ONLINE RESOURCES

New St-chromosome-specific molecular markers for identifying wheat–*Thinopyrum intermedium* derivative lines

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Introduction

Many grass species in Triticeae serve as important gene pools for forage and cereal crops breeding. Extensive natural and artificial interspecific hybridization have given rise to different ploidy of Triticeae species (Wang *et al.* 1994). The St genome was defined in diploid *Pseudoroegneria* species, and is a donor genome of at least seven genera including *Douglasdeweya*, *Roegneria*, *Elytrigia*, *Thinopyrum*, *Elymus*, *Kengyilia* and *Pascopyrum* according to different taxonomic systems (Wang *et al.* 1994; Yen *et al.* 2005). In the past decades, numerous studies on species containing St-chromosome were focussed on genomic relationship and molecular phylogeny based on molecular markers including chloroplast DNA, high-copy nuclear genes, single-copy nuclear genes, as well as genomic *in situ* hybridization (GISH) (Kellogg and Appels 1995; Mason-Gamer *et al.* 2002; Mason-Gamer 2005). Among these species, *Thinopyrum intermedium* with genome constitution of E_1E_2St (Wang *et al.* 1994) or JJ^sSt (Chen *et al.* 1998) was widely used in wheat breeding programme (Chen 2005; Li and Wang 2009).

Molecular markers, particularly genome-specific markers, are useful in identifying the genome constitution of the unknown species, and also provide efficient tools to check the target alien genes transferred to wheat (Schwarzacher *et al.* 1992; Wang *et al.* 2010). Only a few St-chromosome-specific RFLP (Zhang *et al.* 2001), SCAR (Liu *et al.* 2007) and ISSR (Zeng *et al.* 2008) markers were developed to identify genome composition and the individual St-chromosomes. However, the most available markers are lacking in information on the corresponding homologous linkage group relative to wheat.

Based on the orthologous gene conservation between rice and wheat, Ishikawa *et al.* (2007) reported an array of PCR-based landmark unique gene (PLUG) primers, and later confirmed that the PLUG primers could amplify polymorphism among wheat A, B and D genomes due to the intron polymorphisms (Ishikawa *et al.* 2009). Collinear gene relationships among Triticeae species and sequence polymorphism among different species suggested that PLUG markers could be used to identify homologous group relationships between wheat and alien chromosomes. In this study, we produced St-chromosome-specific PLUG and SCAR markers, and successfully used them to detect the St-chromosome in wheat–*Th. intermedium* introgression lines.

Materials and methods

Plant materials

Wheat line ML13 was provided by International Maize and Wheat Improvement Center (CIMMYT). Wheat lines MY11, CN17, CN18 and Chinese Spring (CS), CS-Imperial rye disomic additions CSDA1R–CSDA7R, are maintained at Triticeae Research Institute, Sichuan Agricultural University, China. *Pseudoroegneria spicata* (PI 232131), *Th. intermedium* (PI 440125), *Th. elongatum* (PI 222959), *Dasypyrum villosum* (KD19801), *Secale montanum* (SCND1377) and *Hordeum bogdanii* (CN41966) were offered by National Plant Germplasm System (NPGS), USA. CS *Th. elongatum* disomic additions CSDA1E–CSDA7E were provided by Dr J. Dvorak, University of California, Davis, USA. CS *D. villosum* disomic additions CSDA1V–CSDA7V, wheat–*Th. intermedium* disomic addition lines Z2 (2St-J^s), Z3 (1St), L4 (4St), L6 (2St) and L7 (6St) were provided by Dr B. Friebe, Kansas State University, Manhattan, USA (Chen 2005). Line Z148 was developed from crossing wheat–*Th. intermedium* ssp. *trichophorum* partial amphiploid with wheat line ML-13. An F_2 population was obtained from cross between Z148 and wheat line MY11.

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Molecular marker analysis

Total genomic DNA was prepared from young leaves using the SDS protocol (Yang *et al.* 2005). The PLUG primers design was according to Ishikawa *et al.* (2009). The PLUG-PCR and SCAR-PCR cycle consisted of an initial 5 min denaturation at 95°C, followed by 32 cycles of 95°C for 45 s, 53–63°C for 45 s, and 72°C for 2 min, followed by a final extension at 72°C for 7 min. DNA amplification was performed using an Icycler thermalcycler (Bio-Rad, Hercules, USA). An 8 μL aliquot of the amplification product was analysed by electrophoresis on a 1% agarose gel in 40 mM Tris-acetate. To obtain high levels of polymorphism, an 8 μL aliquot of the product was digested with *Taq*I for 2 h (65°C), *Hpa*II (37°C), or *Hae*III (37°C), respectively. Digested fragments were fractionated by electrophoresis on 3% agarose gel in TAE buffer. Specific PLUG products were cloned and sequenced.

Fluorescence in situ hybridization

Ps. spicata genomic DNA for GISH, and probe pAs1, a 1-kb DNA fragment isolated from *Aegilops tauschii* for FISH, were labelled with digoxigenin-11-dUTP by nick translation according to the manufacturer's instruction (Roche Diagnostics, Indianapolis, USA). The probe was diluted to a final concentration of 1 μg/mL in the hybridization solution and the hybridization mixture was prepared as described by Mukai *et al.* (1993). The digoxigenin labelled genomic DNA signal was detected with fluorescein-conjugated anti-digoxigenin antibody (Roche Diagnostics). The slide was finally mounted in Vectashield antifade solution (Vector Laboratories, Burlingame, USA) with (0.25 μg/mL) propidium iodide for only FITC detection. Photomicrographs of GISH chromosomes were taken using an Olympus BX-51 microscope (Tokyo, Japan).

Table 1. The features of primers used in this study.

Primer	Homologous relationship	Primer sequence	Annealing temperature	Enzyme	Length of St-specific band (bp)
TNAC1001	1S	F: TTCCCATCTCTTGCCATTAAA R: TTTCCGCTTCCTATGATGCTA	57°C	*Taq*I	650
TNAC1010	1S	F: GATGCAACTGCAGGAATGAAG R: TCTCTTCTGAAGCGGTCATGT	57°C	*Taq*I	840
TNAC1019	1AS, 1BL, 1DL	F: AACGTGTCCACCGTCTACATC R: CCAGTGGTCTCTGATTCATCC	57°C	*Hpa*II/*Hae*III	900/700
TNAC1021	1L	F: CTCATGCATGCGTTTGTTAAA R: CCAGCTGAAACAAGCATCTTC	57°C	*Taq*I/*Hpa*II	1240/700
TNAC1026	1L	F: GGGATAGAACTCTGGGACTTCA R: AGTGCCAGGGCATAATACAGC	57°C	*Taq*I/*Hpa*II	700/700
TNAC1038	1L	F: CCACCAGCTTTCCTTACCATA R: ACTGCTCAATCCAACTGGAAA	57°C	*Taq*I/*Hpa*II	700/900
TNAC1085	1L	F: CCAGGCCACATGATAACATTC R: TCATGGTATTCTGCTCCTCCA	57°C	*Taq*I/*Hpa*II/*Hae*III	950/800/1000
TNAC1088	1L	F: GGAATCCTTCCTTGTTGAAGA R: AACCTCCGAGTGAAACACAAA	60°C	*Taq*I/*Hpa*II/*Hae*III	1100/900/800
TNAC1102	2S	F: GGAGAGGTGAAGGACCAACTC R: CCTTGCAGCGTAGTGAGATTT	60°C	*Taq*I/*Hae*III	1000/800
TNAC1176	2S	F: CTTCATGGTTGCTCACGAACT R: CATGCGAAATTTGCTATCCTT	60°C	*Taq*I /*Hae*III	1000/800
TNAC1178	2S	F: TGATACCGAGGCTATCCACAT R: ACATGAACAAGGATCATGCTG	60°C	*Taq*I /*Hae*III	1000/800
TNAC1182	2S	F: CCTTCGTGCTAAGCTCACATT R: CTCACCCACCTGTATGGATTG	60°C	*Hpa*II	950
TNAC1204	2L	F: GAGAGGAATGCGTGAAGTTTG R: AGACCATCTTTCCGGTCTTTG	60°C	*Hae*III	500
TNAC1248	3S	F: ATGATGCAGCAGCAAATTACA R: CTGAGGAGCCTCTCCAACTCT	60°C	*Taq*I	850
TNAC1300	3S	F: TCTGCAGGTTCGGTAGACAAT R: AGTACGGGAGGACGCATGT	60°C	*Taq*I/*Hpa*II	840
TNAC1263	3L	F: TTGAGAAGTACCGCAAGGATG R: CATCCACATCACTGTCACTGTC	60°C	*Taq*I	800
TNAC1383	3L	F: GCGGTCGATCTTCTTCAAGTC R: TCAGATGGACTATGGGAGCAC	60°C	*Taq*I/*Hae*III	1200
TNAC1408	4AS, 4BL, 4DL	F: CAGGAAGTTGGTACCATTGTGA R: CTTGCAGCCTCCTATTGATTC	60°C	*Taq*I	1000
TNAC1663	4AL, 4BS, 4DS	F: CAGATAGACCGGGTGGAATTT R: CGAGGTCTACGTCTTCGAGTC	60°C	*Taq*I/*Hpa*II/*Hae*III	350
TNAC1485	5S	F: CCCAAGTTCACTAACTTCGTTG R: AAATAGTCCTGCATATCTCCTGT	60°C	*Taq*I/*Hae*III	800

Results

St-chromosome-specific PLUG markers

A total of 46 pairs of PLUG primers from both short and long arms of seven homologous groups of wheat chromosomes were used to amplify the genomic DNA of CS and *Ps. spicata*. A total of 39 primer pairs could amplify polymorphism fragments from *Ps. spicata* compared to CS (table 1). The nullisomic–tetrasomic lines of CS and several wheat St-chromosome addition lines were used to localize the polymorphic fragments. As shown in figure 1, three bands with different sizes were amplified from wheat A, B and D chromosomes indicated by the series of CS nullisomic–tetrasomic lines. The results were consistent with those of Ishikawa *et al.* (2009). The additional polymorphic fragments amplified by the primers were putatively located on individual St-chromosomes of the same homologous group, since they represented the syntenic region with different sequences between wheat and rice. A total of 8, 5, 4, 2, 8, 5 and 7 St-chromosome-specific markers were assigned to the homologous groups 1 to 7, respectively (table 1). The distribution of the PLUG markers for St-chromosome-specific amplification with known corresponding rice homology are provided in table 2. The *Th. intermedium* wheat

Table 1 (*contd*).

Primer	Homologous relationship	Primer sequence	Annealing temperature	Enzyme	Length of St-specific band (bp)
TNAC1497	5S	F: ATCAAACCTGACGGTGTTCAG R: CATGCAGACTACAGGTCCAGA	60°C	*Taq*I	700
TNAC1503	5S	F: TGAGGTTGGTTCTCATCTGGA R: CGTTGGAAACAATCTGAATGG	60°C	*Taq*I/*Hae*III	700/700
TNAC1540	5L	F: AACCTCAAGCACTGTCAGCAT R: TTGCAGATCCTCTCAATCTCG	60°C	*Hae*III	800
TNAC1554	5L	F: TTGCTAGCTCAGCACAGTTTG R: TTCTTGGTCACTCTGAGCGTA	60°C	*Hae*III	900
TNAC1559	5L	F: AAACAAGGCCCTGAAACACTT R: CATTGTCAGGCTATGGGACAT	60°C	*Hae*III	1100
TNAC1614	5L	F: AAACAGGGCTTTCAGCTTCTC R: ATCAAGCAGAACAACCTCCAG	60°C	*Taq*I/*Hpa*II/*Hae*III	900/750/950
TNAC1616	5L	F: AGTCAAATTGCCATGTCAACAG R: ACATCACCGCAGTAGGATTTG	60°C	*Taq*I	1100
TNAC1674	6S	F: CCACCACAGAAGCAGATGAAT R: GCTAGATGGCACACCAAGTG	60°C	*Taq*I	1000
TNAC1685	6S	F: ATGGATAGCGGAAGCGACTC R: AGCGTTTCCTCCGGTCTT	60°C	*Taq*I /*Hpa*II/*Hae*III	900/700/650
TNAC1702	6L	F: CATGGAAAGGTTGACAAGGAA R: CTGGATGTTCCATTTCTGCTC	62°C	–	900
TNAC1752	6L	F: GTAGACGATGTCGAGGAGCAT R: CTTCACCAATTTCTCCCATGA	60°C	*Taq*I/*Hpa*II/*Hae*III	650/800/700
TNAC1763	6L	F: CGATTGGCCGTACAACTTTC R: TTGATGACGTTGAAGGGTCTC	60°C	*Taq*I/*Hae*III	700/500
TNAC1805	7S	F: TTCTTTGCTGGTCGTTTCTTG R: CCACGGTAATGTAAGGCACAT	60°C	*Hae*III	400
TNAC1806	7S	F: ATTCCTCGTGAATTGCTGGAT R: TCTGCAGTTAGGGACTTGAAA	60°C	*Taq*I	400
TNAC1926	7S	F: CGTCAGCTACAGCGACATCTA R: AACTTGAGCAGCGTGGTGTT	62°C	–	1400
TNAC1825	7AL, 7BL, 7DL	F: GAAGCGGTTCAGGGTGAC R: ACCCATCACGTTGCTGTAGTC	60°C	*Hpa*II	300
TNAC1867	7AL, 7BL, 7DL	F: GCCTTTCCTTTGGTAGTCTGG R: CGATCCAAATGATCCTGAAGA	60°C	*Hae*III	680
TNAC1903	7L	F: TCGCTTCTTCTGCTTGTTCTT R: CTGCTACTAGGCCACCCAAA	60°C	*Taq*I/*Hpa*II/*Hae*III	500/350/600
TNAC1957	7L	F: TCAACATTTGCAGGATTGTCA R: TTTCACAGGAACCTCTGCATC	62°C	–	800
SCAR_1021	1St	F: AGGTTTGAGGAAAACCAGTA R: CCAGCTGAAACAAGCATCTTC	55°C	–	750
SCAR-1233	2St	F: CGGTGGGATCTATGAAATTA R: ATACCTCCTGAGCTTGGTCCT	55°C	–	850
SCAR-1248	3St	F: TTAGACATCATGAGCACACC R: ATGATGCAGCAGCAAATTACA	55°C	–	850

–, Enzyme digestion is unnecessary for these primers.

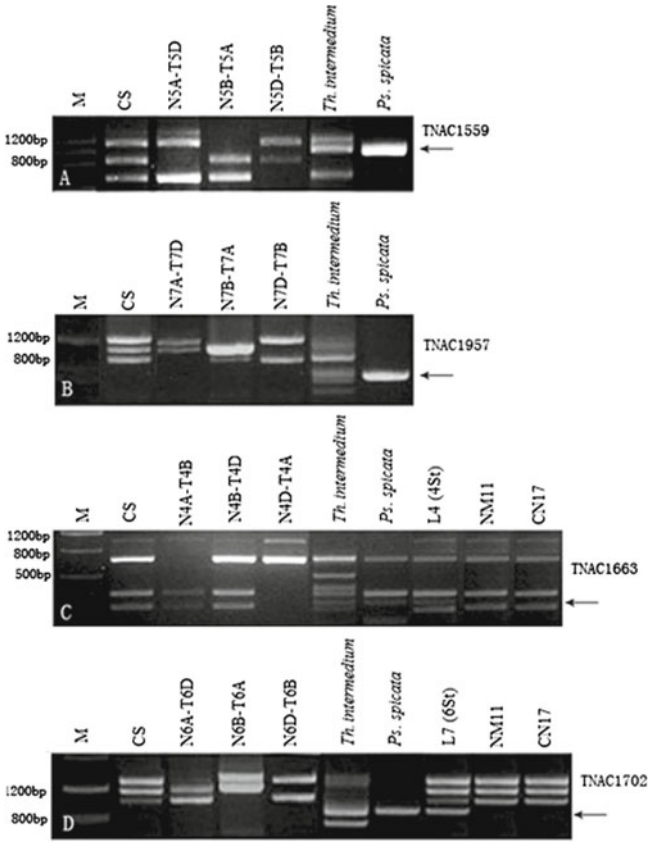

Figure 1. PCR patterns of amplification with the PLUG markers of (A) TNAC1559, (B) TNAC1957, (C) TNAC1663 and (D) TNAC1702. Arrows indicate the St-specific band, and M, marker (DL4500).

addition lines Z2, Z3, L4, L6 and L7 were used to amplify the PLUG primers. The results showed clearly that the alien St-chromosome of Z2, Z3, L4, L6 and L7 can be assigned to homologous groups 2, 1, 4, 2 and 6 respectively (table 2).

Table 2. Number of polymorphic PLUG markers with respect to the homologous group of rice and wheat genomes, on wheat–*Thinopyrum* addition or substitution lines.

Rice group	Wheat group	*Ps. spicata*	Wheat–*Thinopyrum* chromosome addition line					
			Z2	Z3	L4	L6	L7	Z148
5	1	6		6				6
10	1	2		2				2
4	2	5	5			5		
7	2	3	1			1		
1	3	4	1			1		
3	4	2			2			
11	4	2						
9	5	2						
12	5	3						
2	6	5		1			5	1
6	7	4						
8	7	2						

They are consistent with the identification by other methods (Chen 2005).

St-chromosome-specific SCAR markers

Primers TNAC1021, TNAC1233 and TNAC1248 were localized on wheat homologous groups 1, 2 and 3, respectively. However, they amplified the bands with identical size between CS and *Ps. spicata*. These bands were recovered, cloned and sequenced. Sequence alignment results showed that there were several unique regions in genomic DNA of *Ps. spicata* when compared with that of wheat. Three pairs of specific primers, SCAR-1021F/R, SCAR-1233F/R and SCAR-1248F/R (table 1), were designed based on *Ps. spicata* unique sequences. All three pairs of primers could amplify polymorphic products among materials containing St-chromosome(s) and non-St-chromosome materials (CS and nullisomic–tetrasomic lines of CS), indicating that the polymorphic bands could be used as SCAR markers for detecting chromatin of *Ps. spicata* in wheat background. Using St-chromosome addition lines as materials, primers SCAR-1021F/R amplified the specific bands in wheat–*Th. intermedium* addition Z3 (1St), suggesting that SCAR-1021 was 1St-chromosome-specific marker. Meanwhile, SCAR-1233-F/R amplified the specific bands from addition lines Z2 (2St-J^s) and L6 (2St), indicating that SCAR-1233 was 2St-chromosome-specific marker. Because no 3St-chromosome addition line was collected in this study, SCAR-1248-F and SCAR-1248-R were used to amplify CSDA1E–CSDA7E, CSDA1V–CSDA7V and CSDA1R–CSDA7R, the result showed that only CSDA3E, CSDA3V and CSDA3R could amplify polymorphism (figures not shown), and we concluded that SCAR-1248 should be 3St-chromosome-specific marker.

St-specific markers for wheat–Th. intermedium introgression lines

An introgression line Z148 was selected from the progeny of hybrid between wheat and wheat–*Th. intermedium* ssp. *trichorophrum* partial amphiploid (Yang *et al.* 2006). The chromosome constitution of Z148 was determined using the specific markers combined with GISH using *Ps. spicata* as probe. The results revealed that Z148 contains 42 chromosomes with a pair of St-chromosomes (figure 2A). Primers TNAC1001 and TNAC1021 amplified 1St-chromosome-specific bands from Z148, suggesting that Z148 contained a pair of 1St-chromosomes. However, the chromosome 1D specific bands of TNAC1001 and TNAC1021 were absent, indicating that the Z148 lost chromosomes 1D (figure 2B). FISH analysis using pAs1 as probe also showed that Z148 lost chromosome 1D (data not shown). It showed that Z148 is a 1St (1D) substitution line. The results also indicate that the identification of St-chromosomes by St-specific markers was in accordance with that by GISH and FISH methods. Based on the amplification products of the F_2 plants of

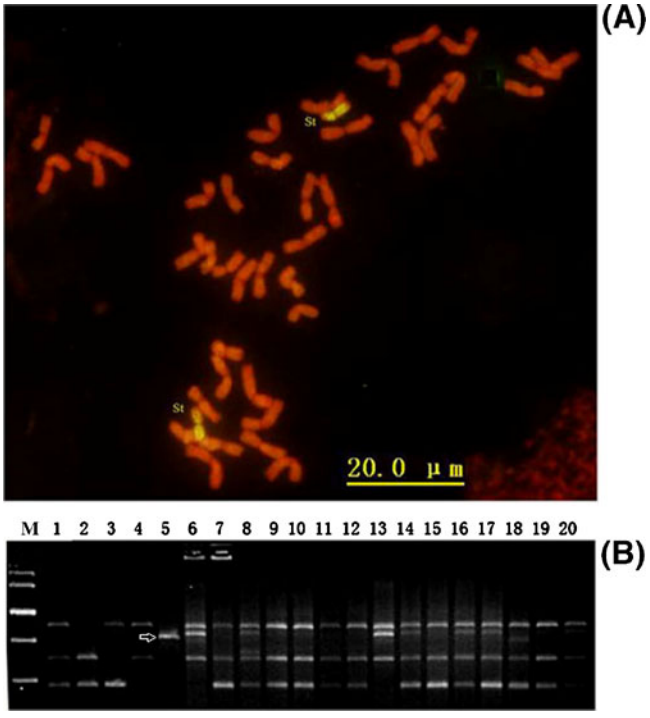

Figure 2. GISH analysis of Z148 with St genome as probe and CS DNA as block (A). PCR amplification in F_2 plants from Z148/MY11 by PLUG primer TNAC1021 (B). 1, CS; 2, N1A-T1D; 3, N1B-T1A; 4, N1D-T1B; 5, *Ps. spicata*; 6, Z148; 7–20, F_2 from Z148/MY11, arrow indicates the 1St-specific band.

hybrid between Z148 and wheat line MY11 (figure 2B) using the specific markers, we found that the target bands can be easily reproduced in the population, which can further identify the substitution and Robertsonian translocation between chromosomes 1St and 1D. At present, we are screening more progenies from wheat–*Th. intermedium* ssp. *trichorophrum* partial amphiploid using the St-chromosome-specific markers.

Discussion

More than 70% of the perennial wild species in Triticeae contain one or more St genome(s), and many of these St-chromosome-containing Triticeae species include allopolyploid combinations with genomes H from *Hordeum*, Y from an unknown donor (Okito *et al.* 2009), P from *Agropyron*, and W from *Australopyrum* (Wang *et al.* 1994). It appears that the St genome is perhaps the core genome of perennial Triticeae grasses (Redinbaugh *et al.* 2000; Arterburn *et al.* 2011). Unlike the other diploid Triticeae species, the diploid St-genome species were not reported to have hybridized to common wheat, so that a complete set of wheat–St-chromosome addition or substitution lines were unavailable. Fortunately, the polyploid St-containing species, like *Th. intermedium*, had been successfully hybridized with wheat. It is thus feasible to transfer the St-genome chromosomes into common wheat using *Th. intermedium* as bridge. GISH analysis probed by *Ps. spicata* genome can be used to detect wheat–*Th. intermedium* substitution or addition lines. Several different St-chromosomes from *Th. intermedium* were transferred to wheat background (Chen 2005; Li and Wang 2009). In the present study, the GISH result (figure 2) also indicated that the produced wheat line Z418 is a substitution line containing a pair of St-genome chromosomes.

Development of St-specific molecular markers allows fast identification of the alien chromosomes in wheat–alien introgression lines carrying St-chromosomes. Molecular markers based on conserved homologous regions from the A, B and D genomes will clearly aid in sequence comparisons of homologous regions of Triticeae genomes (Wang *et al.* 2010). Recently, we detected the sequence variations of *PDHA1* gene in Triticeae species by PLUG primer TNAC1102, and located the conserved homologous regions from linkage group 2 of the A, B and D genomes to corresponding chromosomes 2N from *Ae. ventricosa*, 2V from *D. villosum* and 2R from *S. cereale* (Jia *et al.* 2010). Meanwhile, the PLUG markers with wheat chromosome deletion bin-mapping data will be efficient and easy for the identifying orthologous loci not only across Triticeae genomes but also in Poaceae genomes (Ishikawa *et al.* 2009). In the present study, we obtained 39 pairs of PLUG markers located on homologous groups 1 to 7, and three SCAR primers to identify the St-chromosomes 1St, 2St and 3St. To make sure the St-chromosome-specific markers were consistent to homologous groups, the wheat–*Th. intermedium* addition lines and substitution lines with St-chromosomes were used to test the PLUG markers. The wheat–*Th. intermedium* addition lines Z2 (2St-J^s), Z3 (1St), L4 (4St), L6 (2St) and L7 (6St) were tested (table 2). The results showed that each homologous group marker could be located in the addition line, it supported that our primers could amplify the St-chromosome-specific band of different homologous groups. The molecular results were consistent with those of the *in situ* hybridization using St-genomic DNA as probe. PLUG markers not only distinguish the chromosome group for the St genome but also show presence or absence of the A, B, D genome in wheat. Therefore, they could be widely used in molecular-marker-assisted breeding, comparative gene mapping, chromosome tracing, taxonomic studies and genotypic identification.

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