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Characterization of a new wheat-*Aegilops biuncialis* addition line conferring quality-associated HMW glutenin subunits

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ABSTRACT. In this study, a new disomic addition line, 12-5-2, with 44 chromosomes that was derived from BC_3F_2 descendants of the hybridization between *Triticum aestivum* cv. CN19 and *Aegilops biuncialis* was created and reported. 12-5-2 was immune to both powdery mildew and stripe rust and has stable fertility. Fluorescence *in situ* hybridization and C-banding revealed that 12-5-2 was a $1U^b$ disomic addition line (ADL1U^b). The seed storage protein electrophoresis showed that 12-5-2 presented all high molecular weight glutenin subunits (7 + 8 and 2 + 12) of CN19 and 2 new subunits that were designated Ux and Uy. Additionally, the flour quality parameters

showed that the protein content, Zeleny sedimentation value, wet gluten content, and grain hardness of 12-5-2 were significantly higher than those of its parent CN19. Moreover, 5 pairs of the chromosome $1U^b$-specific polymerase chain reaction-based landmark unique gene markers, TNAC1021, TNAC1041, TNAC1071, TNAC1-01, and TNAC1-04, were also obtained. The new ADL1U^b 12-5-2 could be a valuable source for wheat improvement, especially for wheat end-product quality and resistance to disease.

Key words: *Aegilops biuncialis*; Bread-making quality; C-banding; High molecular weight glutenin

INTRODUCTION

Aegilops species play an important role in the evolution of cultivated wheat and the processes of improving the genetic variation of common wheat (*Triticum aestivum* L.) (McFadden and Sears, 1946). *Aegilops* species carry many agronomically important features such as disease resistance, insect and pest resistance (Gill et al., 1985, 1987; Raupp et al., 1993, 1995; Gradzielewska et al., 2012), drought tolerance (Molnár et al., 2004), salt tolerance (Colmer et al., 2006), and high protein quality (Garg et al., 2009a,b). It was therefore used widely as a valuable source for wheat improvement. Until now, more than 200 wheat-*Aegilops* interspecific hybrid, addition, and translocation lines have been developed, and 53 disease and insect resistance genes have been incorporated into the wheat gene pool from 15 *Aegilops* species (for review see Schneider et al., 2008a).

Aegilops biuncialis (2n = 4x = 28, $U^bU^bM^bM^b$) is a wild species that is closely related to cultivated wheat and has a great number of agronomically useful traits including drought and salt tolerance, disease resistance, and special high molecular weight glutenin subunits (HMW-GSs) (Damania and Pecetti, 1990; Makkouk et al., 1994; Molnár et al., 2004; Colmer et al., 2006; Tan et al., 2009). These useful genes can be incorporated into cultivated wheat by developing addition lines, substitution lines, or translocation lines (Schneider et al., 2005, 2008b; Molnár et al., 2009; Schneider and Molnár-Láng, 2012).

The HMW-GSs that are encoded by many allelic genes are one class of wheat prolamins. Two linked genes designated as x- and y-types are located on the long arms of homeologous group 1 chromosomes. The HMW-GSs have been found to have a major effect on bread-making quality (Payne et al., 1981). Because of the importance of HMW-GSs in wheat quality improvement, a large number of wheat HMW-GS alleles have been cloned (Forde et al., 1985; Thompson et al., 1985; Anderson and Greene, 1989; Wan et al., 2002; Feng et al., 2011). In spite of numerous investigations aimed at finding functionally useful HMW-GSs in wheat, only a very limited number have shown functional differences. Screening for "good quality subunits" in related wild wheat species is therefore important for improving bread-making quality. Only the potential good-quality subunits, Glu-1E from the chromosome 1E addition line of *Agropyron elongatum* (DAL1E) and the Glu-1S subunit from the chromosome 1S addition line of *Aegilops searsii* (DAL1S^s) were identified so far (Garg et al., 2009a,b).

This study was undertaken to identify a new wheat-*A. biuncialis* addition line, evaluate its HMW-GSs from *A. biuncialis* for improving wheat bread-making quality, and select the

polymerase chain reaction (PCR)-based landmark unique gene (PLUG) markers specific to the homeologous group 1 chromosomes of *A. biuncialis*.

MATERIAL AND METHODS

Materials

The wheat-*A. biuncialis* addition line 12-5-2 was created and released by our research group, which was derived from the hybridization between *T. aestivum* cv. Chuannong 19 (CN19), an elite cultivar and *A. biuncialis*. The creation procedure was as follows: F_1 hybrid plants were produced by CN19 x *A. biuncialis*, and then these F_1 hybrids ($ABDU^bM^b$, 2n = 5x = 35) were treated with colchicine at the tillering stage to produce amphiploid plants ($AABBDDU^bU^bM^bM^b$, 2n = 10x = 70), which were backcrossed with wheat and then selfed. Line 12-5-2 was one of the BC_3F_2 descendants. Chinese Spring (CS), CN19, and *A. biuncialis* were collected and preserved by our lab.

Somatic chromosome counts and meiosis analysis

To count somatic chromosomes, seedling root tips were kept in water at 0°C for 24 h and were fixed in ethanol-acetic acid (3:1) for at least 3 days. They were then stained using the conventional Feulgen method. Anthers with pollen mother cells (PMCs) at metaphase I (MI) were fixed in Carnoy's 6:3:1 (ethanol:acetic acid:chloroform) fixative, and meiosis was studied using the conventional acetocarmine procedure.

Giemsa C-banding

The C-banding was carried out according to the protocol that was described by Gill et al. (1991a,b) with the following changes: root tips were pretreated in water at 0°C for 22 h and fixed for 3 days or longer (up to 1 week) at room temperature, and preparations were incubated for 2 min in 0.2 N HCl in a water bath at 60°C and treated in a 5% barium hydroxide solution at room temperature for 7.5 min. Photographs were taken with a cooled CCD camera system (DP70) on an Olympus BX-51 fluorescence microscope.

Fluorescence *in situ* hybridization (FISH)

The clone pTa71, containing a 9.05-kb *Eco*RI fragment of rDNA isolated from wheat (Gerlach and Bedbrook, 1979) was generously provided by Dr. B. Friebe of Wheat Germplasm Resource Center, Department of Plant Pathology, Kansas State University, USA. The plasmid was also labeled with digoxigenin-11-dUTP by nick translation based on the protocols of the manufacturer (Roche Diagnostics Indianapolis, IN, USA). Hybridization was carried out according to Jiang et al. (1996). The detection of digoxigenin was carried out with fluorescein-conjugated anti-digoxigenin Fab fragment (Roche Diagnostics). The slides were finally mounted in vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA) containing 0.25 µg/mL propidium iodide. Slides were examined on an Olympus BX-51 fluorescence microscope. Photographs were taken with a cooled CCD camera system

(DP70 on an Olympus BX-51 fluorescence microscope).

Seed storage protein electrophoresis and quality testing

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate endosperm glutenin proteins according to the procedure of Yang et al. (2001). Quality testing was carried out according to Zhang et al. (2005).

PLUG marker analysis

Total genomic DNA from wheat materials CS, CN19, *A. biuncialis*, and 12-5-2 were extracted as described previously (Zhou et al., 2012). A total of 48 pairs of PLUG markers, including 22 that were group 1 primers in Ishikawa et al. (2009) and 26 (TNAC1-01, TNAC1-02,…, TNAC1-26) that were designed by us, were used in this study. PCR was performed in 25-μL reaction volumes containing the following reagents: 50 ng template DNA, 0.2 mM of each dNTP, 4 μM of each primer, 1.5 mM $MgCl_2$, Taq DNA polymerase buffer, and 1.0 U Taq DNA polymerase (Takara, Japan). The PCR program consisted of a 3-min initial denaturation step at 94°C; 35 cycles with denaturation at 94°C for 45 s, annealing at 57°-60°C for 45 s, and extension at 72°C for 2 min; and a final extension step at 72°C for 5 min. To obtain higher levels of polymorphism, PCR products were digested with the 4-base cutter restriction endonucleases *Taq*I and/or *Hae*III. The products were separated by electrophoresis with 1.2% agarose gels.

RESULTS

Agronomic traits and cytological observation of the 12-5-2 addition line

Line 12-5-2 was obtained from BC_3F_2 descendants of the hybridization between CN19 and *A. biuncialis*. Its agronomic traits were very close to those of its wheat parent CN19. Its adult plant height was around 85 cm, and the number of spikes per plant varied from 4 to 7. Additionally, line 12-5-2 has stable fertility and immunity to wheat powdery mildew and stripe rust under both inoculated and natural conditions in all growth stages. However, the kernels of 12-5-2 changed to be black at harvest stage (Figure 1).

Moreover, the somatic chromosome number per cell of 12-5-2 was 44. It was observed to produce 22 bivalents and had a very low probability of univalents and multivalents in meiotic MI of PMCs. These above results demonstrated that the 12-5-2 line was a disomic addition line and it would act as a resource for a continuous supply of desirable genes from *A. biuncialis* to wheat.

FISH and C-banding of the 12-5-2 addition line

The DNA probe pTa71 was used to detect the presence of satellite chromosomes in line 12-5-2. As illustrated in Figure 2A, 4 chromosome pairs were detected. Compared with the hybridization patterns of pTa71 in wheat (Appels et al., 1980), we know that 1 satellite chromosome pair from *A. biuncialis* was incorporated into line 12-5-2 besides chromosomes 1B, 6B, and 5D.

Figure 1. Kernels of 12-5-2 and CN19.

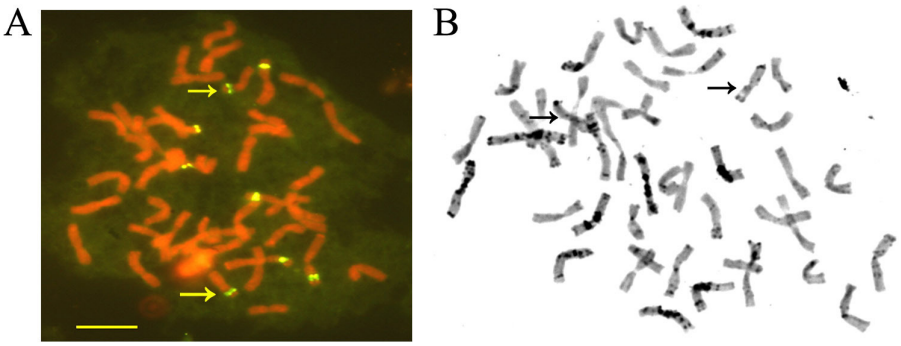

Figure 2. A. FISH on the mitotic chromosomes of 12-5-2 using pTa71 as probe. **B.** Giemsa C-banding of 12-5-2. The chromosome pair $1U^b$ was indicated by arrows. Bar = 10 μm.

In addition, Giemsa C-banding was also used to further identify the satellite chromosomes from *A. biuncialis* in line 12-5-2. Two satellite chromosomes presented a prominently terminal dark band on the long arm (Figure 2B). We also observed that the extra satellite chromosome in 12-5-2 was $1U^b$ according to the standard karyotype of wheat (Gill et al., 1991b) and *A. biuncialis* (Badaeva et al., 2004). Based on the above data, it can be concluded safely that 12-5-2 was a $1U^b$ disomic addition line.

HMW-glutenin and quality testing of 12-5-2

The seed glutenin composition of CS, *A. biuncialis*, 12-5-2, and CN19 was analyzed by SDS-PAGE. As illustrated in Figure 3, both CN19 and CS contained the HMW-GSs of 7 + 8, and 2 + 12, encoded by Glu-B1 and Glu-D1, respectively. Line 12-5-2 exhibited all HMW-GSs (7 + 8 and 2 + 12) of CN19 and 2 new subunits that were designated Ux and Uy, which apparently originated from parent *A. biuncialis*. This result strongly supported the idea that 12-5-2 was a $1U^b$ disomic addition line.

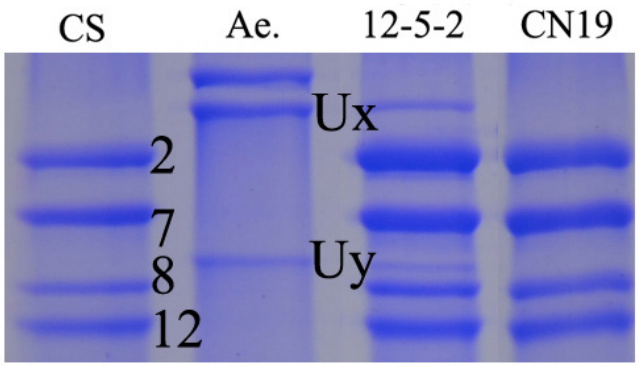

Figure 3. Seed storage protein profiles of 12-5-2. CS = Chinese Spring; Ae. = *Aegilops biuncialis*.

Moreover, the flour quality parameters of protein content, Zeleny sedimentation value, wet gluten content, grain hardness, mixing time, mixing tolerance, water content, and water absorption were tested. The quality data (Table 1) showed that the protein content, Zeleny sedimentation value, wet gluten content, and grain hardness of 12-5-2 were significantly higher than those of its parent CN19. These results indicated that $1U^b$ has a potential to improve wheat end-product quality.

Table 1. Grain and flour characteristics of 12-5-2.

Species	Content of protein (%)	Zeleny sedimentation value (cm^3)	Wet gluten content (%)	Grain hardness	Mixing time (min)	Mixing tolerance (min)	Content of water (%)	Water absorption (%)
CN19	15.1	51.3	33.1	67.9	1.7	2.5	10.5	52.1
12-5-2	20.0	60.9	40.9	75.5	2.0	2.6	10.0	52.4

The chromosome $1U^b$-specific PLUG markers

Five pairs of the chromosome $1U^b$-specific PLUG markers, TNAC1021, TNAC1041, TNAC1071, TNAC1-01, and TNAC1-04, were obtained from 48 pairs of the screening markers (Table 2; Figure 4). As shown in Figure 4, except for 2 $1U^b$-specific bands that were identified in TNAC1071 + *Hae*III (Figure 4A), only one $1U^b$-specific band was observed from each primer + *Taq*I or *Hae*III (Figure 4B-F). These results can be used in further research.

Table 2. Choromosome $1U^b$-specific PLUG markers.

Marker name	Forward sequence/reverse sequence	wEST accession	TIGR ID	Wheat consensus chromosome 1 bin	References
TNAC1021	CTCATGCATGCGTTTGTTAAA CCAGCTGAAACAAGCATCTTC	BU100679	LOC_Os05g23430	C-1L-0.32	Ishikawa et al., 2009
TNAC1041	TCACCACCTCTTTCAGTTGCT GCATCAAGGATGAGGAGTCTG	CK210333	LOC_Os05g42350	1L-0.56-1.00	Ishikawa et al., 2009
TNAC1071	TGGACCTTCTGGTACGACATC CTTCCCACTTTGGTTCTACCC	BT009308	LOC_Os10g32970	1L-0.17-0.47	Ishikawa et al., 2009
TNAC1-01	GGATTTGTACCCATGTGTGT GACTTTTGTGTTCTTTCACGG	BE488529	LOC_Os05g43450	1L-0.47-0.61	Peng et al., 2004 and this study
TNAC1-04	GACATCCACGCGAAGCCATC GACACTAATCATGAGTGGTTG	BF483455	LOC_Os05g09370	1L-0.41-0.47	Peng et al., 2004 and this study

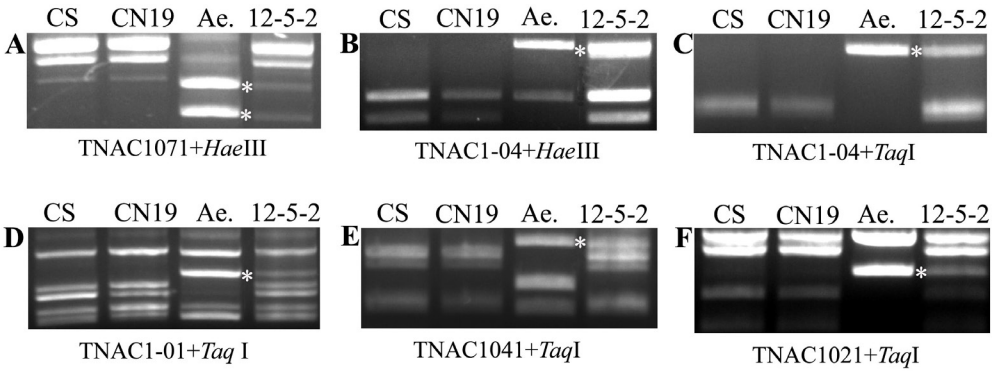

Figure 4. Agarose gel electrophoresis pattern of the PLUG-PCR markers. The chromosome-specific bands were indicated by asterisk. CS = Chinese Spring; Ae. = *Aegilops biuncialis*.

DISCUSSION

The development of wheat-*A. biuncialis* chromosome addition lines allows the study of the genetic effects of individual alien chromosomes in cultivated wheat. In previous studies, 7 wheat-*A. biuncialis* disomic addition lines (2U^b, 2M^b, 3U^b, 3M^b, 5U^b, 6M^b, and 7U^b) were created, and all of these addition lines had reduced fertility compared with that of the parent (Schneider et al., 2005; Schneider and Molnár-Láng, 2012). In this study, a new wheat-*A. biuncialis* disomic addition line 1U^b was created and identified, and the agronomic traits were very close to those of the wheat parent CN19. The addition line 1U^b also has stable fertility and heredity compared with that of the parent, suggesting that the wheat CN19 has compatibility with the donor chromosome 1U^b. Therefore, the addition line 1U^b can be used in wheat breeding directly or as a secondary gene resource.

Very few chromosome-specific molecular markers have been described in *Aegilops* species (Gill et al., 1991a; Zhang et al., 2001; Adonina et al., 2005). As for *A. biuncialis*, only 2 simple sequence repeat (SSR) markers (GWM44 and GDM61) were obtained from 108 wheat SSR markers, which gave specific PCR products from the 2M^b and 3M^b wheat-*A. biuncialis* addition lines (Schneider et al., 2010; Schneider and Molnár-Láng, 2012). In this study, 5 pairs of PLUG markers that were specific to the 1U^b chromosome were obtained from 48 pairs (Table 2; Figure 4). Therefore, it is not difficult to screen and obtain other U^b- and M^b-specific PLUG markers.

Two factors, protein content and protein quality, play a key role in flour bread-making quality. Previous studies indicated that increases in protein content do not always accompany increases in dough strength except for DAL1S^s from *A. searsii* and DAL1E from *Agropyron longatum*. For example, although the DAL1U from *A. umbellulata* contained a large size of x-type HMW-GSs and the protein content was significantly higher than its parent CS, the dough strength was insignificantly different from that of CS (Garg et al., 2009a,b), suggesting that factors such as β-spiral structures of repeat units, conservation of repeats, and interactions between different glutenins and gliadins from different sources may be more important contributors to dough strength. In this study, the protein content, Zeleny sedimentation value, wet gluten content, and grain hardness of 12-5-2 were significantly higher than those of its parent CN19. This indicates a positive effect of gluten proteins from *A. biuncialis* on dough strength.

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