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Development of multiplex-PCR systems for genes related to flour colour in Chinese autumn-sown wheat cultivars

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RESEARCH ARTICLE

Abstract

In wheat kernel, multiple gene loci, *Psy-A1*, *Psy-B1, TaZds-A1*, *TaZds-D1*, *TaLox-B1*, *Ppo-A1* and *Ppo-D1* are implicated in the expression of flour colour traits. In this study, eight functional markers, covering seven gene loci, were used to develop three multiplexed polymerase chain reaction (PCR) systems for marker-assisted selection (MAS) of desirable flour colour, i.e. PCR-I (*YP7B-1*/*YP7B-2*/*YP2A-1*), PCR-II (*YP2D-1*/*PPO18*) and PCR-III (*YP7A*/*LOX18*/*STS01*). Genotyping of 20 control wheat cultivars by these multiplex-PCR systems completely matched the results from the eight single PCR amplifications. Multiplex-PCR typing was successfully established for a total of 155 Chinese autumn-sown wheat cultivars. In multiplexed PCR-I, the occurring frequencies for three alleles (*Psy-B1a*, *Psy-B1b* and *Psy-B1c*) at the *Psy-B1* locus were 33.5, 58.7, and 7.7%; while two alleles (*TaZds-A1a* and *TaZds-A1b*) at *TaZds-A1* were 40.0 and 60.0%, respectively. In multiplexed PCR-II, the occurring frequencies, *TaZds-D1a* and *TaZds-D1b* (98.7 and 1.3%) at the *TaZds-D1* locus, *Ppo-A1a* and *Ppo-A1b* (39.4 and 60.6%) at the *Ppo-A1* locus were obtained, respectively. In multiplexed PCR-III, the occurring frequencies for two predominant alleles at each locus was obtained, *Psy-A1a* and *Psy-A1b* (55.5 and 44.5%) at *Psy-A1* locus, *TaLox-B1a* and *TaLox-B1b* (29.7 and 70.3%) at *TaLox-B1* locus, *Ppo-D1a* and *Ppo-D1b* (45.8 and 54.2%) at *Ppo-D1* locus, respectively. The multiplexed PCRs developed in this study are useful for MAS for wheat flour colour improvement. The genotyping results obtained in this study provide valuable information for DNA-based identification of wheat cultivars.

Keywords: common wheat, functional markers, multiplexed PCR system, lipoxygenase gene, phytoene synthase gene, polyphenol oxidase gene, ζ-carotene desaturase gene

1. Introduction

Flour colour of wheat is one of the most essential quality traits for different end uses (Fu, 2008; Geng *et al.*, 2010; Kumar, *et al.*, 2011). For example, for white salted noodles and steamed bread that are widely consumed in China, Japan and Korea, a bright white or creamy white colour is desired (Crosbie *et al.*, 1998; Fu, 2008; Geng *et al.*, 2010). For yellow alkaline noodles, which are widely adopted in the local cuisine of Malaysia, Singapore, Indonesia, Thailand and southern China, a bright yellow colour is preferred (Crosbie *et al.*, 1998; Fu, 2008). Additionally, time-dependent discolouration (browning) is also an important trait for end-products' quality, particularly for Asian noodles (He *et al.*, 2007). Therefore, it is important to breed cultivars with different colour traits to meet the aforementioned different requirements.

The colour of wheat flour and its end product depends on internal factors, such as the yellow pigment content (YPC) and the activity of polyphenol oxidase (PPO) and lipoxygenase (LOX), etc. (He, 2008; Sun, 2005; Wang, 2009), as well as external factors, including the environment conditions during grain formation, flour extraction rate in milling, etc. (Mares and Panozza, 1999). Though it is hard to improve flour colour property via various external factors,

the desirable YPC and activity of PPO and LOX can be selected via marker-assisted selection (MAS) in wheat high quality breeding programs (Mares and Campbell, 2001).

Despite environmental factors, YPC is mainly controlled by genetic factors. Parker *et al.* (1998) found the heritability of it up to 0.67 in common wheat. Carotenoids are the primary components of flour yellow pigment (Adom *et al.*, 2003; Della Penna and Pogson, 2006; Miskelly, 1984; Wang *et al.*, 2009a). More than ten enzymic steps are involved in the carotenoids synthetic pathway (Hirschberg, 2001). Phytoene synthase (PSY), catalysing the synthesis of phytoene, is generally accepted as the rate-limiting step in the pathway (He *et al.*, 2009; Lindgren *et al.*, 2003). Meanwhile, carotene desaturase (ZDS) is another critical enzyme participating in the two-step desaturation from phytoene to lycopene (Cong *et al.*, 2009). The existed genetic studies presented that genes involved in the carotenoid biosynthetic pathway are good candidates for a large proportion of variations in wheat endosperm colour (Cong *et al.*, 2010). Major quantitative trait loci (QTL) for PSY genes have been mapped on the long arms of group 7 chromosomes (7A and 7B) (He *et al.*, 2008, 2009; Wang *et al.*, 2009a). Past studies showed that, *Psy-A1* and *Psy-B1* are two major loci for PSY coding genes, accounting for about 27-60% and 48-61% of phenotypic variance, respectively (He *et al.*, 2009; Kuchel *et al.*, 2006; Mares and Campbell, 2001; Parker *et al.*, 1998). The ZDS genes are located on the group 2 chromosomes (2A and 2D) (Zhang *et al.*, 2011; Dong *et al.*, 2012). Functional markers *YP7A* at *Psy-A1* locus, *YP7B-1*, *YP7B-2* at *Psy-B1* locus, *YP2A-1* at *TaZds-A1* locus, and *YP2D-1* at *TaZds-D1* locus were developed to effectively distinguish the allelic variations of cultivars with different YPC (Dong *et al.*, 2012; He, 2008; Liu *et al.*, 2012; Zhang *et al.*, 2011).

Despite the critical enzymes in the carotenoid synthetic pathway, two oxidases, i.e. LOX and PPO, are participating in the bleach and darkening reaction of wheat flour and its end-products, respectively. The YPC of wheat is determined by both the synthesis of yellow carotenoid pigments and the LOX-catalysed degradation. To date, the QTL for LOX genes were mainly detected on the homeologous group 4 and 5 chromosomes (Geng *et al.*, 2011; Hart and Langston, 1977; Hessler *et al.*, 2002; Li *et al.*, 1999). Functional markers *LOX16* and *LOX18* at the *TaLox-B1* locus on chromosome 4BS were developed (Geng *et al.*, 2012).

PPO plays a major role in the undesirable time-dependent darkening of Asian noodles, steamed bread, pan bread and pasta (He *et al.*, 2007; Mayer and Harel, 1979; Nilthong *et al.*, 2012). It is also an essential quality parameter of wheat malt (Wang *et al.*, 2014). Previous studies in wheat have shown that two major genes, *Ppo-A1* and *Ppo-D1*, determining PPO activity, are located on the long arms of wheat homeologous chromosomes 2A and 2D, respectively (Anderson and Morris, 2001; Demeke *et al.*, 2001; Jimenez and Dubcovsky, 1999; Mares and Campbell, 2001; Udall, 1996). Functional markers, *PPO18* and *STS01*, were developed to distinguish the allelic variations of cultivars with different PPO activities at the *Ppo-A1* and *Ppo-D1* loci, respectively (Beecher *et al.*, 2012; He *et al.*, 2007; Nilthong *et al.*, 2012; Si *et al.*, 2012; Sun *et al.*, 2005; Wang *et al.*, 2008a,b).

Since multiple genes are associated with the colour of wheat flour and its end-products, molecular detection has been successfully applied for targeting the favourable colour. Compared with the direct estimation through milling, molecular detection can identify the genes without the spatiotemporal interferences (Goutam *et al.*, 2013; Wang *et al.*, 2012). However, multiple loci detection by traditional PCR is time-consuming and costly. In contrast, multiplexed PCR, as an efficient and economical technology that can simultaneously identify several alleles in one PCR amplification, is suitable for identifying multiple loci related to flour colour. To date, several multiplexed PCR systems for the detection of genes related to flour colour have been reported (Wang *et al.*, 2012; Zhang *et al.*, 2008). But the multiplexed PCR assay developed by Zhang *et al.* (2008) only comprises *Ppo-A1* and *Ppo-D1* loci; the multiplexed PCR system established by Wang *et al.* (2012) only includes *Psy-A1* and *Ppo-D1* loci. Considering the involvement of multiple genes in flour colour, allelic variation in one or two loci can hardly explain this complex trait. Therefore, multiplexed PCR systems covering more relevant loci, i.e. *Psy-A1*, *Psy-B1*, *TaZds-A1*, *TaZds-D1*, *TaLox-B1*, *Ppo-A1* and *Ppo-D1*, are crucial for improving MAS in wheat quality breeding.

Chinese autumn-sown wheat cultivars contribute more than 90% to national wheat production and acreage (He *et al.*, 2010a,b). White salted noodles and steamed bread, preferring bright or creamy white colour, account for about 80% of national wheat consumption (He *et al.*, 2003) in China. However, the improvement of wheat colour quality had not been launched in China until early 21st century (He, 2008), thus flour colour of leading cultivars rarely met the requirements of the markets and consumers (Liu *et al.*, 2002). Under this scenario, benzoyl peroxide (BPO) had been widely used as a flour bleaching agent in China for decades until the release of a new standard of state 'wheat flour' (GB1355-2005) where it was prohibited considering its detrimental effects to human health (Tian, 2007). Although a set of cultivars with high level of whiteness had been bred and cultivated successively in China with the prohibition of BPO addition, the general whiteness levels of flour from Chinese leading cultivars still could not meet the markets and consumers' demand for white salted noodles and steamed bread (Fan *et al.*, 2012; Xin *et al.*, 2009). Therefore, breeding and releasing more cultivars with improved natural flour colour is one of the important objectives for Chinese wheat quality

improvement programs. The characterisation of the seven loci related to flour colour in Chinese autumn-sown wheat cultivars would provide valuable information for wheat breeding and production. Thus, the objectives of this study were to: (1) develop multiplexed PCR systems as effective and economical methods for the identification and selection of favourable alleles related to flour colour; and (2) provide information on allelic compositions in 155 Chinese autumn-sown cultivars, to facilitate breeders, both in China and abroad, in designing crosses aimed at improving wheat colour quality.

2. Materials and methods

Plant materials and DNA extraction

A total of 155 Chinese autumn-sown wheat cultivars used in this study are major cultivars in China. Some of them are also used as core parents in Chinese wheat breeding programs. Among the 155 cultivars, 104 were released before the year 2005, referred as 'old cultivars', and 51 were released afterwards, referred as 'new cultivars'. Twenty cultivars were selected as controls for establishing and validating the multiplexed PCR systems (Table 1), and their allelic combinations at the target loci were confirmed by eight single PCRs. The other 135 cultivars were investigated only by the developed multiplex-PCR typing. Genomic DNA was extracted from about five-day-old wheat seedling leaves using a cetyltrimethyl ammonium bromide method (Stewart and Via, 1993) in triplicates.

Single PCR amplifications

Allelic combinations at the seven loci of the 20 controls were detected by eight single PCR amplifications. All the primers were synthesised by Sangon Biotech Co., Ltd. (Shanghai, China P.R.), based on the primer sequences published in literature (Table 2).

Single PCR amplification was performed with a reaction volume of 20 µl containing 50 ng of template DNA. For *YP7A*, *YP7B-1*, *YP7B-2*, *YP2A-1*, *LOX18* and *PPO18*, 20 mM of Tris-HCl (pH=8.4), 20 mM of KCl, 100 µM of each dNTP, 1.5 mM of $MgCl_2$ and 1 unit of *Taq* DNA polymerase (TRANSGEN Biotech Co., Ltd., Beijing, China P.R.) were included, with primer concentrations of 5, 6, 6, 5, 10 and 10 pmol, respectively. For *YP2D-1* and *STS01*, 10 µl MasterMix (BIOSCI Biotech Co., Ltd., Hangzhou, China P.R.) and 10 pmol of each primer were included. The reaction conditions were 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, 59-65 °C (Table 2) for 45 s, and 72 °C for 30-70 s, with a final extension of 8 min at 72 °C.

Table 1. The selected 20 control cultivars and their genotyping results at the seven loci related to wheat flour colour by eight single PCRs.

Cultivar	*Psy-A1*	*Psy-B1*	*TaZds-A1*	*TaZds-D1*	*TaLox-B1*	*Ppo-A1*	*Ppo-D1*
Ningdong 6	a	a	b	b	b	a	a
Zhoumai 16	a	b	b	a	b	a	a
Zhoumai 17	a	a	a	a	b	a	a
Xinong 979	b	b	a	a	a	b	b
Xinong 88	b	b	b	a	b	b	b
Shaan 354	a	c	a	a	b	a	b
Xiaoyan 54	b	b	a	a	b	b	b
Xuzhou 25	b	c	b	a	b	a	b
Xinong 6028	b	a	a	a	b	b	a
Xinong 8727	b	b	b	a	b	b	b
Xifeng 27	a	a	b	b	a	b	a
Fengchan 3	b	b	b	a	a	b	a
Zhoumai 23	a	b	b	a	b	a	b
Mianyang 31	a	b	b	a	b	b	a
Shaannong 512	b	c	a	a	b	b	b
Mianyang 19	b	b	b	a	b	a	a
Changwu 521	b	b	a	a	a	a	b
Xiaoyan 216	b	a	b	a	b	b	b
Xinong 2208	b	b	a	a	b	b	b
Xiaoyan 166	b	b	a	a	b	a	b

'a', 'b' or 'c' indicates different alleles at each gene locus.

Table 2. Primer sequences and the relevant information for the seven flour colour related loci.

Locus	Marker	Primer sequence (5'-3')	Fragments (bp)	Annealing temperature (°C)	Allele	Reference
Psy-A1	*YP7A*	F: GGACCTTGCTGATGACCGAG	194	63	*Psy-A1a*	He, 2008
		R: TGACGGTCTGAAGTGAGAATGA	231		*Psy-A1b*	
Psy-B1	*YP7B-1*	F: GCCACAACTTGAATGTGAAAC	151	59	*Psy-B1a*	
		R: ACTTCTTCCATTTGAACCCC	156		*Psy-B1b*	
	YP7B-2	F: GCCACCCACTGATTACCACTA	428	62	*Psy-B1c*	
		R: CCAAGGTGAGGGTCTTCAAC				
TaZds-A1	*YP2A-1*	F: CCCTAAGGAAGCCGAGCAAAT	183	60	*TaZds-A1a*	Dong *et al.*, 2012
		R: GTGAGAGTACTAATGTTATGACCG	179		*TaZds-A1b*	
TaZds-D1	*YP2D-1*	F: GTGGGATCCTGTTGCTTATGC	–	64	*TaZds-D1a*	Zhang *et al.*, 2011
		R: GTAGATTATCCAAGCCAACTGCC	981		*TaZds-D1b*	
TaLox-B1	*LOX18*	F: ACGATGTGAGTTGTGACTTGTGA	–	65	*TaLox-B1a*	Geng *et al.*, 2012
		R: GCGCGGATAGGGGTGC	791		*TaLox-B1b*	
Ppo-A1	*PPO18*	F: AACTGCTGGCTCTTCTTCCCA	685	63	*Ppo-A1a*	Sun *et al.*, 2005
		R: AAGAAGTTGCCCATGTCCGC	876		*Ppo-A1b*	
Ppo-D1	*STS01*	F: CGCCGACCATTTCAACAA	560	64	*Ppo-D1a*	Wang *et al.*, 2008a
		R: AGAAGGACCACAAGCCGTAG	–		*Ppo-D1b*	

PCR products were separated by electrophoresis in a 1.5% agarose gel and then stained with ethidium bromide and visualised using UV light, whereas the PCR products of *YP7B-1* and *YP2A-1*, were separated by a 6% denaturing polyacrylamide gel and resolved by silver staining.

Development of multiplexed PCR systems and genotyping of flour colour genes

In order to produce clear and bright PCR bands with expected fragment sizes in multiplexed PCR systems, several attempts were made to optimise the systems. First, markers with similar PCR amplification and electrophoretic conditions were included in the same multiplexed PCR system. For example, *YP7B-1* and *YP2A-1* were included in the same multiplex-PCR typing based on their similar annealing temperatures and electrophoretic conditions. Second, more markers were added into an established system to test their performance, facilitating more loci been genotyped in one system. For example, marker *YP7B-2* was successfully added into the *YP7B-1*/*YP2A-1* system, so that one PCR-based assay could investigate both the *Psy-B1* and *TaZds-A1* loci. Finally, a series of adjustments on PCR parameters were made to optimise the reaction systems, e.g. screening the optimal annealing temperature, determining optimal concentrations of each primer pairs, etc.

If the results of multiplexed PCR systems in the 20 control cultivars completely match those assayed by single PCRs, they are proven to be effective and reliable. The rest cultivars (135 Chinese autumn-sown wheat cultivars) were then genotyped with the newly developed multiplexed systems. The allelic combinations at target loci in 155 cultivars were confirmed by at least two replicates with consistent tested results. Analyses of relevant data were performed by the inserted functions in MS Excel 2007 (Microsoft, Redmond, WA, USA).

3. Results

Single PCR amplifications

In order to confirm the allelic combinations at the seven target loci, functional markers *YP7A*, *YP7B-1*, *YP7B-2*, *YP2A-1*, *YP2D-1*, *LOX18*, *PPO18* and *STS01* were used to perform eight single PCR amplifications in the 20 cultivars, respectively. The results are illustrated in Table 1, and the electrophoresis of PCR products in 11 out of the 20 controls are shown in Figure 1.

At the *Psy-A1* locus, *YP7A* gave a 194-bp fragment, indicating the presence of *Psy-A1a* allele in 7 cultivars, and a 231-bp fragment, indicative of *Psy-A1b* allele in 13 cultivars (Table 1). At the *Psy-B1* locus, *YP7B-1* amplified a 151-bp fragment for *Psy-B1a* allele in 5 cultivars, a 156-bp fragment for the other allele (*Psy-B1b*) in 12 cultivars, and non-PCR product in 3 cultivars (Table 1). Then, *YP7B-2* was applied, which result in a single band of 428-bp for *Psy-B1c* allele in 3 cultivars in which *YP7B-1* failed to amplify any PCR product (Table 1). Similarly, *YP2A-1*, *YP2D-1*, *LOX18*, *PPO18* and *STS01* were used individually to discriminate the remaining loci (Figure 1D-1H and Table 1). The results showed that all the target alleles at the seven loci could be identified in the selected cultivars, suggesting that these

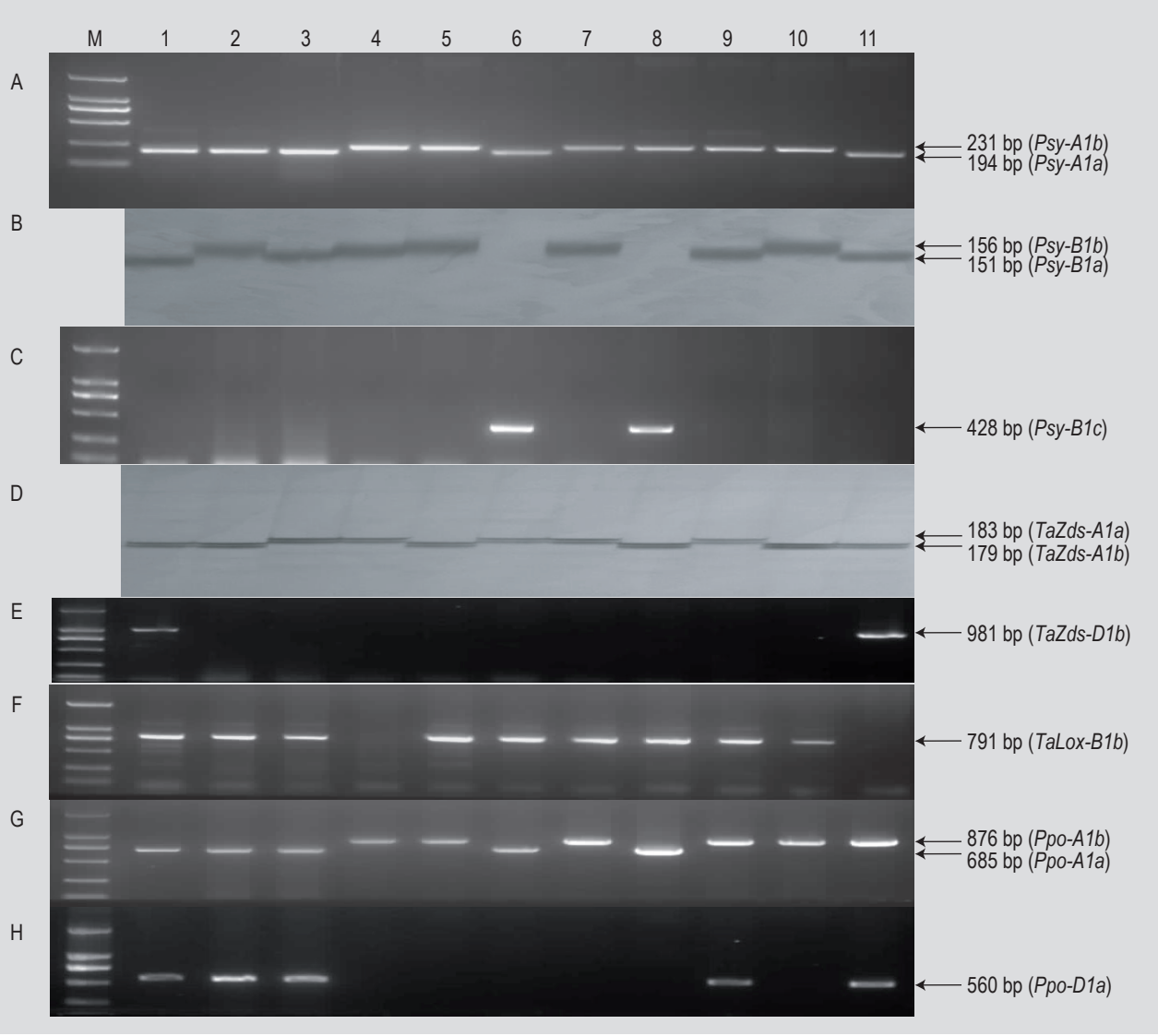

Figure 1. Electrophoresis of PCR products in a subset of the 20 cultivars amplified at each locus by eight single primer sets. (A-H) Cultivars amplified by markers *YP7A*, *YP7B-1*, *YP7B-2*, *YP2A-1*, *YP2D-1*, *LOX18*, *PPO18*, *STS01*, respectively. M = marker DL 2000; 1 = Ningdong 6; 2 = Zhoumai 16; 3 = Zhoumai 17; 4 = Xinong 979; 5 = Xinong 88; 6 = Shaan 354; 7 = Xiaoyan 54; 8 = Xuzhou 25; 9 = Xinong 6028; 10 = Xinong 8727; 11 = Xifeng 27.

wheat cultivars could be used as controls for establishing the multiplexed PCR systems.

Development and validation of multiplexed PCRs

Three multiplexed PCR systems including seven target loci were developed for genotyping 15 alleles related to flour colour. Multiplexed PCR-I was developed by functional markers *YP7B-1*, *YP7B-2* and *YP2A-1*, identifying three alleles (*Psy-B1a*, *Psy-B1b*, *Psy-B1c*) at the *Psy-B1* locus and two alleles (*TaZds-A1a* and *TaZds-A1b*) at the *TaZds-A1* locus, respectively. Multiplexed PCR-II was developed by two specific markers (*YP2D-1* and *PPO18*), two alleles at each locus could be discriminated: *TaZds-D1a* or *TaZds-D1b* at the *TaZds-D1* locus, *Ppo-A1a* or *Ppo-A1b* at the *Ppo-A1* locus. Multiplexed PCR-III system was established by *YP7A*, *LOX18* and *STS01*, genotyping six alleles: *Psy-A1a* or *Psy-A1b* at the *Psy-A1* locus, *TaLox-B1a* or *TaLox-B1b* at the *TaLox-B1* locus, and *Ppo-D1a* or *Ppo-D1b* at the *Ppo-D1* locus, simultaneously. The PCR components, amplification and electrophoretic conditions of the newly developed multiplexed PCRs were shown in Table 3. Figure 2 showed the band patterns of 11 out of the 20 controls using the multiplexed PCRs. The results of the three multiplexed PCR assays in 20 cultivars completely matched those results obtained from eight single PCRs, indicating that the multiplexed PCR assays developed in this study were effective and reliable.

Table 3. The specific PCR components and electrophoretic conditions of each multiplex PCR system.

Multiplex PCR system	PCR-I	PCR-II	PCR-III
Objective loci	*Psy-B1/TaZds-A1*	*TaZds-D1/Ppo-A1*	*Psy-A1/TaLox-B1/Ppo-D1*
Allelic variations	*Psy-B1a/Psy-B1b/Psy-B1c* *TaZds-A1a/TaZds-A1b*	*TaZds-D1a/TaZds-D1b* *Ppo-A1a/Ppo-A1b*	*Psy-A1a/Psy-A1b* *TaLox-B1a/TaLox-B1b* *Ppo-D1a/Ppo-D1b*
Markers	*YP7B-1/YP7B-2/YP2A-1*	*YP2D-1/PPO18*	*YP7A/LOX18/STS01*
PCR components			
MasterMix (BIOSCI) µl	–	10	–
10×buffer (µl)	2	–	2
Concentration of dNTPs (µmol/l)	200	–	225
Concentration of primers for each gene (pmol)	6/6/5[a]	14/6[b]	5/10/10[c]
Taq DNA polymerase (U)	1.5	–	1.5
Template DNA (ng)	150-200	150-200	150-200
PCR amplification conditions			
Pre-denaturation	95 °C, 5 min	95 °C, 5 min	95 °C, 5 min
Denaturation	95 °C, 45 s	95 °C, 45 s	94 °C, 1 min
Annealing	59 °C, 45 s	63 °C, 45 s	64 °C, 1 min
Extension	72 °C, 30 s	72 °C, 1 min	72 °C, 90 s
Cycles	35	34	36
Final extension	72 °C, 10 min	72 °C, 5 min	72 °C, 8 min
Electrophoretic conditions			
Agarose (%)	–	2	2
Denaturing polyacrylamide gel (%)	6	–	–
Voltage (V)	2,000	100	100
Time (h)	1	1.5	1.5

[a] Concentration of primers, *YP7B-1*, *YP7B-2* and *YP2A-1*, respectively.
[b] Concentration of primers, *YP2D-1* and *PPO18*, respectively.
[c] Concentration of primers, *YP7A*, *LOX18* and *STS01*, respectively.
– Establishing the system without the reagent.

Characterisation of 155 Chinese autumn-sown wheat cultivars by the newly developed multiplexed PCR systems

Using multiplexed PCR-I, alleles *Psy-B1a*, *Psy-B1b* and *Psy-B1c* were found in 52, 91 and 12 cultivars, respectively; *TaZds-A1a* and *TaZds-A1b* were found in 62 and 93 cultivars, separately (Table 4 and Supplementary Table S1). Using multiplexed PCR-II, *TaZds-D1b* was detected only in Ningdong 6 and Xifeng 27, while *TaZds-D1a* was identified in other 153 cultivars; *Ppo-A1a* and *Ppo-A1b* were detected in 61 and 94 cultivars, respectively (Table 4 and Supplementary Table S1). Using multiplexed PCR-III, *Psy-A1a* and *Psy-A1b* were identified in 86 and 69 cultivars, separately; *TaLox-B1a* and *TaLox-B1b* were investigated in 46 and 109 cultivars, respectively; *Ppo-D1a* and *Ppo-D1b* were found in 71 and 84 cultivars, respectively (Table 4 and Supplementary Table S1).

Generally, the alleles *Psy-B1b*, *TaZds-A1b*, *TaZds-D1a*, *Ppo-A1b*, *Psy-A1a*, *TaLox-B1b*, and *Ppo-D1b,* identified at the seven gene loci, showed higher occurring frequencies in the 155 genotyped cultivars (Table 4). The occurring frequencies of alleles *Psy-A1b*, *Psy-B1b*, *TaZds-A1a*, *TaZds-D1b*, *TaLox-B1a*, *Ppo-A1b* and *Ppo-D1a* were different between the old (104) cultivars and new (51) cultivars. Specifically, alleles *Psy-A1b*, *Psy-B1b*, *TaLox-B1a* occurred with higher frequencies in new cultivars, whereas alleles *TaZds-A1a*, *Ppo-A1b* and *Ppo-D1a* more frequently occurred in old cultivars (Table 5). Besides, the occurring frequencies of most major allelic combinations listed in Table 6 were higher in new cultivars than the old ones, except for the allelic combination *Psy-B1b*/*TaZds-A1a*. No additional differences in allelic frequencies were observed between the cultivars released from the two periods. Moreover, non-significant allelic variations were found associated with cultivars distributed in different provinces of China (detailed information not presented).

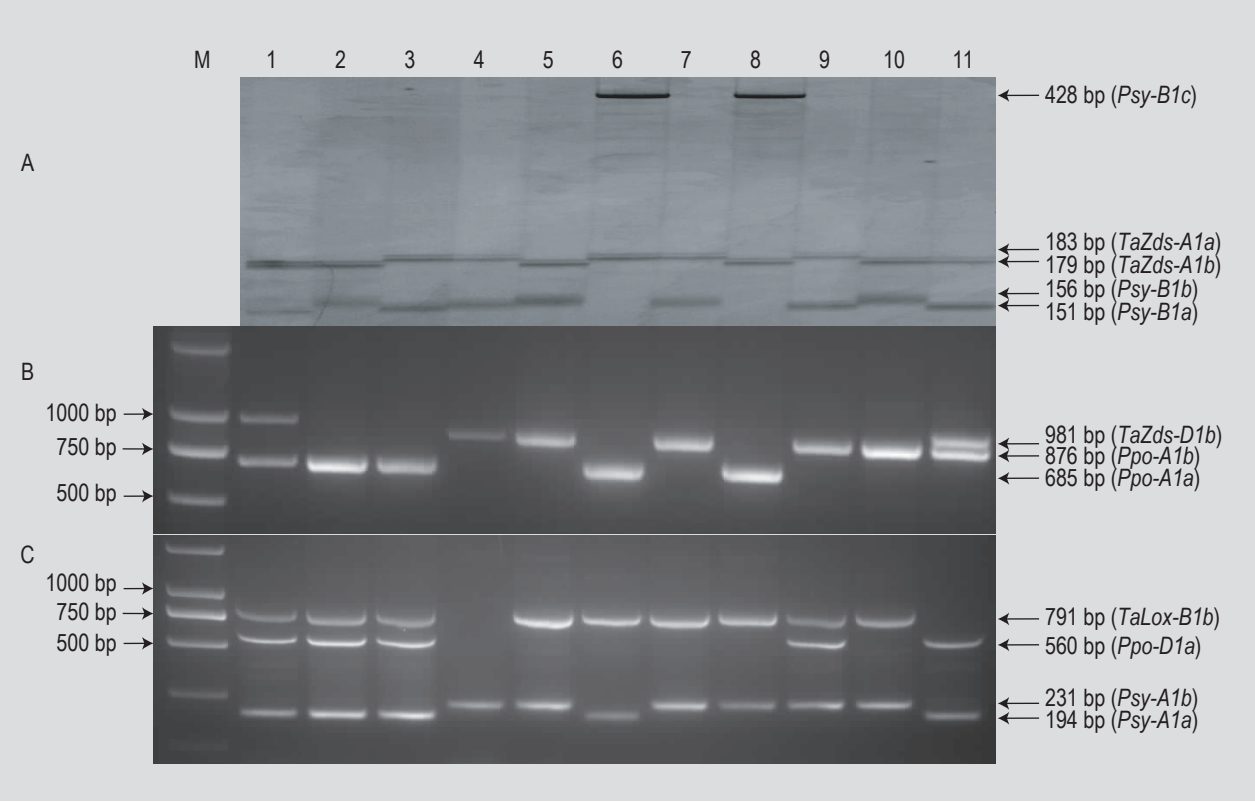

Figure 2. Electrophoresis of PCR products of selected control cultivars amplified by the developed multiplexed PCR systems. (A) Multiplexed PCR-I for *Psy-B1* and *TaZds-A1* loci with five alleles. (B) Multiplexed PCR-II for *TaZds-D1* and *Ppo-A1* loci with four alleles. (C) Multiplexed PCR-III for *Psy-A1*, *TaLox-B1* and *Ppo-D1* loci with six alleles. M = marker DL 2000; 1 = Ningdong 6; 2 = Zhoumai 16; 3 = Zhoumai 17; 4 = Xinong 979; 5 = Xinong 88; 6 = Shaan 354; 7 = Xiaoyan 54; 8 = Xuzhou 25; 9 = Xinong 6028; 10 = Xinong 8727; 11 = Xifeng 27.

Table 4. Results of 155 Chinese autumn-sown wheat cultivars detected by the new multiplexed PCR systems.

Multiplex PCR system	Allele	Frequency (%)	Phenotype based on the previous studies[1]
I	*Psy-B1a*	33.5	medium YP content (He, 2008)
	Psy-B1b	58.7	lower YP content (He, 2008)
	Psy-B1c	7.7	higher YP content (He, 2008)
	TaZds-A1a	40.0	lower YP content (Dong *et al.*, 2012)
	TaZds-A1b	60.0	higher YP content (Dong *et al.*, 2012)
II	*TaZds-D1a*	98.7	higher YP content (Zhang *et al.*, 2011)
	TaZds-D1b	1.3	lower YP content (Zhang *et al.*, 2011)
	Ppo-A1a	39.4	higher PPO activity (Sun *et al.*, 2005)
	Ppo-A1b	60.6	lower PPO activity (Sun *et al.*, 2005)
III	*Psy-A1a*	55.5	higher YP content (He 2008)
	Psy-A1b	44.5	lower YP content (He, 2008)
	TaLox-B1a	29.7	higher LOX activity (Geng *et al.*, 2012)
	TaLox-B1b	70.3	lower LOX activity (Geng *et al.*, 2012)
	Ppo-D1a	45.8	lower PPO activity (Wang *et al.*, 2008a)
	Ppo-D1b	54.2	higher PPO activity (Wang *et al.*, 2008a)

[1] YP = yellow pigment; LOX = lipoxygenase; PPO = polyphenoloxidase.

Table 5. Occurring frequencies of alleles tested in 155 cultivars released before and after 2005.

Allele	Frequencies of alleles (%)	
	Before 2005	After 2005
Psy-A1b	42.3	49.0
Psy-B1b	57.7	60.8
TaZds-A1a	44.2	31.4
TaZds-D1b	1.0	2.0
TaLox-B1a	25.0	39.2
Ppo-A1b	63.5	54.9
Ppo-D1a	51.0	35.3

Table 6. Occurring frequencies of mostly allelic combinations in 155 cultivars released before and after 2005.

Allelic combination	Frequencies of allelic combinations (%)	
	Before 2005	After 2005
Psy-A1b/*Psy-B1b*	30.8	31.4
Psy-A1b/*TaZds-A1a*	18.3	19.6
Psy-A1b/*TaLox-B1a*	9.6	13.7
Psy-B1b/*TaZds-A1a*	28.8	19.6
Psy-B1b/*TaLox-B1a*	13.5	25.5
TaZds-A1a/*TaLox-B1a*	11.5	15.7
Psy-A1b/*Psy-B1b*/*TaZds-A1a*	13.5	13.7
Psy-A1b/*Psy-B1b*/*TaLox-B1a*	6.7	11.8
Psy-A1b/*TaZds-A1a*/*TaLox-B1a*	3.8	7.8
Psy-B1b/*TaZds-A1a*/*TaLox-B1a*	7.7	11.8
Psy-A1b/*Psy-B1b*/*TaZds-A1a*/*TaLox-B1a*	2.9	7.8

4. Discussion and conclusions

Efficiency of the developed multiplexed-PCR typing

It has been acknowledged that flour colour is an essential property for both breeding and processing of wheat. Generally, YPC, LOX and PPO activity in wheat kernels are physiological-biochemical traits, which are controlled by multiple genes and difficult to be directly selected (Geng, 2010; He, 2008; Sun, 2005). Traditional test methods are time consuming, special instruments-dependent, expensive, and requiring high quantity of samples (Geng, 2010; Sun, 2005; Wang *et al.*, 2012). PCR-based assays, in contrast, can directly identify the genes controlling phenotypic traits by molecular markers, without any interference from the environment (Wan *et al.*, 2008), and hence can be performed during any of the wheat growth stages (Ma *et al.*, 2003). As a more effective and rapid way for genotyping cultivars, multiplexed PCR with several pairs of primers would facilitate the use of single PCR reaction for discriminating various gene loci (Ma *et al.*, 2003). Using the three multiplexed assays developed in this study, 15 alleles at 7 flour colour related gene loci could be simply genotyped by 3 PCR-based reactions rather than 8 single PCR amplifications, which will reduce test time and sample requirements by one third to a half for early wheat breeding. The results of the 20 controls tested by the three multiplexed PCR systems were consistent with those detected via single PCR, demonstrating that the multiplex-PCR typing is accurate and reliable.

Evaluation of the multiplexed PCR assays

Wheat flour colour was controlled by multiple gene loci (Maphosa, 2013). In order to improve the selecting efficiency and accuracy in breeding, multiple gene loci related to flour colour should be considered simultaneously.

The three multiplexed PCR systems developed covering nearly all the known gene loci related to flour colour, providing an effective tool for identifying and selecting cultivars and lines with optimised alleles in MAS breeding for different flour colour requirements. For example, regarding Chinese white salt noodle and steamed bread, a white or creamy white colour is needed, therefore cultivars with lower YPC, higher LOX activity and lower PPO activity are preferred (Geng, 2010; He, 2008; Sun, 2005), and the corresponding allelic combinations are *Psy-B1b*/*TaZds-A1a* (multiplexed PCR-I), *Ppo-A1b*/*TaZds-D1b* (multiplexed PCR-II) and *Psy-A1b*/*TaLox-B1a*/*Ppo-D1a* (multiplexed PCR-III). In contrast, when yellow alkaline noodle is considered, a bright yellow colour is desired, thus cultivars with higher YPC, lower LOX activity and PPO activity are needed (Geng, 2010; He, 2008; Sun, 2005), and the corresponding allelic combination should be *Psy-A1a*/*Psy-B1c*/*TaZds-A1b*/*TaZds-D1a*/*TaLox-B1b*/*Ppo-A1b*/*Ppo-D1a*.

Obviously, it is difficult for a cultivar or line to contain all the favourable alleles, so the target material should obtain as many favourable alleles as possible. Meanwhile, attention should also be paid to different genotypes contributing to the same phenotype at different levels. For example, *Ppo-A1b* is preferred than *Ppo-D1a* due to its higher phenotypic effect (Si *et al.*, 2012).

Although several other functional markers related to flour colour were reported, they were not included in this study for the following reasons: (1) the PCR products of *Psy-A1e*, *Psy-A1p*, *Psy-A1q*, *Psy-A1r* and *Psy-A1s* could only be distinguished by digestion with *Mva*I using *csPSY* marker (Howitt *et al.*, 2009), which may interfere the multiplexing results of the PCR-based assay; (2) the involving of marker *LOX16* would cause primer-primer interactions in this study

(relevant result was not provided); (3) *STS-H* was not used in this study due to the fact that markers *STS01* and *STS-H* are co-dominant markers (Wang *et al.*, 2008b), and *STS01* has already been successfully applied in several studies (Si *et al.*, 2012; Wang *et al.*, 2009b); and (4) only a few wheat cultivars and lines contained homoeologous alleles *Psy-A1c*, *Psy-B1d*, *Psy-B1e* and *Psy1-D1g* which were distinguished by markers *YP7A-2*, *YP7B-3*, *YP7B-4* and *YP7D-1*, *YP7D-2* or *YP7D-3*, separately (He, 2008; Wang, 2009). For example, no tested materials in 217 wheat accessions and only three materials in 342 CIMMYT spring wheat accessions were identified containing allele *Psy-A1c*; only 2 with *Psy-B1d* in 217 wheat accessions and 2 with *Psy-B1e* in 324 CIMMYT spring wheat accessions were found (He, 2008); only 2 tested materials were investigated carrying *Psy1-D1g* in 209 wheat accessions and 341 CIMMYT spring wheat accessions (Wang, 2009); and no relevant alleles mentioned above were found in our initial genetic analysis studies of Chinese wheat germplasms (relevant result was not provided).

Therefore, the three multiplexed PCRs developed can now be effectively used to supplement conventional breeding for accumulating the superior alleles in relation to good flour colour, and for eliminating or greatly reducing the undesirable alleles through MAS in early stages of breeding program.

Distribution of alleles at the tested loci in Chinese autumn-sown wheat cultivars

The genotyping results showed that the proportion of cultivars with *Psy-B1b* allele is much higher than cultivars with *Psy-B1c*, which is consistent with the breeding aim for improving flour whiteness in China (Zhang *et al.*, 2010). However, the lower proportions of *Psy-A1b* and *TaZds-A1a* are undesirable for white salt noodles and steamed bread. *TaZds-D1b* was detected only in Ningdong 2 and Xifeng 27, exhibiting a low genetic diversity at the *TaZds-D1* locus, which is consistent with the study of Zhang *et al.* (2011), reporting that no *TaZds-D1b* allele was detected in all the tested CIMMYT wheat lines and only 4 *TaZds-D1b* allele was detected in 217 wheat accessions. The allele *TaLox-B1b* is related to better storage stability, which is in accordance with the larger proportion of cultivars containing this allele among the tested materials (Liavonchanka and Feussner, 2006), and this can explain the lower whiteness observed in Chinese wheat to some extent. Cultivars with *Ppo-A1b* accounted for more than a half, while cultivars with *Ppo-D1a* were slightly less common than those with *Ppo-D1b*.

China, as a main supporter to the BPO prohibition, brings out the new standard (GB1355-2005), which requires breeders to breed cultivars with high whiteness (Tian, 2007). Based on previous reports, a series of cultivars with high whiteness have been bred and cultivated successively in recent years (Fan *et al.*, 2012; Xin *et al.*, 2009). It can be seen that the occurring frequencies of most of the alleles related to whiteness (*Psy-A1b*, *Psy-B1b*, *TaZds-D1b*, *TaLox-B1a*) were higher in the new cultivars than the old cultivars (Table 5). However, the alleles *TaZds-A1a*, *Ppo-A1b* and *Ppo-D1a* in the new cultivars were lower than the old cultivars (Table 5). For the eleven allelic combinations related to whiteness (*TaZds-D1b* only occupied a small proportion and could be overlooked), occurring frequencies of ten of those in new cultivars were higher than old cultivars (Table 6). These genotyping results illustrated the improvement of flour colour traits of Chinese wheat cultivars in the current breeding progress.

Therefore, wheat cultivars with whiteness-related alleles at *Psy-A1*, *TaZds-A1*, *TaZds-D1* and *Ppo-D1* loci should be reinforced in future breeding of wheat cultivars for white salted noodles and steamed bread. Two approaches could be employed in this regard, *viz.*, to screen current cultivars and lines for favourable alleles using the multiplexed PCR systems developed in this study, or to use the cultivars with ideal alleles identified in this study as parents in cross schemes with high-yielding wheat cultivars to develop new cultivars with improved flour colour traits.

In this study, three multiplexed PCR systems were developed as effective and reliable methods for screening desired genotypes. Genotyping results of the 155 cultivars are useful for both farmers and breeders to select ideal cultivars for cultivation and breeding. It is also advised that more efforts are needed for the improvement of wheat flour colour in China in the future.

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Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/QAS2015.0609.

Table S1. Allelic variations at *Psy-A1*, *Psy-B1*, *TaZds-A1*, *TaZds-D1*, *TaLox-B1*, *Ppo-A1* and *Ppo-D1* loci in 155 Chinese autumn-sown wheat cultivars identified by multiplexed PCR assays.

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