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Whole genome development of intron targeting (IT) markers specific for *Dasypyrum villosum* chromosomes based on next-generation sequencing technology

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Abstract *Dasypyrum villosum* (Dv), a wild relative of wheat, is an important and useful gene resource for wheat improvement. A large number of wheat-Dv aneuploid lines harboring whole or fragments of Dv chromosomes have been developed. However, the lack of sufficient molecular markers hindered accurate identification of Dv chromatin, especially when the introgressed fragments are small. Development of molecular markers covering the whole Dv genome and evenly distributed on different chromosome regions is not only useful for the detection of the introgressed alien chromatin in wheat background, but also provides evidence of the syntenic relationship between homoeologous chromosomes. In the present study, in order to develop high density and evenly distributed molecular markers on individual Dv chromosomes, genomic DNA of Dv leaves was sequenced and assembled. Sequence assemblies of all wheat chromosomes were first used to identify exon–exon junctions and localize introns in Dv. Intron length polymorphisms suitable for designing Dv primers flanking introns were evaluated, and a total of 1624 intron targeting (IT) markers was designed. By using the Chinese Spring, the *Triticum durum*-Dv amphiploid and the Dv sequenced DNA libraries, 841 IT molecular markers specific for Dv chromosomes were developed, with maximum efficiency up to 51.79%. We assigned the 841 IT markers to seven Dv chromosomes (1V–7V) using seven wheat–Dv chromosome addition and substitution lines: 135 to 1V, 175 to 2V, 120 to 3V, 89 to 4V, 140 to 5V, 71 to 6V, and 111 to 7V, respectively. Using *T. aestivum*-Dv telosomic and whole arm translocation lines, they were further located on the short or long chromosome arms. These specific markers for individual chromosomes of Dv provided efficient tools for the characterization of structural variation involving the individual chromosome of Dv, as well as for the selection of useful genes located on individual Dv chromosome in breeding programs.

Keywords *Triticum aestivum* L. · *Dasypyrum villosum* L. · Intron-targeting markers · Alien chromosome line · Next-generation sequencing

Key message The V-genome specific IT markers were mined to facilitate the identification and utilization of Dv chromatin.

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Introduction

Dasypyrum villosum (L.) (Candargy, Dv; 2*n* = 14, VV) (L.) Schur [syn. *Haynaldia villosa* (L.) Schur], a wild relative of wheat, has been used to introgress genes that confer resistances to powdery mildew, rusts, take-all, eyespot, wheat spindle streak mosaic virus (WSSMV),

tolerances to drought and frost, good tiller ability, and high grain protein content (Blanco et al. 1987; Murray et al. 1994; Chen et al. 1995; Grądzielewska 2006; De Pace et al. 2011). A large amount of common wheat-Dv cytogenetic materials, including amphiploid, disomic and ditelosomic addition lines, and disomic substitution lines, as well as translocation lines that display the expression of those useful genes have been developed (Grądzielewska 2006; De Pace et al. 2011). For example, the translocation line T6VS·6AL has both powdery mildew (*Pm21* on 6VS) and stripe rust (*Yr26* on 1B introduced from *Triticum turgidum*) resistance. The introduction of 6VS (and corresponding absence of 6AS) has no significant negative effect on yield or quality traits (Chen et al. 1995; Li et al. 2005b, 2007), and T6VS•6AL lines are widely used as parents in wheat breeding programs. More genes have been transferred into wheat by the development of alien translocation lines, including wheat yellow mosaic (WYM) resistance gene *Wss1* (Zhang et al. 2005; Zhao et al. 2013), stem rust resistance gene *Sr52* (Qi et al. 2011), grain softness genes (Zhang et al. 2010, 2012), seed storage protein genes (Zhao et al. 2010; Vaccino et al. 2010; Zhang et al. 2014), yield-related genes (Zhang et al. 2015), the powdery mildew resistance gene *Pm55* (Zhang et al. 2016b) and the cereal cyst nematode resistance gene *CreV* (Zhang et al. 2016a). Such wheat lines represent excellent resources, and greatly enrich the genetic basis of wheat.

To improve the efficiency of gene transfer from Dv to wheat, it is necessary and important to develop molecular markers linked to useful genes for accelerating wheat plant breeding by marker-assisted selection (MAS). The development of a large number of molecular markers specific for Dv will help to accurately trace the introduced alien chromatin carrying useful alien genes. Although it is a diploid genome, the outcrossing breeding system of Dv makes its genome very complex. Several different types of Dv chromosome-specific molecular markers have been reported, including random amplified polymorphic DNAs (RAPD) (Qi et al. 1996; Li et al. 2005a), restriction fragment length polymorphism (RFLP) (Qi et al. 1996; Li et al. 2005a), simple sequence repeat (SSR) (Zhang et al. 2006), sequence characterized amplified region (SCAR) (Liu et al. 1999; Cao et al. 2006; Chen et al. 2006), conserved-intron scanning primers (CISPs) (He et al. 2013), and expressed sequence tags (EST-PCR) (Wang et al. 2007; Cao et al. 2009; Chen and Chen 2010; Zhao et al. 2012; Zhang et al. 2012; Chen et al. 2013; He et al. 2013; Zhao et al. 2014; Zhang et al. 2015; Bie et al. 2015; Zhang et al. 2016a). However, the number of molecular markers specific for Dv chromosomes is still very limited. Therefore, there is an urgent need to mine more molecular markers, especially those having a wider genomic distribution and amenable to high throughput genotyping, in order to identify alien introgression and translocation lines and also aid molecular genetics and breeding studies involving Dv.

Introns are an attractive source of polymorphism for marker development because insertions/deletions and base substitutions are more common within intron than within exon sequences (Kimura 1983). Intron length polymorphism has been considered as a convenient and reliable source of informative markers with high interspecies transferability (Poczai et al. 2013). Based on the orthologous gene conservation between rice and wheat (Ishikawa et al. 2007), a set of PCR-based landmark unique gene (PLUG) primer pairs were designed; subsequent analysis showed that PLUG markers are suitable not only for detecting the polymorphisms among wheat A, B and D genomes (Ishikawa et al. 2009), but also for identifying the homology between wheat and alien chromosomes. Thus, they are useful in MAS, comparative genomics, alien chromosome tracing, taxonomic studies and genotyping (Poczai et al. 2010; Li et al. 2013; Hu et al. 2012; Zhan et al. 2013).

Next-generation high-throughput DNA sequencing techniques (NGS) have opened opportunities for sequencing an entire plant genome at low cost, allowing fast characterization of genome structure at high resolution and detailed comparative study across species (Egan et al. 2012). All wheat chromosomes have been sequenced using NGS and their gene contents has been determined (International Wheat Genome Sequencing Consortium 2014). These new genomic resources have opened an avenue for efficient high-throughput marker development, high-density mapping, gene cloning, comparative genomics, functional studies and phylogenetic analyses.

In this paper, the Dv genome was sequenced and assembled to develop more specific gene markers. Based on blasting against the genome sequences of *Triticum aestivum* cv Chinese Spring (Poczai et al. 2013), intron length polymorphisms were identified and chromosome-specific molecular markers for Dv were successfully developed and allocated on specific Dv chromosome arms.

Materials and methods

Plant materials

Triticum durum-Dv amphiploid (AABBVV), *T. aestivum*-Dv disomic addition lines [DA1V, 3V–7V, in a Chinese Spring (CS) background] and *T. aestivum* -Dv disomic substitution line DS2V (in a Xiangmai1 background), *T. aestivum* -Dv translocation lines involving different Dv chromosome arms, including T1VL·1DS(CS), T1VS·1DL(CS), T2VL·2DS(CS), T2VS·2DL(CS), T4VL·4DS(CS), T4VS·4DL(CS), T5VS·5DL(CS), T6VL·6AS(CS) and T6VS·6AL(Yangmai 5) were developed by the Cytogenetics Institute, Nanjing Agricultural University (hereafter CINAU). The Dv (accession No. 91C43) was introduced from Cambridge Botanical Garden (Cambridge, UK).

NGS and assembling of Dv

About 24 μg genomic DNA from young leaves of Dv was used to create the eight shotgun DNA-seq libraries with 626–636 bp and 311–321 bp inserted sizes, respectively. The libraries were sequenced in a single lane of Illumina HiSeq 2500 platform. De novo assembly of the Illumina paired-end reads was performed using the software SOAPdenovo (version 2.0) using k-mer size of 31, which provided the assembly with the best sequence coverage and N50 size to generate Dv genome scaffolds.

Sequence resources for primer design

DNA sequences used in this work included the Dv assembly (SRP077844), assemblies and annotated genes of 21 chromosomes of CS (*T. aestivum* Ensembl plants, Release 30, http://plants.ensembl.org/index.html).

Homology and alignment analysis

The flowchart for designing the Dv chromosome-specific markers is shown in Fig. S1. In the first step, we chose a set of annotated genes on the subgenomic D genome of CS to calculate the exon–exon junction sizes in the genomic sequences of individual homoeologous groups of wheat chromosomes (WHG1–WHG7) and 1V–7V chromosomes of Dv, respectively. In the second step, all genes were compared with the genomic sequences of individual homoeologous groups of wheat chromosomes (WHG1–WHG7) and chromosomes 1V–7V of Dv using a local Blastn program, respectively. All genes matching individual homoeologous groups of wheat chromosomes (WHG1 to WHG7) and appropriate Dv 1V–7V assemblies and possessing at least one predicted exon–exon junction were selected. Intron sizes of the corresponding genes were then calculated and compared against each other. Genes whose intron sizes in Dv 1V–7V chromosomes differed by at least 10% from those in corresponding allele of individual wheat homoeologous groups (WHG1–WHG7) were chosen for marker design, respectively. The primer pairs were designed in the exons flanking the targeted introns.

Intron targeting primer design

Intron targeting (IT) primers were designed in the exon regions flanking a targeted intron using the online software Primer 3 (version 4.0, http://frodo.wi.mit.edu/primer3//primer3/). The designed primers were expected to amplify the genomic DNA both in common wheat and Dv. The PCR setting for these primers is: melting temperature 55–65 °C (optimum: 60 °C), primer length 18–25 bp (optimum: 20), the estimated sizes of the amplified fragments were approximately 50 bp more than the targeted introns. All primers were synthesized by Shanghai Invitrogen Biotechnology Company (Shanghai, China).

DNA extraction and PCR

Genomic DNA was extracted from 2 g fresh leaves of plants at three-leaf stage with the SDS-phenol-chloroform method according to Sharp et al. (1989) and Devos et al. (1992) and purified to eliminate RNA, amylase and other unwanted compounds. The DNA purity and concentration was assessed with microplate reader (M200, TECan, Männedorf, Switzerland). Each DNA sample was finally diluted to approximately 50 ng/μl and stored at −20 °C until use.

PCR amplification was carried out in a 10-μl reaction volume containing 40 ng genomic DNA, 2 μM each of the primer pairs, 2.5 mM each dNTPs, 2.5 mM $MgCl_2$, 1 × PCR buffer (10 mM Tris-HCl, pH 8.5, 50 mM KCl), and 0.5 U *Taq* DNA polymerase with a PTC-200 thermal cycler (Bio-Rad, Hercules, CA). The amplification

was conducted at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, annealing of different primers at 50, 55, or 60 °C for 50 s at a ramp rate of 0.5 °C/s, 72 °C for 1 min 10 s, and a final extension at 72 °C for 10 min. PCR products were resolved in 8% non-denaturing polyacrylamide gels (Acr: Bis = 19: 1 or 39: 1) and the band patterns were visualized by silver staining (Bassam and Gresshoff 2007).

Results

Amplification patterns of different IT markers

In our previous study, we developed IT markers specific for chromosome arms 4VS and 6VS of Dv by chromosome sorting and NGS. In the present paper, we focused on the remaining arms of Dv chromosomes. A total of 1624 primer pairs was designed, including 265 for 1V, 332 for 2V, 211 for 3V, 151 for 4VL, 328 for 5V, 138 for 6VL and 199 for 7V (Table 1). They were used for amplification in CS, Dv and *T. durum*-Dv amphiploid. The results indicated that the majority (97.84%) of these primer pairs were suitable for producing PCR amplicons in all the three materials. Only 841 primer pairs (51.79%) amplified specific polymorphic fragments that were common in Dv and *T. durum*-Dv amphiploid, indicating they were specific for the genome of Dv. We also found that 35 primer pairs had no amplicons in any of the three materials.

Table 1 Number of intron targeting (IT) markers obtained by PCR amplification of the nuclear DNA of CS, Dv, *Triticum durum*-Dv amphiploid, using primer pairs specific for introns of the Dv chromosome arms

Chromosome/ chromosome arm	Number of markers	Number of markers having amplicons in the genomic DNA template of Chinese Spring (CS), Dv and *T. durum*-Dv amphiploid	Number of markers having no amplicons in the genomic DNA template of Chinese Spring (CS), Dv and *T. durum*-Dv amphiploid
1VL	180	170	10
1VS	85	85	0
2VL	200	191	1
2VS	132	129	3
3VL	140	138	2
3VS	71	71	0
4VL	151	148	3
5VL	246	241	5
5VS	82	81	1
6VL	138	134	4
7VL	105	102	3
7VS	94	91	3
Total number	1624	1589	35

Allocation of IT markers to specific Dv chromosomes

To allocate specific markers on Dv chromosomes, the 841 primer pairs were used for amplification in different wheat-Dv addition/substitution lines involving different Dv chromosomes. If a primer pair amplified a distinct PCR product in Dv, *T. durum*-Dv amphiploid and an alien chromosome line involving one of the Dv chromosomes, while not in *T. durum* and CS, it might serve as a specific marker for this alien chromosome. To summarize, we assigned 135, 175, 120, 89, 140, 71, and 111 IT specific markers to 1V, 2V, 3V, 4V, 5V, 6V and 7V, respectively (Tables S1, S2).

Some of the amplification results for the 21 primer pairs are shown in Fig. 1. The representative three types of IT markers corresponding to the sequence information in subgenome AA, BB, DD and VV, the sequences of forward and reverse primers, and the intron sizes are given in Tables 2 and 3, respectively. For example, as shown in Fig. 1a, primer CINAU954 produced three bands with different sizes in Chinese Spring (AABBDD), presumably on AA, DD and BB, respectively, according to the predicted amplified size of the flanking intron. The primers also produced a band in Dv (VV) that has different size (500 bp) from those in CS, indicating this band is V genome specific. When amplified in *T. durum*-Dv amphiploid (AABBVV), CS-Dv addition/substitution lines (DA1V, DA3V–7V and DS2V), the primer pair produced the same band in DA1V and *T. durum*-Dv amphiploid as that in Dv (VV); thus, we assigned this 500 bp band to chromosome 1V.

We classified the developed IT markers into different types based on the observed two, three or four band patterns on the polyacrylamide gel. Type I markers evidenced four bands, three of which each contained an amplicon from one of the orthologous introns in the three chromosomes of the same homoeologous group of CS (WHG1 to WHG7), and the fourth band containing

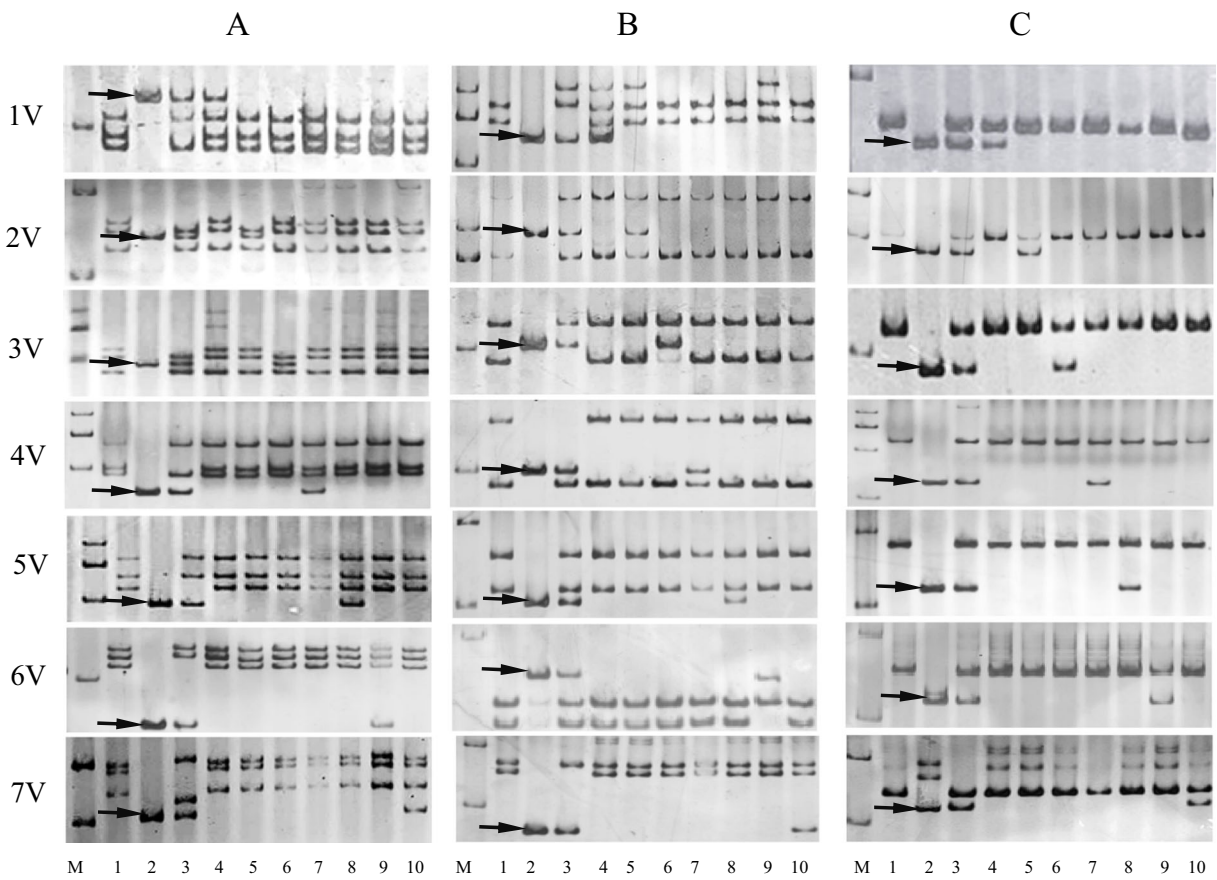

Fig. 1a–c Bands in non-denaturing 8% polyacrylamide gels electrophoresis evidencing PCR products of intron targeting (IT) markers specific for individual chromosome of Dv. Lanes: *M* Marker, *1* Chinese Spring (CS), *2* Dv, *3 T. aestivum*-Dv amphiploid, *4* DA1V, *5* DS2V, *6* DA3V, *7* DA4V, *8* DA5V, *9* DA6V, *10* DA7V. **a** The amplification of representative Type I IT markers (CINAU954, CINAU1068, CINAU1205, CINAU1331, CINAU1454, CINAU1532, CINAU1566) specific for Dv chromosome of 1V, 2V, 3V, 4V, 5V, 6V and 7V from top to bottom, respectively. **b** The amplification of representative Type II IT markers (CINAU862, CINAU1010, CINAU1162, CINAU1325, CINAU1459, CINAU1542, CINAU1661) specific for Dv chromosome of 1V, 2V, 3V, 4V, 5V, 6V and 7V from top to bottom, respectively. **c** The amplification of representative Type III IT markers (CINAU893, CINAU1067, CINAU1161, CINAU1295, CINAU1474, CINAU1517, CINAU1608) specific for Dv chromosome of 1V, 2V, 3V, 4V, 5V, 6V and 7V from top to bottom, respectively. *Arrows* Amplified bands specific for Dv individual chromosomes

an amplicon from the orthologous intron in the Dv-chromosome syntenic to that wheat homoeologous group. For example, CINAU954 is 1V specific, and amplified fragments specific for 1V-, 1A- 1B- and 1D- simultaneously (Fig. 1). These markers are co-dominant, and are useful to simultaneously trace the alien Dv chromosome and its wheat homoeologous group chromosomes.

Type II markers evidenced three bands: one band containing amplicons of the same size from orthologous introns (Tables 3, S3, S4) in any of two chromosomes of the same wheat homoeologous group, the second band containing the amplicon from the third chromosome of that homoeologous group, and the third band containing an amplicon from the orthologous intron in the Dv-chromosome syntenic to that wheat homoeologous group. For example, CINAU1010 is specific for 2V and amplified fragments specific for 2V-, and any two of wheat chromosomes 2A- 2B- or 2D- (Fig. 1).

Type III markers evidenced two bands: one band containing amplicons of the same size from introns (Tables 3, S3, S4) on three homoeologous chromosomes of the same wheat homoeologous group, and the second band containing an amplicon from a Dv-chromosome corresponding to that wheat homoeologous group. For example, CINAU1161 is specific for 3V and amplified

Table 2 Catalog of some of the Type I, II, and III markers displaying nucleotide sequence homoeology to sequence scaffolds in the chromosomes of the A, B, D, and V genomes. CS Chinese Spring

Chromosome	Marker no.	Scaffold of A subgenome of CS	Scaffold of B subgenome of CS	Scaffold of D subgenome of CS	Scaffold of Dv	Type
1V	CINAU954	1A	1B	1D	C305877432	TypeI
	CINAU862	1A	1B	IWGSC_CSS_1DL_scaff_2267931	C305885216	TypeII
	CINAU893	1A	1B	1D	C305878460	TypeIII
2V	CINAU1068	2A	IWGSC_CSS_2BL_scaff_7952142	2D	C304433822	TypeI
	CINAU1010	IWGSC_CSS_2AL_scaff_6389182	2B	2D	C305880242	TypeII
	CINAU1067	2A	IWGSC_CSS_2BL_scaff_7935396	2D	C304590456	TypeIII
3V	CINAU1205	3A	3B	3D	C305884212	TypeI
	CINAU1162	3A	3B	IWGSC_CSS_3DL_scaff_6953496	C305302174	TypeII
	CINAU1161	3A	3B	IWGSC_CSS_3DL_scaff_6953491	C305881762	TypeIII
4V	CINAU1331	4A	4B	4D	C305821372	TypeI
	CINAU1325	4A	4B	4D	C304069526	TypeII
	CINAU1295	4A	4B	IWGSC_CSS_4DL_scaff_14442531	C305863640	TypeIII
5V	CINAU1454	5A	5B	IWGSC_CSS_5DS_scaff_2741529	C305873938	TypeI
	CINAU1459	IWGSC_CSS_5AS_scaff_1131244	5B	IWGSC_CSS_5DS_scaff_2767297	C304112234	TypeII
	CINAU1474	IWGSC_CSS_5AS_scaff_1524785	5B	5D	C305649954	TypeIII
6V	CINAU1532	6A	IWGSC_CSS_6BL_scaff_1790846	6D	C305818034	TypeI
	CINAU1542	6A	IWGSC_CSS_6BL_scaff_4369299	6D	C305884056	TypeII
	CINAU1517	IWGSC_CSS_6AL_scaff_5828160	IWGSC_CSS_6BL_scaff_4218867	6D	C305694850	TypeIII
7V	CINAU1566	IWGSC_CSS_7AL_scaff_4550761	7B	IWGSC_CSS_7DL_scaff_41304	C305342276	TypeI
	CINAU1661	7A	7B	7D	C305815232	TypeII
	CINAU1608	IWGSC_CSS_7AL_scaff_4398763	7B	7D	C305832338	TypeIII

Table 3 Sequences of forward and reverse primers for identifying Type I, II, and III IT markers for syntenic introns in the homoeologous chromosomes of the A, B, D, and V genomes

Chromosome	Marker no.	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Intron size of A genome	Intron size of B genome	Intron size of D genome	Intron size of V genome	Type	Same size production in CS
1V	CINAU954	GTTTCAGGGAGGCT GCATTG	CCCGAGCACTCATT AACAACC	194	143	158	228	Type I	
	CINAU862	AGCCTGTCTCGGTG GTAAAA	CTGGAGTAGACAGT GCGTTTG	591	590	524	497	Type II	A and B
	CINAU893	AAGAAAGGAAGGCT GGAAGAG	GCTTCTGCCGCCAT TAACT	130	130	130	99	Type III	
2V	CINAU1068	GGAAGCAGTGGGAG AAGAGT	ATCATCTCAGGACC TGCAGC	116	81	128	103	Type I	
	CINAU1010	CACGGTTGGATTCA GGAAGC	CAGAACTTTGCTCC ACCAATG	1152	861	617	713	Type II	
	CINAU1067	GCCCTTCATCCTCGTCGG	AAGTCGAGGAACCA GAAGCC	106	103	119	88	Type III	
3V	CINAU1205	TCTGACAATAGCAG AATCACAGG	GGAACAAGAGCAGG CAATGT	436	357	439	414	Type I	
	CINAU1162	ACAATTTGTGCGCCATGG	TGCACTTGTTTGAG ATACTCCA	208	125	127	142	Type II	A and B
	CINAU1161	TGTGTACCTTGTGC TTTTCCA	GCCTTGCGTTGATT ACACCA	199	199	199	146	Type III	
4V	CINAU1331	AGCTCACAAAGAAG GAACAACA	TTCCGTCAAAACTC CAAGGC	553	391	426	331	Type I	
	CINAU1325	TCTCGCTAACCTAA GGAGCA	GACGATCGGTGACA GGACAT	130	165	247	154	Type II	A and B
	CINAU1295	GGTGGTAGTTCAAG CAAGACT	TCGCCTTGGAGATA TGTCGT	474	466	459	242	Type III	
5V	CINAU1454	GCTGTCATGCGCAC TATCAT	TGGCGTACAAGAAA TTCTCAGG	513	629	433	382	Type I	
	CINAU1459	TCAAGCCTTATCAA ACCAGATGT	TCCTGTTTCTGCTG CTACCA	187	269	266	159	Type II	
	CINAU1474	GGCTCCTTACAAGA TCAAGTGG	GCCCTCGAAGTGCA TTAAGA	223	222	222	99	Type III	
6V	CINAU1532	CTGATGACTGCCAA TGAATTTCT	CAATGCCTCTCGAC CAACTT	191	214	209	80	Type I	
	CINAU1542	GCAAGATAATGGCA CGATTCCT	AGTCACGTTCTTGT TTGCCT	71	99	93	124	Type II	
	CINAU1517	GAAGCTCTGGAATC ATGGCG	CATGCCAGTTGAAC TCCAGG	228	229	227	157	Type III	
7V	CINAU1566	CTCTTCCACCTACA CTCCAAG	TAGCCCACCGAATC ACAGG	519	467	505	419	Type I	
	CINAU1661	TGCCACACATGCTT GATAATGA	GCAATGGGATCCTC AAGTTGG	7,590,429	244	240	83	Type II	A and B
	CINAU1608	GCTCTATCAGGTCA CAACAATCT	AGCAATTCTCCACC CTCACA	231	231	228	194	Type III	

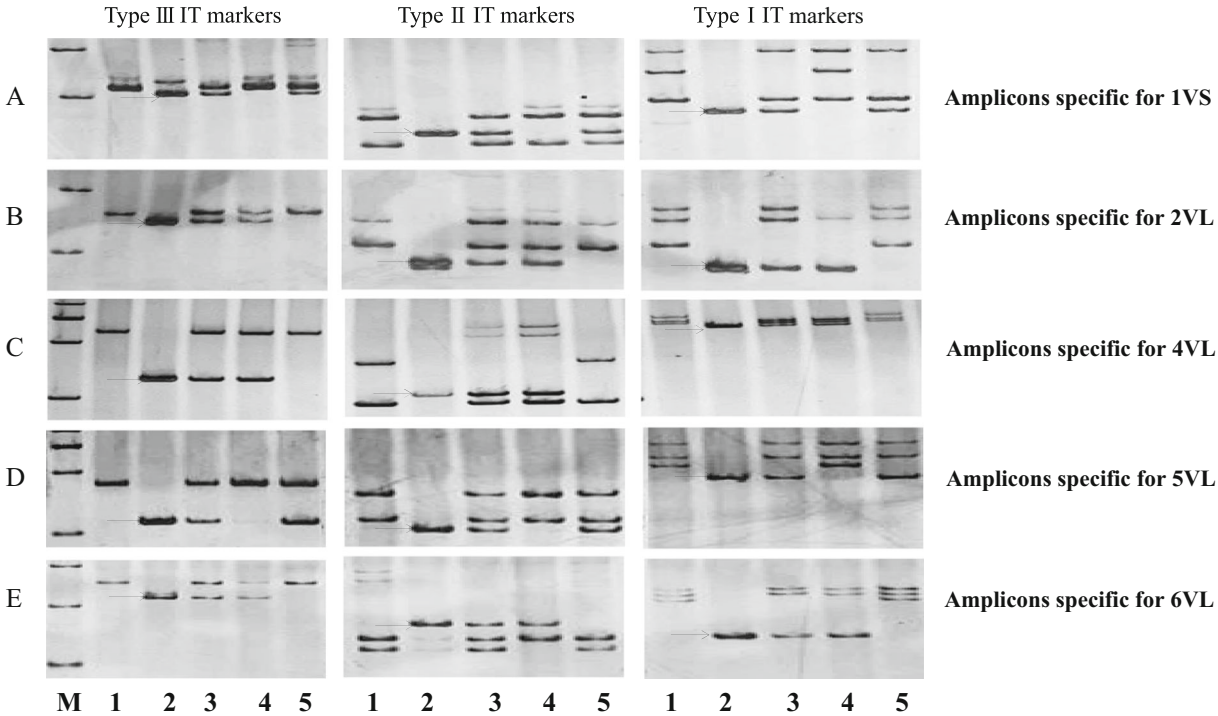

Fig. 2a–e Bands in non-denaturing 8% polyacrylamide gel electrophoresis evidencing PCR products of IT markers specific for individual chromosome arms of Dv. Lanes: *M* Marker; *1* CS; *2* Dv; *3 T.aestivum*-Dv amphiploid; *4*, *5* different *T.aestivum*-Dv translocations. **a** Lanes: *4* T1VL·1DL, *5* T1VS·1DS. The amplification of different representative IT markers (CINAU944, CINAU939, CINAU967 from left to right panels, respectively) specific for chromosome arm 1VS. **b** Lanes: *4* T2VL·2DS, *5* T2VS·2DL. The amplification of different representative IT markers (CINAU1011, CINAU1015, CINAU1013 from left to right panels, respectively) specific for chromosome arm 2VL. **c** Lanes: *4* T4VL·4DS, *5* T4VS·4DL. The amplification of different representative IT markers (CINAU1295, CINAU1325, CINAU1319 from left to right panels, respectively) specific for chromosome arm 4VL. **d** Lanes: *4* DA5V, *5* T5VS·5DL. The amplification of different representative IT markers (CINAU1404, CINAU1363, CINAU1454 from left to right panels, respectively) specific for chromosome arm 5VL. **e** Lanes: *4* T6VL·6AS, *5* T6VS·6AL. The amplification of different representative IT markers (CINAU1517, CINAU1541, CINAU1532 from left to right panels, respectively) specific for chromosome arm 6VL

a specific fragments from 3V-, and another fragment from CS chromosomes (possibly one of the 3A or 3B or 3D), or two CS chromosomes of (3A and 3D, 3D and 3B, or 3A and 3D); or all three chromosomes (3A, 3D and 3B) (Fig. 1).

Allocation of the IT markers to specific arms of Dv chromosomes

To further map these specific IT markers on specific arms of individual Dv chromosomes, all the IT markers specific for Dv chromosomes were used for amplification in different alien translocation lines. Using chromosome 2V as an example, if the amplification of a primer pair specific for 2V generated a common polymorphic band in Dv, *T. durum*-Dv amphiploid, translocation line T2VS·2DL (or T2VL·2DS), but not in CS, it is considered as a specific marker for chromosome arm 2VS (or 2VL)(Fig. 2, Tables S3, S4). Using those criteria, we assigned 47, 57, 44, 3, 51 and 51 IT specific markers to chromosome arms 1VS, 2VS, 3VS, 4VS, 5VS and 7VS, respectively. Similarly, 88, 118, 76, 86, 89, 71, and 60 IT specific markers were assigned to chromosome arms 1VL, 2VL, 3VL, 4VL, 5VL, 6VL and 7VL, respectively (Tables S1, S2).

Discussion

In recent years, the great accumulation of genetic markers and a large number of DNA sequences have made the study of comparative genomics in the grass family more feasible. It is found, in most cases, that the linear order (colinearity) of genetic markers is well

conserved and is retained at the molecular level (microcolinearity) (Gale and Devos 1998). Sponteneous substitution usually occurs among chromosomes belonging to the same homoeologous groups; thus, the identified *T. aestivum*-Dv substitution lines combined with isozyme analysis have been used successfully for assigning the added V genome chromosomes to specific homoeologous groups (Liu et al. 1995). RFLP and microsatellite molecular markers further confirmed the homoeology relationship between Dv and wheat chromosomes (Qi et al. 1999; Zhang et al. 2006). Zhao et al. (2014) developed EST-based molecular markers and proved that chromosome 4V is highly homoeologous to wheat homoeologous group 4 chromosomes.

However, the above types of molecular markers developed for Dv chromosomes have some disadvantages. RFLPs are dependent on radioactive probes and are also time-consuming. SSRs have low transferability among wild relatives and their application in wheat breeding is limited by the lack of locus specificity (Mullan et al. 2005). Out of 276 SSR markers, 148 could amplify polymorphic amplicons between Dv and CS, but only 7 (*wmc49* on 1BS, *wmc25* on 2BS, *gdm36* on 3DS, *gdm145* on 4AL, *wmc233* on 5DS, *wmc256* on 6AL and *gwm344* on 7BL) amplified specific polymorphic DNA fragments from chromosome 1V to 7V, respectively (Zhang et al. 2006). Wheat EST bin maps have been explored as a source for development of specific markers for alien chromosomes, but the polymorphic rate is very low (Qi et al. 2007). For example, Zhao et al. (2014) designed a total of 607 EST-based primer pairs, but only 58 (polymorphic rate being 9.23%) could amplify bands specific for chromosome 4V. A total of 240 EST-based STS primer pairs was designed, and only 13 (polymorphic rate being 5.42%) were specific for chromosomes of Dv (Cao et al. 2009). In the present study, we developed 841 chromosome-specific IT markers for Dv based on NGS technology, and the polymorphic rate was as high as 51.79%, indicating this is a highly efficient approach for developing chromosome-specific markers with higher success rate, specificity, stability and lower cost.

The IT markers discovered in this study are based on the sequence conservation of orthologous genes. All annotated genes from subgenomic D genome of CS were compared with genomic sequences of Dv using a local Blastn program. We found that most genes located on homoeologous group 1 to 7 wheat chromosomes could hit the genes from the corresponding homoeologous 1 to 7 Dv chromosomes, respectively. Using different alien translocation lines previously identified by FISH and molecular markers in our laboratory, these specific IT markers were mapped to specific arms of individual Dv chromosomes. The 1VL (88), 3VL (76), 5VL (89), 6VL (71) and 7VL (60) IT markers were derived from the long arm of the wheat homoeologous group 1, 3, 5, 6, and 7, respectively (Table S2). The IT markers for the 1VS (47), 3VS (44), 5VS (51) and 7VS (51) were derived from the wheat 1S, 3S, 5S and 7S arm, respectively.

However, we also observed some exceptions. For chromosome 2VL, all 118 IT markers were derived from the long arm of wheat group 2, while for 57 IT markers for 2VS, 56 were derived from short arm of wheat group 2, and one (CINAU975) was from the long arm of wheat group 2. For chromosome 4V, all 86 4VL IT markers were derived from the long arm of wheat group 4, and three 4VS IT markers (CINAU1275, CINAU1290 and CINAU1349) were also derived from the wheat 4L. This confirmed that Dv and *T. aestivum* had a close phylogenetic relationship, and suggested that their collinear regions were highly conserved, reinforcing the homoeologous relationship between Dv and wheat chromosomes (Table S5) (Qi et al. 1999; Zhang et al. 2006). The four exceptions indicated there present complex syntenic relationships between the wheat and Dv genomes, which may be the results of chromosomal rearrangements that most likely occurred during the karyotype evolution of Dv.

The IT markers developed in this study will dramatically increase the density of the Dv physical and cytological map to detect structural changes in Dv chromosomes or arms. Furthermore, some of the IT markers are co-dominant and hence will be very helpful in identifying chromosome changes both for the alien chromosomes and wheat chromosomes in a large population. In breeding program, they can be used to distinguish the homozygous and heterozygous individuals by MAS.

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Author's contribution W.X.E., W.H.Y. and X.J. designed experimental plan. Z.X.D., W.X. and Y.C.X. performed all experiments. X.J., W.Y.F., C.A.Z. and X.L.P. carried out sequence analysis and designed IT markers. C.P.D. contributed to the development of the germplasms used in the experiments. Z.S.Z. managed the materials in the field. W.H.Y., W.X.E. and Z.X.D. wrote the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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