-B1SSR. Variation at Psy-A1SSR showed significant association with YPC, whereas no association was observed with Psy-B1SSR. Marker-assisted introgression of Psy-A1SSR*e* allele from PDW 233, to durum wheat cultivars MACS 3125 and HI 8498 resulted in improvement of YPC. Backcrossed $BC_3F_{2:4}$ and $BC_2F_{2:3}$ lines selected using Psy-A1SSR showed 89 to 98% gain in YPC over recurrent parents indicating robustness of marker. The marker can thus be utilized in marker-assisted improvement of YPC in durum wheat cultivars.

Keywords Durum wheat · Yellow pigment content · Phytoene synthase · Marker-assisted breeding

Introduction

The yellow pigments (carotenoids) present in endosperm impart bright yellow color to semolina and pasta. Higher the yellow pigment content (YPC), better the appearance and acceptability of the pasta in the market. This makes the color of the pasta one of the important quality parameters for the pasta industry (Troccoli et al. 2000) and hence, it has been included in several durum wheat quality improvement programs (Clarke et al. 1998; Dick and Youngs 1988; Di Fonzo et al. 2005; Pfeiffer and Payne 2005). Considering their significance, chemical composition of yellow pigments has been studied extensively with the help of high-performance liquid chromatography (HPLC) by several researchers. Hentschel et al. (2002) found that all-*trans*-lutein and zeaxanthin accounted around 30 to 50% of the yellow pigment quantity and hence concluded that there are still some more compounds in durum wheat not yet identified that contribute considerably to the yellow color of the grain extracts. With an improved normal-phase HPLC procedure, lutein was detected as a major component along with α+β-carotene, β-cryptoxanthin, and zeaxanthin in trace quantity in durum wheat (Fratianni et al. 2005; Panfili et al. 2004).

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Since YPC shows complex inheritance, several efforts have been made to identify the genetic components controlling the trait. Major QTL controlling YPC were reported on chromosomes 7AL and 7BL in durum as well as bread wheat (Blanco et al. 2011; Colasuonno et al. 2014; Elouafi et al. 2001; Mares and Campbell 2001; Parker et al. 1998; Patil et al. 2008; Pozniak et al. 2007; Roncallo et al. 2012; Zhai et al. 2016a; Zhang et al. 2008). In further studies on these QTL, *Psy-A1* and *Psy-B1* encoding *Phytoene synthase* involved in carotenoid biosynthesis were identified to be linked with QTL for YPC on chromosome 7A and 7B, respectively (He et al. 2008; Pozniak et al. 2007). Moreover, allelic variation at *Psy-A1* and *Psy-B1* was also studied to test the effect of various alleles on the trait. In durum wheat, eight and seven alleles of *Psy-A1* and *Psy-B1*, respectively, have been identified (He et al. 2009; Singh et al. 2009). In studies on bread wheat, 11 alleles of *Psy-A1*, five of *Psy-B1*, and four of *Psy-D1* have been reported (Crawford et al. 2011; Ficco et al. 2014; He et al. 2008, 2009; Howitt et al. 2009; Wang et al. 2009). These reports provided considerable evidence that allelic variation at *Psy-A1*, *Psy-B1*, and *Psy-D1* is associated with variation in yellow pigment content in wheat. Genome-wide association studies also supported involvement of *Psy-A1* and *Psy-B1* in determination of YPC in bread wheat (Zhai et al. 2018). Studies on transcription of *Psy1* homoeologs and their functional characterization using reverse genetic tools provided in-depth insight into function and transcriptional regulation of *Psy1* in bread and durum wheat (Qin et al. 2016; Zhai et al. 2016b). In recent studies, two paralogs of *Psy1*, namely *Psy2* and *Psy3*, were also identified and mapped on short and long arm, respectively, of group 5 chromosome of wheat. *Psy2* was associated with yellow index in tetraploid wheat (Colasuonno et al. 2017), whereas, *Psy3* showed elevated expression in leaves and roots when plants were exposed to abiotic stresses (Dibari et al. 2012; Flowerika et al. 2016; Li et al. 2008).

Marker-assisted breeding (MAB) has become popular among wheat breeders across the world during the last 20 years, mainly because it improves precision for selection even at early generation stage and also enables breeders to pyramid desirable genes in a single genetic background. MAB has been convincingly utilized to introgress genes for disease resistance, plant height, and quality traits in elite wheat background (Kumar et al. 2011; Randhawa et al. 2013; Tyagi et al. 2014; Vasistha et al. 2016; Vishwakarma et al. 2016; William et al. 2007). Genes for grain protein content (*Gpc-B1*), gluten strength (*x7* of *Glu-B1*; *x1*, *xNull* and *x2** of *Glu-A1*; *x2*, *x5*, *y10* and *y12* of *Glu-D1*) and low cadmium uptake (*Cdu1*) have been targeted in MAB for wheat quality improvement (de Bustos et al. 2001; Randhawa et al. 2013; Tyagi et al. 2014; Vishwakarma et al. 2016; William et al. 2007). Although several gene/allele-specific markers have been reported with their potential use in improvement of YPC in durum wheat, reports on marker-assisted development of advanced wheat breeding lines with improved YPC are lacking (Zhai et al. 2016c). Since majority of the Indian durum wheat cultivars have low YPC with an average of 4.5 ppm (Santra et al. 2006), deployment of DNA-based markers in breeding program can lead to overall improvement of YPC in Indian durum cultivars.

In our earlier study, we identified a major QTL, *QYp.macs-7A* for YPC in PDW 233/Bhalegaon 4 population (Patil et al. 2008). *Psy-A1* in PDW 233 and Bhalegaon 4 was also characterized and investigated for its association with *QYp.macs-7A* and variation in YPC; however, its linkage with *QYp.macs-7A* could not be established due to lack of polymorphism in coding regions and introns of *Psy-A1* between PDW 233 and Bhalegaon 4. Moreover, the dominant SCAR markers reported in the study may not be useful in selection in early generations due to chances of false positives. The objectives of the present study were (a) to analyze flanking regions of *Psy-A1* and *Psy-B1* in PDW 233 and Bhalegaon 4 for polymorphism to develop *Psy-A1*- and *Psy-B1*-specific co-dominant markers for selection of high YPC, (b) to test association between identified markers and variation in YPC in diverse germplasm and RIL populations, and (c) to test the utility of identified marker in marker-assisted backcross breeding for improvement of YPC in Indian durum wheat cultivars.

Material and methods

Plant material

PDW 233/Bhalegaon 4 RIL population reported in our earlier study (Patil et al. 2008) was used for identification and mapping of marker for YPC. The population was evaluated for YPC across five environments (Table 1) and same phenotype data was used to estimate marker-trait association. The marker was further validated on two sets of $F_{2:6}$ RILs populations developed

Table 1 Yellow pigment content (ppm) in durum wheat accessions and RIL populations

RIL populations/Durum wheat accessions	No. of samples	Year	Location	Parents		RILs			Standard deviation
				P_1	P_2	Minimum	Maximum	Mean	
PDW 233[a]/Bhalegaon 4[b,*]	140	2001–2002	Pune	9.36	4.27	3.23	9.90	6.27	1.39
		2002–2003	Indore	8.98	3.10	2.44	9.44	5.12	1.83
		2002–2003	Pune	8.25	3.24	1.62	8.51	4.97	1.57
		2002–2003	Karnal	8.66	4.61	1.92	12.97	6.21	1.89
		2003–2004	Pune	7.56	2.84	2.12	11.15	4.61	1.88
MACS 3125[a]/UC 1114[b]	92	2012–2013	Pune	3.57	7.42	2.00	7.15	4.34	1.44
Bijaga Yellow[a] /Castelporziano[b]	237	2017–2018	Pune	4.27	5.91	1.48	7.30	3.21	0.90
Durum wheat accessions	222		Pune	–	–	2.95	9.91	5.74	1.21
CIMMYT breeding lines	189	2012–2013		–	–	4.00	9.91	5.77	1.13
Durum cultivars	12	2005–2006		–	–	3.26	8.80	5.69	1.85
Local durums	14	2005–2006		–	–	2.95	6.53	4.91	0.95
Less cultivated tetraploid species	7	2005–2006		–	–	5.20	8.82	7.04	1.57

*Phenotype data reported earlier in Patil et al. 2008

[a] P_1

[b] P_2

from durum wheat crosses MACS 3125/UC 1114 and Bijaga Yellow/Castelporziano. MACS 3125 and Bijaga Yellow are durum wheat cultivars released for cultivation in the states of Maharashtra and Karnataka, respectively, while Castelporziano (PI347331) is a mutant derived from *Triticum durum* cv. Cappelli. UC 1114 is durum wheat selection containing *dicoccoides Gpc-B1* functional allele, obtained from Prof. J. Dubcovsky, University of California, Davis, CA. In addition to these populations, a set of 222 tetraploid wheat genotypes comprising 189 durum breeding lines, 14 Indian local durums, and 12 durum cultivars as well as 7 accessions of less cultivated species (*T. carthlicum* and *T. polonicum*) was also used to test association between identified marker and yellow pigment content. The durum wheat breeding lines included entries from the 37th and 45th IDYN (International Durum Yield Nursery) as well as IDSN (International Durum Screening Nursery), from CIMMYT, Mexico. Detailed parentage and selection history of cultivars and breeding lines is given in Supplementary Table S1.

Field trials

PDW 233/Bhalegaon 4 RIL population was planted in randomized block design (RBD) with two replications across five different environments as given in Table 1. Details of field trials and year × location combinations are reported in Patil et al. (2008). Other two RIL populations MACS 3125/UC 1114 and Bijaga Yellow/Castelporziano were planted in augmented design during 2012–2013 and 2017–2018, respectively, at the Pune location. Out of 222 tetraploid wheat accessions, durum cultivars, local durums, and less cultivated tetraploid species were planted in RBD with two replications at Pune location during 2005–2006, while, breeding lines from CIMMYT were sown in RBD with two replications during 2012–2013. Similarly, selected $BC_3F_{2:3}$ and $BC_2F_{2:3}$ lines carrying Psy-A1SSR*e* allele developed through marker-assisted breeding were sown in replicated trial with RBD during 2013–2014, 2014–2015, and 2015–2016 along with recurrent parent, donor, and best checks. All the field trials were conducted under irrigated high fertility conditions.

Evaluation of yellow pigment content

Yellow pigment content in the grains was evaluated as described in Santra et al. (2003). Briefly, 125 mg of flour was suspended in 625 μL of n-butanol saturated with water. The samples were mixed thoroughly and allowed to stand for 16 h in dark for pigment extraction. The samples were centrifuged at 10000 g for 5 min and the absorbance of supernatant was measured at 440 nm. In

marker-assisted selection experiments, yellow index (YI) of whole meal was measured in terms of b^* values using CR 410 Chroma Meter, (Konica Minolta; Symons and Dexter 1991).

Development of marker

Earlier, we had sequenced 2336 bp region of *Psy-A1* in parental genotypes PDW 233 and Bhalegaon 4 (Patil et al. 2008). The primers for this sequencing analysis were designed using GenBank accessions (DQ642439, `DQ642440, DQ642443, DQ642444, EU096090) of Phytoene synthase gene sequences reported in durum wheat (Pozniak et al. 2007; Zhang and Dubcovsky 2008). Continuing the work further, flanking 5′ upstream (942 bp) and 3′ downstream regions (399 bp) in PDW 233 and Bhalegaon 4 were sequenced. A 1488 bp fragment spanning promoter region and exon 1 was amplified using primers Psy-7A5′F1 (5′ CTACTCCTACAGATGAGGAGC3′) and Psy-7ARg (5′GCTCAAGAAAAACAGAGTATCCAC3′). Similarly, 3′ downstream region was amplified using primers Psy-7AFd (5′AAAATGATGCTACG TGTAGTTCG3′) and Psy-7A3′R (5′CATCTTCT CGCATCCATTCCTC3′). Amplicons were sequenced on ABI PRISM® 3100-*Avant* Genetic Analyzer. Sanger sequencing reads were aligned using ClustalW multiple alignment application available in BioEdit version 7.2.5 (Hall 1999) to obtain consensus sequences of *Psy-A1* for PDW 233 and Bhalegaon 4. The consensus sequences were aligned to find out differences, if any, using multiple sequence alignment program Multalin (Corpet 1988) available at http://multalin.toulouse.inra.fr/multalin/. To target observed microsatellite variation in the promoter region of *Psy-A1*, a pair of primers (Psy-1SSRF: 5′GTCCATCCATCCCT TTCCAGG3′; Psy-1SSRR: 5′ATGCGAGG ACAAAGTCCAGTG3′) was designed. PCR reaction contained 250 nM of each primer, 0.2 mM of each deoxynucleotide, 10 mM Tris–HCl pH 9.5, 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 U *Taq* polymerase, and 40–60 ng of template DNA. The thermal cycling conditions were 94 °C for 4 min followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C followed by 72 °C for 5 min. PCR products were separated on 6% denaturing polyacrylamide gel and visualized by silver stain. Since the primer pair Psy-1SSRF/R also amplified microsatellite variation in promoter of *Psy-B1*, it was also investigated for sequence variation using primers Psy-1SSRF: 5′GTCCATCCATCCCTTTCCAGG3′ and Psy-7BR 5′GCTCCATGATCATCAGAACATG3′. Previously reported markers Psy1-A1_STS (Singh et al. 2009) and YP7A-2 (He et al. 2009) for *Psy-A1* were also tested on 66 accessions to correlate their allelic frequencies with Psy-1SSR marker. PCR amplification conditions for Psy1-A1_STS and YP7A-2 were as described in the original references.

Marker-assisted selection using Psy-A1SSR

To test utility of the Psy-A1SSR marker in improvement of YPC content using marker-assisted selection (MAS), Psy-A1SSR*e* allele from PDW233, a cultivar with high YPC, was transferred to two popular Indian durum cultivars with low YPC viz. MACS 3125 (released for Maharashtra state) and HI 8498 (released for Central Zone of India) using backcross breeding approach. BC_1F_1, BC_2F_1, BC_3F_1, and BC_3F_2 plants were screened for the presence of Psy-A1SSR*e* allele using Psy-A1SSR marker. Detail marker-assisted breeding protocol is given in Supplementary Fig. 1. Selected $BC_3F_{2:3}$ lines in MACS 3125 background and $BC_2F_{2:3}$ lines in HI 8498 background were evaluated for YI, YPC, grain yield, grain characteristics, flowering time, and spike traits in replicated trial with randomized block design conducted for 3 and 2 years, respectively.

Data analysis

Arithmetic means, standard deviation, and coefficient of variation were calculated using SPSS 11.0. Single marker regression analysis for YPC was carried out using linear regression model in SPSS package version 11.0. Rare alleles with frequency < 3% in wheat accessions were not considered for single marker regression analysis. In MAS trials, the one-way ANOVA was carried out using MS excel office 10 (Microsoft Office 10). The improved lines in the background MACS 3125 and HI 8498 were compared with recipient genotypes applying LSD test at $\alpha = 0.05$.

Results

Yellow pigment content in durum wheat

Total 222 durum wheat accessions including durum wheat cultivars, Indian local durums, breeding lines,

and less cultivated tetraploid relatives showed a wide range in YPC from 2.95 to 9.91 ppm. Average YPC was 5.69, 4.91, and 5.77 ppm in cultivars, local durums and breeding lines from CIMMYT, respectively (Table 1). Less cultivated tetraploid wheat showed comparatively higher average YPC (7.04 ppm) than the other classes of durum wheat studied. In MACS 3125/UC 1114 and Bijaga Yellow/Castelporziano, RIL populations YPC varied from 2 to 7.15 ppm and 1.48 to 7.30 ppm with the average of 4.34 ppm and 3.21 ppm, respectively. YPC in PDW 233/Bhalegaon 4 population ranged from 1.62 to 12.97 ppm with an average of 5.44 ppm as reported earlier (Patil et al. 2008). PDW 233, showing highest average YPC 8.36 ppm among durum cultivars, was further used as a donor in marker-assisted introgression of *Psy-A1* in MACS 3125 and HI 8498.

Marker development and mapping of *Psy-A1*

Primer pair Psy-7A5′F1/Psy-7ARg amplified a 1488 bp DNA segment spanning part of exon-1 and 942 bp region upstream to start codon in PDW 233 and Bhalegaon 4. Similarly, Psy-7AFd/Psy-7A3′R amplified 878 bp fragment covering part of exon-6 and 584 bp downstream to stop codon. These fragments were sequenced and complete sequence of *Psy-A1* including coding regions (sequenced earlier, Patil et al. 2008) and flanking regions was derived in PDW 233 (4424 bp; GenBank accession MG733357) and Bhalegaon 4 (4312 bp; GenBank accession MG733358). While no sequence variation in 3′ downstream region (399 bp) was observed between PDW 233 and Bhaleagon 4, promoter region (942 bp) of *Psy-A1* showed presence of a microsatellite at 560 bp upstream to start codon, with variation in number of TC repeats between both the parents. PDW 233 has di-nucleotide repeat $(TC)_{21}$ as compared to $(TC)_{25}$ in Bhalegaon 4 (Fig. 1a). The primer pair Psy-1SSRF/Psy-1SSRR targeting variation in this microsatellite region was used to genotype and map *Psy-A1* in the PDW 233/Bhalegaon 4 RIL population. This primer pair amplified 236 bp and 244 bp microsatellite fragments linked to *Psy-A1* in PDW 233 and Bhalegaon 4, respectively (Fig. 1b). This microsatellite variation present in promoter region of *Psy-A1* was named as Psy-A1SSR, which was mapped on long arm of chromosome 7A within the QTL for YPC, *QYp.macs-7A* (Supplementary Fig. 2). Same primer pair also amplified another 201 bp monomorphic band in PDW 233 and Bhalegaon 4 (Fig. 1b). Using nullisomic/tetrasomic lines in cv. Chinese Spring, 201 bp amplicon was assigned to chromosome 7B. In sequence analysis, the amplicon showed a complex microsatellite- $(TC)_5(AG)_9$ at 545 bp upstream to the start codon of *Psy-B1* in PDW 233 and Bhalegaon 4 (GenBank accessions MG733359 and MG733360).

Association of Psy-1SSR with yellow pigment content

Psy-A1SSR showed significant association with YPC in PDW 233/Bhalegaon 4 population across all the five environments (R^2 24.70% to 62.70%, $P < 0.001$). The marker was mapped within the major QTL for YPC *QYp.macs-7A* in PDW 233/Bhalegaon 4 population. Psy-B1SSR was monomorphic in the population. Since the primer pair Psy-1SSRF/Psy-1SSRR targeted microsatellite variation at *Psy-A1* and *Psy-B1* in single PCR reaction, effect of both the homoeologs on YPC was tested in two different RIL populations segregating for both the homoeologs of the microsatellite. In single marker analysis, Psy-A1SSR showed significant association with YPC in MACS 3125/UC 1114 (R^2 23.76%, $P < 0.001$) and Bijaga Yellow/Castelporziano (R^2 36.90%, $P < 0.001$) RIL populations (Table 2). However, in both the populations, variation in YPC explained by microsatellite linked to *Psy-B1* was not significant. When tested in a set of 222 durum wheat accessions, Psy-1SSR showed eight and seven alleles of microsatellite homoeologs linked to *Psy-A1* and *Psy-B1*, respectively. Psy-A1SSR*e* was the most predominant allele among 222 accessions (frequency 86.9%); however, its frequency was still higher (97.4%) in advanced breeding lines in IDSN and IDYN from CIMMYT. In contrast, its frequency was only 24.2% in cultivars and local durums. Psy-A1SSR*e* was present in very few cultivars such as PDW 233, WH 896, DWR 1006, and HI 8627 that are selected or derived for durum lines from CIMMYT. Psy-A1SSR*a*, *c*, and *d* were observed in durum cultivars and local durums with frequencies 9.1%, 18.2%, and 27.3% respectively. Psy-A1SSR*f*, *g*, and *h* were observed in less cultivated tetraploid wheat. Among Psy-B1SSR alleles, *d* allele showed the highest occurrence (frequency 67.7%) followed by *c* allele (frequency 23.1%) in 222 accessions. However, since the overall frequency of Psy-A1SSR*a*, *b*, *f*, *g*, and *h* alleles was < 3%, these were not considered for regression analysis. Results from regression analysis with Psy-A1SSR*c*, *d*, and *e* alleles showed significant association (R^2 8.75%, $P < 0.001$) between Psy-A1SSR and YPC

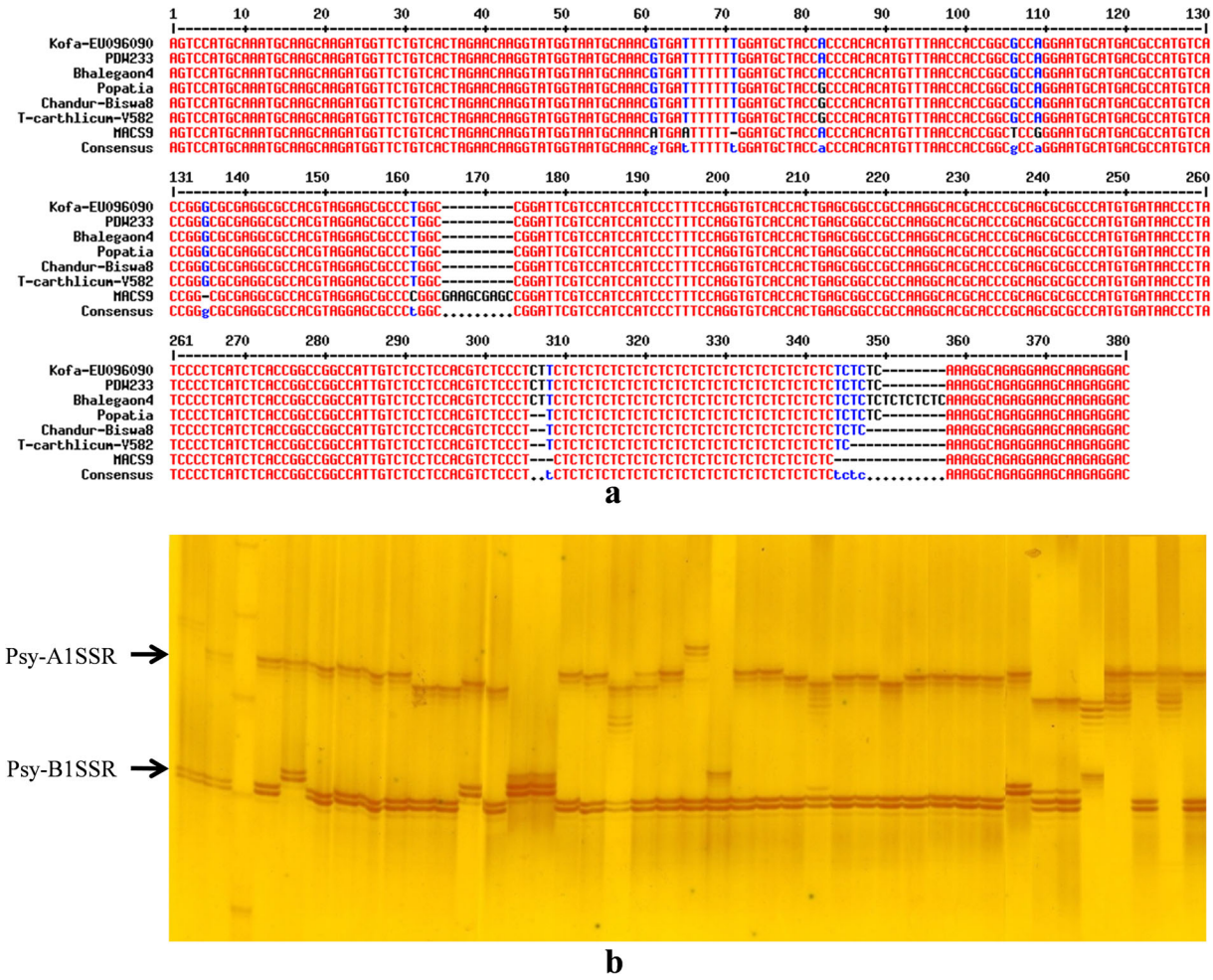

Fig. 1 Microsatellite variation in promoter region of *Psy-1* genes in durum wheat. **a** Variation in TC repeats at Psy-A1SSR region; **b** allelic variation at microsatellite Psy-1SSR observed in durum wheat accessions

in durum wheat accessions. Association of Psy-B1SSR with YPC was not significant (Table 2; Supplementary Fig. 3). The results were also confirmed by analysis of variance, where allelic variation at Psy-A1SSR showed significant association with YPC (Supplementary Table S2). Further, a post hoc multiple comparison test was applied to compare mean YPC values for alleles Psy-A1SSR*c*, *d*, and *e*. The significance of the difference between two alleles was tested by least significant difference test and also validated by applying Bonferroni adjustment method to eliminate significance by chance. The accessions with a predominant allele Psy-A1SSR*e*, showed significantly higher ($P < 0.01$) YPC (5.85 ppm) than the accessions with Psy-A1SSR*c* and *d* alleles (Table 3 and Supplementary Table S3). Mean YPC difference was not significant for allele *c* and *d*. Psy-A1SSR*a*, *b*, *f*, *g*, and *h* were observed as rare alleles with very low frequencies (< 3%) which do not permit a valid comparison for mean YPC of these alleles, hence could not be compared with other alleles for their association with the trait.

Allelic frequencies of Psy-A1SSR were compared with co-dominant markers for *Psy-A1* reported earlier. Majority of the accessions with Psy-A1SSR*a*, *d*, and *e* allele showed *Psy1-A1l* allele with Psy1-A1_STS (Singh et al. 2009) and *Psy1-A1c* allele with YP7A-2 marker (He et al. 2009). Most of the accessions carrying Psy-A1SSR*c* and Psy-A1SSR*f* alleles showed *Psy1-A1a* and *Psy1-A1o* alleles, respectively, with Psy1-A1_STS and YP7A-2 markers. The least frequent alleles Psy-A1SSR*g* and *h* did not show any association with reported

Table 2 Regression analysis of Psy-1SSR associated with yellow pigment content in durum wheat

RIL populations/Durum wheat accessions	Year	Location	Marker	R^2%	Significance
PDW 233/Bhaleagon 4*	2001–2002	Pune	Psy-A1SSR	31.90	$P < 0.001$
	2002–2003	Indore		62.70	$P < 0.001$
	2002–2003	Pune		58.10	$P < 0.001$
	2002–2003	Karnal		24.70	$P < 0.001$
	2003–2004	Pune		34.70	$P < 0.001$
MACS 3125/UC 1114	2012–2013	Pune	Psy-A1SSR	23.76	$P < 0.001$
			Psy-B1SSR	0.60	ns
Bijaga Yellow/Castelporziano	2017–2018	Pune	Psy-A1SSR	36.90	$P < 0.001$
			Psy-B1SSR	1.60	ns
Durum wheat accessions	2005–2006/2012–2013	Pune	Psy-A1SSR$^{\$}$	8.75	$P < 0.001$
			Psy-B1SSR$^{\$}$	1.44	ns
			Psy1-A1_STS$^{\#}$	0.01	ns
			YP7A-2$^{@}$	2.90	$P < 0.05$

*Phenotype data reported in Patil et al. 2008 was used for single marker regression analysis with Psy-1SSR

$^{\$}$ $N = 222$; $^{\#}$ $N = 116$; $^{@}$ $N = 163$

alleles of *Psy-A1* gene. Linkage disequilibrium (LD) between Psy-A1SSR, Psy1-A1_STS, YP7A-2, and nearby SSR was estimated. It was observed that LD estimated as r^2 was significant for pair-wise comparison among Psy-A1SSR, Psy1-A1_STS, YP7A-2, and gpw4050 (Supplementary Fig. 4).

Improvement of yellow pigment content by marker-assisted breeding

To test the utility of the marker for improvement in YPC content, Psy-A1SSR*e* allele from PDW 233 was introgressed in the background of two durum cultivars

Table 3 Yellow pigment content in tetraploid wheat carrying different alleles of Psy-1SSR

Locus	Alleles (bp)	Number of samples	Yellow pigment content (ppm)				
			Mean	Std. deviation	Std. error	Minimum	Maximum
Psy-A1SSR	*a*	3	3.36	0.36	0.21	2.95	3.61
	b	1	3.61	–	–	3.61	3.61
	c	7	4.35	0.78	0.29	3.26	5.52
	d	12	5.05	0.90	0.26	3.78	6.87
	e	192	5.85	1.05	0.08	4.00	9.91
	f	3	6.41	1.13	0.65	5.58	7.70
	g	2	8.05	1.09	0.77	7.28	8.82
	h	2	7.15	0.91	0.65	7.80	6.50
Psy-B1SSR	*a*	5	5.15	0.90	0.40	3.78	5.91
	b	2	6.93	4.21	2.97	3.96	9.91
	c	51	5.51	1.36	0.19	2.95	8.82
	d	152	5.79	0.99	0.08	3.61	9.13
	e	9	5.99	1.09	0.36	4.57	7.80
	f	2	6.26	1.10	0.78	5.48	7.04
	g	1	7.50	–	–	7.50	7.50
	Total	222	5.74	1.21	0.08	2.95	9.91

with low YPC, MACS 3125 (YPC 3.57 ppm; *b** 9.5) and HI 8498 (YPC 3.26 ppm; *b** 9.9) using backcross breeding approach and only foreground screening with Psy-A1SSR marker (Supplementary Fig. 1). MACS 3125 is a high yielding durum wheat cultivar while HI 8498 is a popular durum cultivar notified for Central Zone in India. All the BC_1F_1 plants carrying Psy-A1SSR*e* were compared for morphological traits with recipient parent. Since PDW233 flowers later than recipient parents, healthy plants with flowering time similar to recurrent parent were identified and back crossed with recurrent parent. Same procedure was followed up to BC_3F_1 (for MACS 3125 background) and BC_2F_1 (for HI 8498 background). In case of MACS 3125, out of 230 BC_3F_2 plants, 57 homozygous for Psy-A1SSR*e* allele were selected and 12 families were selected based on early flowering time. $BC_3F_{2:4}$ and $BC_3F_{2:5}$ families were grown in replicated trials in 2013–2014 and 2014–2015 along with recurrent parent and donor. Grains were harvested and YI (*b** value) of these lines were measured using whole meal. These were compared with MACS 3125 for *b** value using LSD at $\alpha = 0.05$. All these lines were also evaluated for agronomic traits such as grain yield, thousand grain weight, spike length, spikelets per spike, grains per spike, and grain weight per spike. Based on 2-year data for agronomical traits and elevated YI, five best lines were identified (Table 4). YI (*b** value) in these improved lines ranged from 11.5 to 11.8 with an average of 11.65 which was 22.6% higher than *b** value of recurrent parent MACS 3125, whereas, grain yield and other agronomic traits were at par with MACS 3125. YPC in marker-assisted introgressed lines was confirmed by microestimation method as described in Santra et al. (2003). YPC in these lines increased significantly (6.16–7.7 ppm) and showed an average

Table 4 Comparison of recurrent parent, donor, and MAB-derived lines for YPC and agronomic traits

Introgressed lines	Genotype			YPC (ppm)[$]	Yellow index (*b**)	Thousand Kernel Weight (g)	Grain yield (q/ha)	Spike length (cm)	Spikelets per spike	Grains per spike	Grain weight per spike (g)
	PsyA1SSR	Psy1-A1_STS	YP7A-2								
MACS 3125/PDW 233											
MACS 3125	*c*	*Psy1-A1l*	*Psy1-A1c*	3.57	9.5	43.5	46.3	7.4	17.3	42.6	1.9
PDW 233	*e*	*Psy1-A1l*	*Psy1-A1c*	8.56	11.5	44.3	44.0	7.0	18.1	52.6	2.1
MACS 3125-2	*e*	*Psy1-A1l*	*Psy1-A1c*	6.37	11.5	38.2	47.3	7.4	16.8	54.1	2.0
MACS 3125-4	*e*	*Psy1-A1l*	*Psy1-A1c*	7.70	11.8	37.5	42.6	7.1	16.9	52.9	2.2
MACS 3125-5	*e*	*Psy1-A1l*	*Psy1-A1c*	6.16	11.5	44.9	42.4	6.9	16.6	41.9	1.7
MACS 3125-8	*e*	*Psy1-A1l*	*Psy1-A1c*	6.74	11.7	44.7	41.6	7.2	16.6	43.4	2.0
MACS 3125-12	*e*	*Psy1-A1l*	*Psy1-A1c*	6.75	11.8	39.3	41.9	6.7	16.8	45.6	1.9
LSD (0.05)				1.92	1.8	10.5	13.7	1.4	3.2	8.6	0.6
HI 8498/PDW 233											
HI 8498	*c*	*Psy1-A1a*	*Psy1-A1a*	3.26	9.9	45.3	41.7	6.4	17.2	41.2	2.0
PDW 233	*e*	*Psy1-A1l*	*Psy1-A1c*	8.57	12.2	42.0	41.4	7.2	17.6	45.7	2.1
HI 8498-1	*e*	*Psy1-A1l*	*Psy1-A1c*	7.08	11.9	46.8	46.6	6.4	16.4	41.3	2.0
HI 8498-7	*e*	*Psy1-A1l*	*Psy1-A1c*	5.00	11.8	46.3	50.6	6.5	15.9	42.5	2.1
HI 8498-8	*e*	*Psy1-A1l*	*Psy1-A1c*	7.46	12.3	48.4	49.6	6.3	16.0	42.9	2.2
HI 8498-9	*e*	*Psy1-A1l*	*Psy1-A1c*	6.33	11.8	49.6	40.8	6.8	16.7	40.1	2.2
LSD (0.05)				1.69	1.4	5.4	8.2	0.6	1.9	6.6	0.6

[$] Determined from harvest of selected fixed, introgressed lines during season 2016–2017

88.9% increase over recurrent parent MACS 3125 (3.57 ppm).

In case of HI 8498, 30 BC_2F_2 plants homozygous for Psy-A1SSR*e* allele were selected out of 125 individuals and advanced into $BC_2F_{2:3}$ families. Out of these 30 families, nine healthy $BC_2F_{2:3}$ families with flowering time similar to recurrent parent were selected. These selected families were advanced up to $BC_2F_{2:6}$ stage and simultaneously tested for 3 years in a replicated trial in 2013–2014 ($BC_2F_{2:4}$), 2014–2015 ($BC_2F_{2:5}$), and 2015–2016 ($BC_2F_{2:6}$) along with recurrent parent and donor. Based on 3-year data for agronomical traits, elevated YI, best four lines were selected (Table 4). YI (*b** value) in these selected lines ranged from 11.8 to 12.3 with an average of 11.96 which was 20.27% higher than HI 8498. YPC in marker-assisted introgressed lines was confirmed by microestimation method as described in Santra et al. (2003). YPC in these lines increased significantly (range 5.00 to 7.46 ppm) and showed an average 98% increase over recurrent parent HI 8498 (3.26 ppm). Grain yield and agronomic traits in these lines were at par with HI 8498. Selected lines in both the backgrounds showed improved YPC and comparable agronomic traits and grain yield, thus demonstrating utility of the marker for durum wheat end-use quality improvement.

Discussion

Variation at Psy-A1SSR is associated with yellow pigment content in tetraploid wheat

Several studies have reported involvement of *Psy-A1* and *Psy-B1* loci in determination of YPC and development of PCR-based markers for their selection in bread and durum wheat (Crawford et al. 2011; Ficco et al. 2014; He et al. 2008, 2009; Howitt et al. 2009; Pozniak et al. 2007; Ravel et al. 2013; Singh et al. 2009; Wang et al. 2009). However, most of the reported markers are specific to certain alleles or haplotypes of A, B, and D homoeologs of *Psy-1*, that results into increase in number of analyses for screening of breeding lines. Therefore, development of a robust marker that can be tagged to multiple homoeologs and multiple alleles, at the same time, is warranted to accelerate selection for desired homoeologs/alleles of *Psy-1* in breeding program and also to reduce cost involved in marker analyses. In our earlier study, we had identified QTL *QYp.macs-7A* in RIL population developed from PDW 233/Bhalegaon 4 cross. Since the QTL falls in same region where *Psy-A1* was reported in previous study (Pozniak et al. 2007), DNA sequences of exon and intron regions of *Psy-A1* were analyzed in parents (Patil et al. 2008). Although PDW 233 and Bhalegaon 4 showed contrasting YPC, no sequence variation at *Psy-A1* was observed between them and hence association of QTL *QYp.macs-7A* and *Psy-A1* could not be established. Due to the sequence similarity between PDW 233 and Bhalegaon 4, *Psy-A1*-specific reported markers Psy1-A1_STS (Singh et al. 2009) and YP7A-2 (He et al. 2009) showed monomorphic *Psy1-A1l* and *Psy1-A1c* alleles, respectively, in both the parents; and could not be used to map *Psy-A1* in the population. Monomorphic alleles for markers Psy1-A1_STS and YP7A-2 were also observed in parental genotypes of other two populations namely Castelporziano, Bijaga Yellow, MACS 3125, and UC 1114, although they showed contrasting YPC. Thus, same allele of *Psy-A1* identified by Psy1-A1_STS and YP7A-2 seems to be contributing differently to YPC in PDW 233, Bhalegaon 4, Castelporziano, Bijaga Yellow, MACS 3125, and UC 1114. These observations also highlighted the need of robust marker that can be useful in diverse genetic backgrounds.

Here, we report microsatellite variation in the promoter region of both the homoeologs of *Psy-1*. Considering its tight linkage with *Psy-1*, this variation was used to develop functional marker which could be used for selection of various alleles of A as well as B homoeolog of *Psy-1*. The marker was mapped within QTL *QYp.macs-7A*, confirming its association with YPC in PDW 233/Bhalegaon 4 population (Supplementary Fig. 2). The marker was also validated for its association with variation in YPC in two more RIL populations (Bijaga Yellow/Castelporziano; MACS 3125/UC 1114) and 222 tetraploid wheat accessions using single marker regression analysis. Moreover, the PCR primer pair targeting the functional marker amplified both the homoeologs of microsatellite linked to *Psy-A1* and *Psy-B1* in single PCR reaction. Using this marker, eight alleles of Psy-A1SSR and seven alleles of Psy-B1SSR in tetraploid wheat accessions could be differentiated suggesting its usefulness in wide range of genetic backgrounds for selection of either or both the homoeologs of *Psy-1* simultaneously. Recent reports suggest that transcript levels of *Psy-1* homoeologs are associated with variation in YPC in wheat (Qin et al. 2016) and maize (Fu et al. 2013). Putative light response element (LRE),

gibberellin response element (P Box), MeJA response element (MeJA), and abscisic acid response element (ABRE) were identified as potential *cis*-regulatory elements in the promoter region of *Psy-A1* in durum wheat. The sequence variation in the promotor region was predicted to be associated with differential expression of *Psy-1* homoeologs and thereby variation in YPC in the endosperm. Sequence alignment showed that the Psy-A1SSR identified in present study does not fall in any of these elements identified by Qin et al. (2016), though it is flanked by two LRE. Microsatellite variation at UTR is known to be associated with change in transcription level of many genes in human (Bagshaw 2017) and Arabidopsis (Zhang et al. 2006). Recently, repeat length variation in 5' UTR of *myo*-inositol monophosphatase gene was found to be associated with seed size (Dwivedi et al. 2017) as well as phytic acid content and drought tolerance in chickpea (Joshi-Saha and Reddy 2015). Similarly, microsatellite-mediated enhanced expression of *Tryptophan decarboxylase* gene due to polymorphic $(GA/CT)_n$ repeat present in 5′UTR was reported in *Catharanthus roseus* (Kumar and Bhatia 2016). The microsatellite variation identified in the promoter of *Psy-1* homoeologs in present study has provided a unique site for functional marker development, based on the several earlier reports its role as potential key regulatory element in modulation of the gene expression could be speculated. However, further experimentation would be necessary to prove the role of microsatellite variation in modulating *Psy-1* expression.

In the present study, *Psy-A1* showed association with YPC in three RIL populations as well as in 222 tetraploid wheat accessions. The accessions of less cultivated species of tetraploid wheat, viz. *T. carthlicum* and *T. polonicum*, with Psy-A1SSR*f*, *g*, and *h* alleles showed high YPC and can be used as donors for improvement of YPC in durum wheat. Singh et al. (2014) has reported that gene for YPC derived from wild wheat *Lophopyrum ponticum* improved YPC by 24%, but lowered thousand grain weight, test weight, and grain hardness in durum wheat. Therefore, one should be cautious about undesirable linkage drag, if any, associated with these alleles present in the less cultivated tetraploid species while using them in breeding program. Although *Psy-A1* was associated with YPC, *Psy-B1* did not explain variation in YPC in any of the plant material used in present study including CIMMYT durum wheat lines. Similar results were reported by He et al. (2009) in CIMMYT spring wheat lines, where no significant difference in YPC was observed among the CIMMYT lines carrying four different alleles of *Psy-B1*. In a recent study, Campos et al. (2016) also reported that variation in *Psy-A1* alone exhibit significant difference in yellowness in Mediterranean landraces and modern cultivars. These results suggested that selection of desirable allele of *Psy-A1* alone is sufficient to improve YPC in durum wheat. However, the other homoeolog *Psy-B1* is also important when the donor is from different genetic background such as Kofa (Pozniak et al. 2007; Zhang and Dubcovsky 2008), Chinese winter wheat (He et al. 2009) and bread wheat Apache (Ravel et al. 2013), where *Psy-B1* has been associated with variation in YPC.

Psy-A1SSR marker tags a major QTL for YPC having *Psy-A1* as the candidate gene. Besides *Psy-A1*, another QTL for YPC was mapped at about 40 cM proximal to *Psy-A1* on chromosome 7A in durum wheat (Blanco et al. 2011; Elouafi et al. 2001; Zhang and Dubcovsky 2008). Similarly, a marker-trait association (MTA) affecting YPC was identified at 181 Mbp away from *Psy-A1* in a genome-wide association study (Zhai et al. 2018). Short arm of chromosome 7A also showed presence of QTL as well as MTA affecting YPC in bread and durum wheat (N'Diaye et al. 2017; Roncallo et al. 2012; Zhai et al. 2016a). All these reports suggest existence of three candidate genes affecting YPC on chromosome 7A. Selection for these QTL and MTA may lead to further additive improvement in YPC in durum wheat breeding lines.

Deployment of Psy-A1SSR in marker-assisted breeding for improved YPC

Marker-assisted breeding has contributed significantly in improvement of traits governed by single major gene (Randhawa et al. 2013; William et al. 2007) such as plant height, resistance to wheat rusts caused by *Puccinia* species, and resistance to insects, particularly, Russian wheat aphid, Hessian fly, and wheat midge. Similarly, some complex polygenic traits like grain protein content and gluten strength that determine end-use quality of wheat as well as resistance to Fusarium head blight and spot blotch have also been successfully improved in wheat by MAB for major gene/QTL contributing significantly to the target trait (de Bustos et al. 2001; Tyagi et al. 2014; Vasistha et al. 2016; Vishwakarma et al. 2016; William et al. 2007). In the present study, improvement in a polygenic trait YPC

was demonstrated by Psy-A1SSR-assisted introgression of *Psy-A1* in two cultivars MACS 3125 and HI 8498 using PDW 233 as a donor. Marker-assisted foreground selection for Psy-A1SSR*e* allele coupled with selection based on morphological and agronomic traits at the later stages resulted into development of elite durum lines with YPC significantly higher than recurrent parents MACS 3125 and HI 8498.

In many durum wheat breeding programs, selection for elevated yellow pigment content is practiced in advanced breeding lines in later generations by estimation of whole meal color and semolina color. However, the use of PCR markers will be useful for precise selection in early generations and can be used instead of trait evaluation, thus saving time and resources required for analyses. Both MACS 3125 and HI 8498 are popular durum wheat cultivars but low YPC limits their pasta-making potential. Marker-assisted introgression of superior Psy-A1SSR*e* allele from PDW 233 resulted into improvement of YPC in both the backgrounds. The improved lines showed significantly higher YPC and *b** values without altering other agronomic traits and grain yield potential in both the backgrounds.

Conclusion

We have developed a robust DNA-based marker that can simultaneously tag A and B homoeologs of *Psy-1* gene associated with YPC in durum wheat. The marker was also validated for its association with YPC in two RIL populations and a set of 222 tetraploid wheat accessions. The utility of newly developed marker in marker-assisted breeding program to improve YPC in two durum wheat cultivars MACS 3125 and HI 8498 was also demonstrated. The results show that with MAB, value addition of elite cultivars for specific traits such as end use quality is possible. Use of marker Psy-A1SSR in breeding will be useful to enhance YPC in durum wheat cultivars and can result in the release of better quality durum wheat from future breeding programs.

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