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ORIGINAL ARTICLE

New molecular markers and cytogenetic probes enable chromosome identification of wheat-*Thinopyrum intermedium* introgression lines for improving protein and gluten contents

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Abstract

Main conclusion **New molecular markers were developed for targeting *Thinopyrum intermedium* 1St#2 chromosome, and novel FISH probe representing the terminal repeats was produced for identification of *Thinopyrum* chromosomes.**

Thinopyrum intermedium has been used as a valuable resource for improving the disease resistance and yield potential of wheat. A wheat-*Th. intermedium* ssp. *trichophorum* chromosome 1St#2 substitution and translocation has displayed superior grain protein and wet gluten content. With the aim to develop a number of chromosome 1St#2 specific molecular and cytogenetic markers, a high throughput, low-cost specific-locus amplified fragment sequencing (SLAF-seq) technology was used to compare the sequences between a wheat-*Thinopyrum* 1St#2 (1D) substitution and the related species *Pseudoroegneria spicata* (St genome, $2n = 14$). A total of 5142 polymorphic fragments were analyzed and 359 different SLAF markers for 1St#2 were predicted. Thirty-seven specific molecular markers were validated by PCR from 50 randomly selected SLAFs. Meanwhile, the distribution of transposable elements (TEs) at the family level between wheat and St genomes was compared using the SLAFs. A new oligo-nucleotide probe named Oligo-pSt122 from high SLAF reads was produced for fluorescence in situ hybridization (FISH), and was observed to hybridize to the terminal region of 1St#L and also onto the terminal heterochromatic region of *Th. intermedium* genomes. The genome-wide markers and repetitive based probe Oligo-pSt122 will be valuable for identifying *Thinopyrum* chromosome segments in wheat backgrounds.

Keywords Grain quality · In situ hybridization · Molecular markers · *Thinopyrum intermedium* · Wheat

Introduction

The autoallohexaploid wheatgrass, *Thinopyrum intermedium* (Host) Barkworth and D.R. Dewey has been hybridized extensively with wheat and has proved to be a valuable resource for enhancing the disease resistance and yield potential of wheat (Li and Wang 2009). Numerous wheat-*Thinopyrum* chromosome addition and substitution lines, as well as partial amphiploids, have been developed and represent essential genetic stocks for studying the expression of novel genes in the *Th. intermedium* genomes (Chen 2005; Li and Wang 2009). The *Th. intermedium* chromosomes contained genes with positive effects for gain quality in wheat background (Niu et al. 2011). However, the complex genomic composition of *Th. intermedium* has been investigated for decades and various hypotheses have been proposed (Chen et al. 1998; Mahelka et al. 2011, 2013). The chromosome sets of *Th. intermedium* were recently designated as the J, J^S, and St genomes based on the results of genomic in situ hybridization. These studies indicated that the J genome was related to both *Th. elongatum* and *Th. bessarabicum*; however, the J^S genome appeared to show the

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characteristics of a modified *Th. elongatum/Th. bessarabicum* genome (Chen et al. 1998; Chen 2005). The designation of St genome, which shows a high degree of similarity to that of *Pseudoroegneria,* has been widely accepted by researchers. Recently, we isolated the genomic specific long terminal repeat (LTR) sequence and discriminated the J^S and J genome, respectively, by FISH (Liu et al. 2009; Tang et al. 2011). However, individual chromosomal identification of the *Th. intermedium* genomes needs to be completed.

Recent investigations using molecular-based techniques on large and complex crop genomes, such as wheat and *Thinopyrum* species, have revealed the presence of multiple homoeologous gene copies, chromosomal rearrangements and amplification of repetitive DNA. Data generated from these studies has led to molecular marker development and also chromosome identification (Edwards et al. 2013). The latest next generation sequencing (NGS) technology has enabled considerable progress towards understanding the complex genomes of Triticeae species. The bioinformatic tools designed to deal with growing quantities of genomic data continue to develop (Edwards and Batley 2010). With the help of NGS techniques and bio-informatics analysis, a specific-locus amplified fragment sequencing (SLAF-seq) technique has been recently developed as a high-resolution strategy for large scale de novo discovery and genotyping of SNP (Sun et al. 2013). SLAF-seq is similar to restriction site associated DNA (RAD) sequencing, but pair-end reads of SLAF-seq can somewhat increase marker specificity and accuracy (Zhang et al. 2013). Thus, the SLAF provided high throughput, high accuracy and low-cost tools for the complex genome mapping of Triticeae species (Chen et al. 2013; Zhang et al. 2015; Xia et al. 2015).

Our previous studies developed wheat-*Th. intermedium* ssp. *trichophorum* chromosome 1St#2 substitution and translocation lines, which contained novel agronomically important genes (Hu et al. 2011; Li et al. 2013, 2015a). With the aim to precisely trace the 1St#2 chromosomes by marker assisted selection in wheat breeding programs for improving grain quality, the SLAF-seq technology was applied to wheat-*Th. intermedium* 1St#2 introgression lines and its parents. By comparison of genome-wide SLAFs between wheat and *Thinopyrum* species, we selected a number of 1St#2 specific markers and analyzed repetitive sequences for developing a new FISH probe for *Th. intermedium* chromosome identification.

Materials and methods

Plant materials

A wheat-*Th. intermedium* ssp. *trichophorum* partial amphiploid TE-3 (Yang et al. 2006) was used as the donor in the transfer of *Thinopyrum* chromosomes to wheat. Line AS1677 ($2n = 42$) contained a pair of *Thinopyrum* chromosomes as a 1St#2 (1D) substitution line which was developed from the crosses of wheat cultivar ML-13 to the partial amphiploid TE-3 (Hu et al. 2011; Li et al. 2013). Translocation lines E9-2 ($2n = 42$, T1DL.1St#2S) and E1233 ($2n = 42$, T1DS.1St#2L) were developed from crosses between AS1677 with the wheat cultivar MY11 (Li et al. 2013). Line X479 is a double 1St#2 (1B) plus $4St/4J^S$ (4B) substitution, and Line X482 is a double $4St/4J^S$ (4D) plus 6St (6D) substitution line; both were selected from the progenies of the cross between TE-3 and wheat cultivar MY26 (Li et al. 2015a). *Pseudoroegneria spicata* PI 232131 (St genome, $2n = 2x = 14$) and *Th. intermedium* PI440028 (StJ^sJ genome, $2n = 6x = 42$) were obtained from the National Small Grains Collection at Aberdeen, Idaho, USA. Wheat-*Th. intermedium* disomic addition lines Z3 were provided by Dr. B. Friebe, Kansas State University, USA.

SLAF high-throughput sequencing

The DNA samples from Chinese Spring wheat (CS), *Ps. spicata* and AS1677 were used to conduct SLAF-seq. The detailed protocols of DNA Digestion, PCR fragment amplification, fragment selection, library construction were as previously described by Sun et al. (2013). A library of products with appropriate sizes of 300–500 bp was sequenced on an Illumina HiSeq 2500 sequencing platform (Illumina, San Diego, CA, USA) at Biomarker Technologies Corporation in Beijing.

Fluorescence in situ hybridization (FISH)

Seedling root tips were collected and then treated with nitrous oxide followed by enzyme digestion, using the procedure of Han et al. (2006). The synthesized oligo-nucleotide probes Oligo-pSc119.2, Oligo-pTa535, Oligo-pTa71 and Oligo-$(GAA)_7$ were used for identifying the wheat chromosomes according to the description of Tang et al. (2014). A new oligo-probe, Oligo-pSt122 (5′ GGCT CACATT AGGGAAGAAT CGGTGAACAA AGAAAA-GACA AATTCACCGT ATAGAGCT 3′) was synthesized and labeled with 5′ 6-carboxyfluorescein (FAM) based on high copy number of SLAF reads sequences. The protocol of non-denaturing FISH by the synthesized probes was described by Fu et al. (2015). After the oligo-based FISH, the sequential Giemsa C-banding was done according to Yang et al. (2009). Photomicrographs of FISH and C-banded chromosomes were taken with an Olympus BX-51 microscope equipped with a DP-70 CCD camera.

Development of 1St#2 chromosome-specific markers

The SLAF fragments of AS1677 and *Ps. spicata* ($2n = 2x$) were selected by sequence specificity comparison. The sequences with good quality from *Ps. spicata* and AS1677 were first compared with the CS sequences acquired by SLAF-seq, and they were then compared with the sequences on www.ncbi.nlm.nih.gov and www.cerealsdb.uk.net. Finally, the specific sequences of AS1677 and *Ps. spicata* were compared and the 1St#2 chromosome-specific sequences of *Th. intermedium* were obtained.

Transposable element (TE) comparison

To determine the sequence composition, the SLAF reads (SSR excluded) were subjected to BLAST analyses against the repetitive element sequences using complete TREP, a database for Triticeae repetitive elements from http://wheat.pw.usda.gov/ITMI/Repeats (Wicker et al. 2002). This curated database includes known copies rather than consensuses of 584 TE families mostly from barley and cultivated wheat. Complete TREP thus included variation within TE families from species related to those investigated here. BLASTN searches identified hits showing 80 % similarity with sequences from databases and selected hits with e values $<10E^{-6}$. As BLASTN retrieved numerous hits, BLASTX search was not required. Classification of TE families into classes, orders, super-families, and families was consistent with the report of Wicker et al. (2007).

Verifying molecular marker analysis

DNA was extracted from young leaves of *Th. intermedium*, TE-3, lines AS1677, E1233, E9-2 and CS (Yang et al. 2006). Polymerase chain reaction (PCR) was performed in an iCycler Thermal Cycler (Bio-RAD Laboratories, Emeryville, CA) in a 25 µl reaction, containing 10 mmol Tris–HCl (pH 8.3), 2.5 mmol $MgCl_2$, 200 µmol of each dNTP, 100 ng template DNA, 0.2 U Taq polymerase (Takara, Japan) and 400 nmol of each primer. The cycling parameters were 94 °C for 3 min for denaturation; followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min; and a final extension at 72 °C for 10 min. The amplified products were separated by 1.5 % agarose gel as described by Hu et al. (2012).

Grain quality tests

Grain samples for agronomic traits observations were collected from two field replications per genotype per location at the Xindu Experimental Station, Chengdu, China during the 2013–2015. The hardness of grains and 1000 grain weight was determined by the Single Kernel Characterization System (SKCS 4100, Perten Instruments, Springfield, IL, USA) from 300 grains of each samples following manufacturer's protocol. The grains were milled using the Brabender Quadramat junior milling system (Brabender Instruments, South Hackensack, NJ, USA). The grain protein content was measured with a near-infrared (NIR) spectrometer (6500, Foss 12 Systems, Silver Spring, MD, USA) according to the approved methods 46-12 (AACC 2000). The measurement and calibration of wet gluten content by NIR spectrometer referred the report of Li et al. (2009).

Results

Characterization of wheat-*Thinopyrum* chromosome 1St#2 derivative lines

Giemsa C-banding of AS1677 chromosomes at mitotic metaphase is shown in Fig. 1a. The *Th. intermedium* ssp. *trichophorum* 1St#2 chromosomes had telomeric bands at the ends of the long arm and substituted for wheat chromosome 1D (Hu et al. 2011). Multi-color FISH by probes Oligo-pSc119.2 (green) and Oligo-pTa535 (red) showed chromosome 1St#2 of line AS1677 with a weak hybridization of Oligo-pTa535 at the end of the short arm, but no clear hybridization of Oligo-pSc119.2 (Fig. 1b). Since the hybridization pattern of Oligo-pTa535 on chromosome 1D showed stronger signals than that for chromosome 1St#2S (Tang et al. 2014), the FISH can identify the wheat-1St#2 translocation lines. As shown in Fig. 1c, d, line E1233 contained a T1DS.1St#2L translocation and line E9-2 carried a T1DL.1St#2S translocation, respectively.

Grain traits observations

Grain characteristics of the 1St#2 (1D) substitution, translocation lines and wheat control MY11 were measured from plants grown under field conditions of 2014 and 2015 seasons. As shown in Table 1, the 1St#2 (1D) substitution line AS1677 showed higher grain hardness than the cultivar MY11, however, both grains of the 1St#2 translocation lines become soft. The contents of kernel protein and wet glutens of the T1DS.1St#2L translocation line E1233 and the AS1677 were significantly higher than those of the wheat cultivar MY11. Our previous study revealed that a *Thinopyrum* specific high molecular weight gluten subunits (HMW-GS) gene, Glu-St#2x located on chromosome 1S#2L, and the 1St#2 (1D) substitution lines appear higher SDS sedimentation value and better solvent retention capacity than those of wheat parents (Li et al. 2013). We thus suggested that chromosome 1St#2L contains novel

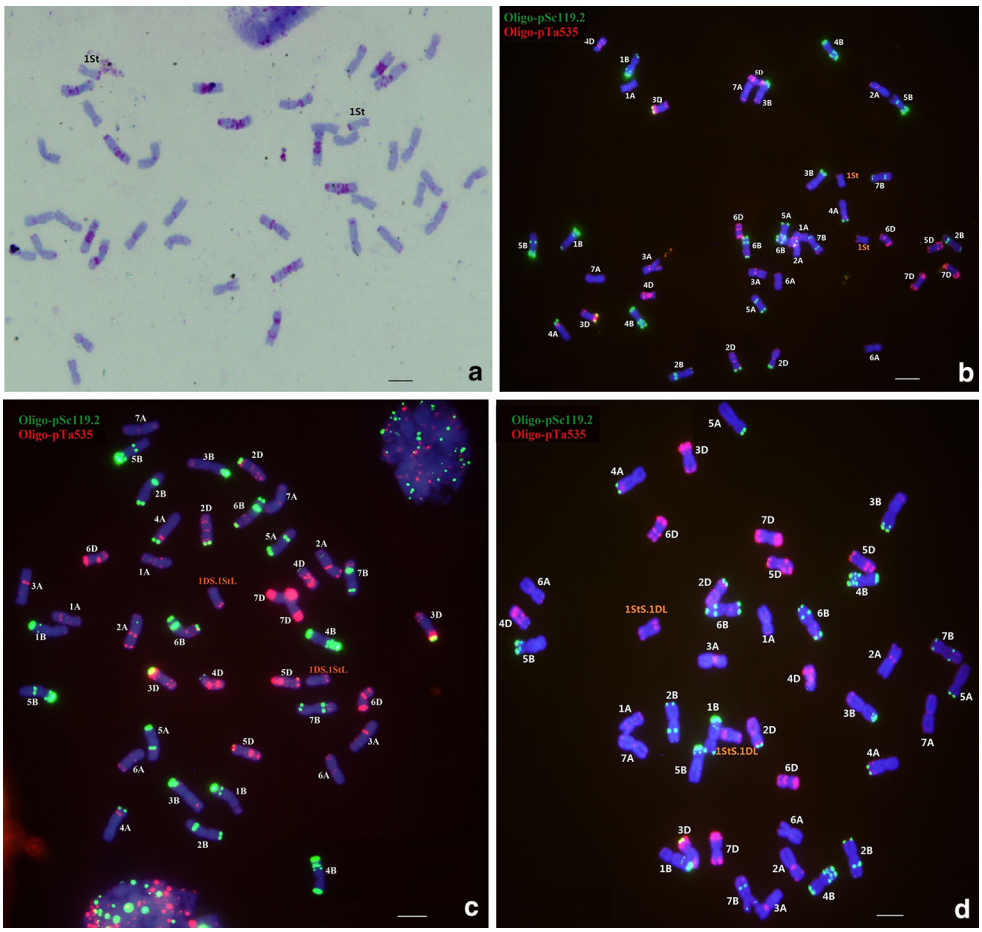

Fig. 1 C-banding and FISH of wheat-*Th. intermedium* ssp. *trichophorum*1St#2 lines AS1677 (**a**, **b**), E1233 (**c**) and E9-2 (**d**). *Bar* shows 10 μm

Table 1 Grain characteristics of tested lines

Lines	Chromosomes	Hardness (SKCS)	1000-grain weight (g)	Diameter (mm)	Protein content (%)	Wet gluten (%)	Test weight (g/L)
MY11	wheat	31.9 (Soft)	35.9	2.9	11.34	25.45	785
AS1677	1St#2(1D)	67.2 (Hard)	40.9	2.9	14.47**	31.89*	796
E1233	1DS.1St#2L	31.0 (Soft)	46.3**	3.0	15.80**	34.85**	779
E9-2	1DL.1St#2S	34.2 (Soft)	28.0*	2.6*	11.92	25.41	775

*, ** Significant difference at $p < 0.05$, and 0.01, respectively

genes for positive effect on end-use quality of wheat improvement.

Th. intermedium 1St#2 chromosome-specific molecular markers

To produce high density molecular markers to target the presence of *Th. intermedium* chromosome 1St#2 based on SLAF-seq, we obtained 1,043,655 and 714,595 reads which resulted in 62,437 and 44,497 effective SLAFs for AS1677 and *Ps. spicata*, respectively. After comparing the CS sequences acquired by SLAF-seq, a subset of 7,691 sequences from AS1677 showing less than 50 % homology to wheat were selected. From this subset, 359 AS1677 sequences with homologies higher than 80 % to those of *Ps. spicata* were obtained. These SLAFs sequences were identified as putative 1St#2 chromosome-specific sequences.

Based on 50 sequences randomly selected from the 359 specific sequences for the 1St#2 chromosome, 50 pairs of primers were designed for developing specific molecular markers. The genomic DNA from CS, AS1677, *Th. intermedium* and *Ps. spicata* were used to test the effectiveness

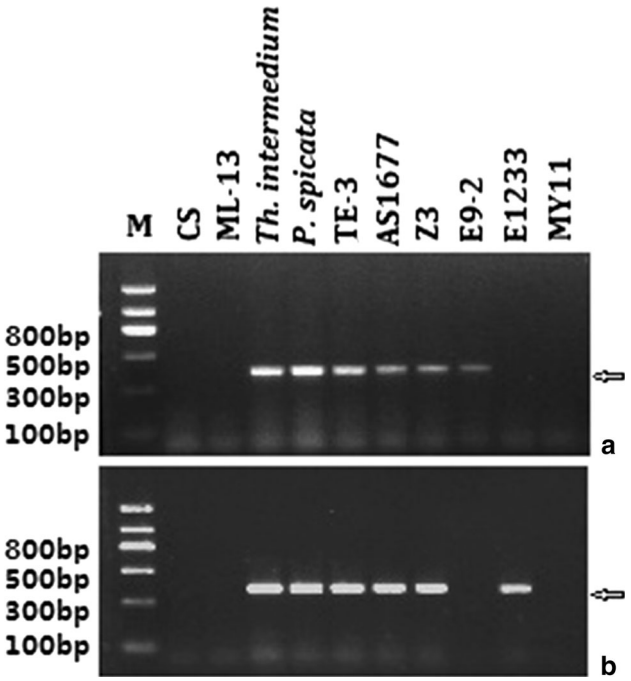

Fig. 2 PCR *patterns* of primers St13947 (**a**) and St27473 (**b**) for wheat-*Th. intermedium* derivatives and its parents

of the PCR primers. A total of 37 primers were found to produce amplicons using DNA from AS1677 and *Th. intermedium*, but no amplification was obtained with CS, which confirmed that the markers are chromosome 1St#2 specific (Fig. 2). The primers are listed in Table 2. To localize the markers onto specific arms of 1St#2, the translocation lines T1DL.1St#2S (E9-2) and T1DS.1St#2L (E1233) were used to amplify the primers. Total of 20 markers were located onto the short arm of 1St#2 and 17 markers located onto the long arm of 1St#2 (Table 2). Therefore, the 37 specific molecular markers of chromosome 1St#2 were developed and can be used to characterize 1St#2 chromosome. The markers are all specific and stable, and will find application in detecting and localizing novel genes on the *Th. intermedium* chromosome 1St#2 in wheat background.

Distribution of repetitive sequences of SLAFs

The total sequences were BLASTN against the databases including plant repeat database (http://www.tigr.org/tdb/e2k1/plant.repeats/) and Triticeae repeats (http://wheat.pw.usda.gov/GG2) for searching the TE and the different kinds of repeats (Wicker et al. 2009). The most abundant LTR retrotransposons of TEs were identified representing 81.36 and 86.42 % in AS1677 and *Ps. spicata*, respectively. Among the LTR retrotransposon super-families, *Ps. spicata* contained 37, 36 to 24 % corresponded to *CACTA*, *Gypsy* and *Copia* elements, respectively, while the wheat AS1677 has the LTR super-families *Copia*, *Gypsy* and *CACTA* with 44, 40, and 12 %, respectively (Fig. 3a).

A total of 44 families were present at more than 0.5 % of the reads, representing the most abundant families. As shown in Fig. 3b, the Angela clade (*Copia* element) contributed the most to the overall genome, with around 14 % of the total number of reads, and was significantly more abundant in AS1677 (23.4 %) than in *Ps. spicata* (14.2 %). Egug (*Gypsy* element) represented as much as 5–6 % of the total number of reads, showing higher proportions in *Ps. spicata* than in AS1677, while Ceraba (*Gypsy* element) showed significantly higher proportions of reads in As1677 than in *Ps. spicata*. The LTR retrotransposon family with the largest difference in *CACTA* element (Conan, Clifford) shows significantly higher proportions of reads in *Ps. spicata* than those in As1677, while the *CACTA* family (Jorge) occurs in higher abundance in AS1677 than those in *P. spicata* (Fig. 3b). It is likely that the different distribution of LTR may provide opportunity to discriminate the *Ps. spicata* genome from the wheat genomes.

Development of *Thinopyrum* specific telomeric repetitive probes

The SLAFs reads of *Ps. spicta* displayed <50 % wheat homology were analyzed by BLAST to tandem repeats of Triticeae species. A 73 bp long fragment with a sequence depth of 85 showed high homology to a part of Clifford family of *CACTA* element, and 58 bp sequence without SSR part was selected to design an Oligo-FISH probe. This 58 bp oligo labeled with 5′ FAM (green) was named Oligo-pSt122. The probes Oligo-pTa535 (red), Oligo-pSc119.2 (green), and Oligo-pTa71 (red) together with Oligo-pSt122 (green) were sequentially hybridized onto the wheat-*Th. intermedium* ssp. *trichophorum* derivatives by ND-FISH. The C-banding and FISH patterns of 1St#2 and 1D from AS1677, E9-2, E1233 are shown in Fig. 4a. The FISH results showed that Oligo-pSt122 only hybridized to terminal regions of 1St#2L, which is identical to heterochromatic C-banding, but it showed no hybridization to wheat chromosomes (Fig. 4a). Furthermore, we conducted the sequential FISH by probes Oligo-pSt122 (Fig. 4b) and C-banding (Fig. 4c) to metaphase cells of *Th. intermedium* PI440028. We found that the FISH signals present on 20 chromosomes of *Th. intermedium* chromosomes. Comparing the FISH patterns and C-banding patterns of PI440028, all FISH signals of the Oligo-pSt122 were identical to all of the telomeric C-band locations.

Our previous study indicated that the lines X479 and X482 were both double substitutions and possessed the chromosome constitutions of 1St#2 (1B) + 4St/4J^S (4B) and 4St/J^S (4D) + 6St (6D), respectively (Li et al. 2015a).

Table 2 PCR primers and location of *Th. intermedium* 1St#2 chromosome-specific markers

Primers No.	Primer sequences(5′–3′)		Chromosome 1St#2 arms
	Forward	Reverse	
St4369	ACACTGGATTATTGCTCAAG	GACAGTTCTTGCAGGTTTTG	1St#2S
St6686	GGTAGCCACACCTAATTTCA	GTAACATGCAGATTAGGTAC	1St#2L
St9554	GCCTGGCACTTGGTGAGAG	CAAGTAAAACTTGTGAACTC	1St#2S
St10540	CATACAGAACCTGTTCAAAGC	GCAGGTGGGTCTACCATGAC	1St#2S
St11027	GCAACACCAACCTCAGTATTC	GCAAGTGCCTGATAATGAAG	1St#2L
St13947	CGAGCTATAGATATGGATAAG	TCACTCTTCGATCATTCAACC	1St#2S
St14982	TTTGACATGTGCACACCAA	GCACTTACAGATAAACATATC	1St#2S
St17388	CCATAACGCATCCAAAGGAT	CTCAGTGCGAATTCTGCTCC	1St#2S
St17853	GTGTTTGGGCAGGAATGCAG	CAAGGCTGATATCCAGTCC	1St#2S
St18269	CTACTCTCTCACAACTTCCA	CCTGCCATATTTGTTATTCG	1St#2S
St18527	CTGTGTGCATACATATACTTTG	TTGTGGCTACTGGTGATTAG	1St#2L
St20853	GATGCCACTAATAAATTGTTGC	GTGACCTTGCAGGTTCGGTG	1St#2L
St21397	CTGATTTCCTCACATATCCAC	CTTACTGTCTACAGCAGTTG	1St#2S
St22006	TGTAGAGGAAGAATTTGAGC	TCGGATTACACTTCTTCATC	1St#2S
St26392	GGACTATCGGACTATTTGAAC	GAGATAGTTTGTGGTAGTGG	1St#2L
St27473	ATTCCAGCAAACAGTAGATTAT	GTGGAATGGAACCTATCTCG	1St#2L
St27989	CACTCTCCCATGTGCAAAC	CGGGATCCCTATACTCAAG	1St#2L
St30824	CGAGAAGAAGTGCAAGCTTG	ATGCGTCCAATCACATAGCG	1St#2L
St34277	GAAATGCAATCGGTTCCTACG	TGCATTAGGCAATTTACACC	1St#2S
St34493	CTTGGATATGCACGGTGAAG	ACAGGGATCTCACGACAGTC	1St#2L
St37528	CATACGCAGACAACGATCAG	CAAGCTACGCGATCAGATGC	1St#2L
St37895	ACACAGAGCAGAAAAATTAG	GTAAATAGAGTTCGAATGTG	1St#2L
St40468	CTGACGCCACCTCTTCTCA	GGCGCTTGATTTGTCAGAAG	1St#2L
St41137	CTCACCAATGCCATCCTCTC	CTTATAGCCCATTTACATCAC	1St#2S
St41431	CATGGTACACTACCAGAATCG	AATCATATTTAGGTTTCCATC	1St#2S
St43202	GAACAACTACTACACGCGTC	GCGTGAGCAATACAATGACC	1St#2L
St44406	TGTCAGAAGTCTCCACAGTCA	CCTGGATGTAATTTCGTGTCT	1St#2L
St44421	CAGTTATACCATTTCCGGTC	GCCAGCACTCACGATGAAC	1St#2S
St44796	GCTGGCAGTACGTTTTTCGC	CATAGAGCAATGCTTTAGGG	1St#2L
St47017	CGAGCGGTGCAAGATATAAC	GGGAGCTAATGCAGTTTGTC	1St#2S
St51158	GTATACTGGTTATACCTTTGC	CAATTCCACCAGCACTCATG	1St#2S
St51282	GGATATTGCTATGGACTAAC	GTAAATATCAATACGGTGTG	1St#2L
St51347	CAAAGTCTGGAGACGTACTTC	GCGCAACTGAAGTTTGAATG	1St#2S
St51507	ATGTAGTGCTAACTTGTACC	CATGGACTCCAATATGCCAT	1St#2S
St51828	CACCACACAGAGTTACAAATG	GATTTTGCTCCCCGACTCG	1St#2S
St171464	CAAGCGCTTATATATACGTG	TTGGTCCCTGAACTCTTGCA	1St#2L
St281111	TGGTTCATGCCGGATCCATA	CACTTTCTATGCTAAGCTCG	1St#2S

As shown in Fig. 5a-c, we clearly found that Oligo-pSt122 hybridized only to the *Th. intermedium* chromosomes of X479, as expected. The Oligo-pSt122 signals appeared on telomeric regions of long arm of 1St#2, which is identical to the heterochromatin C-banding of 1St#2 in AS1677 (Fig. 4a). Furthermore, the Oligo-pSt122 signals were located at the end of the long arm in chromosomes 4St/J^S and 6St (Fig. 5d–f). Since the 6St was lacking any of the Oligo-pTa535, Oligo-pSc119.2, or Oligo-$(GAA)_7$ signals, the specific hybridization of Oligo-pSt122 to *Th. intermedium* chromosomes will be helpful for identification of 6St chromosomes. Furthermore, chromosome spreads of wheat-*Th. intermedium* partial amphiploid TE-3 (54 chromosomes) were also hybridized (Fig. 5g–i). The

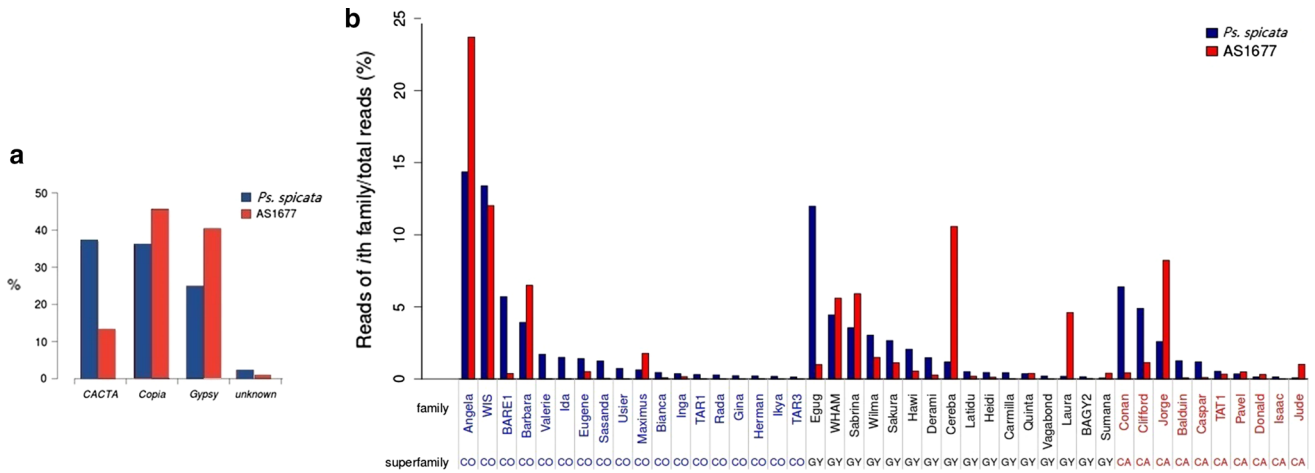

Fig. 3 Distribution of major LTR retrotransposon super-families (**a**) and LTR families (**b**). The CO, GY, CA referred *Copia*, *Gypsy* and *CACTA* family, respectively

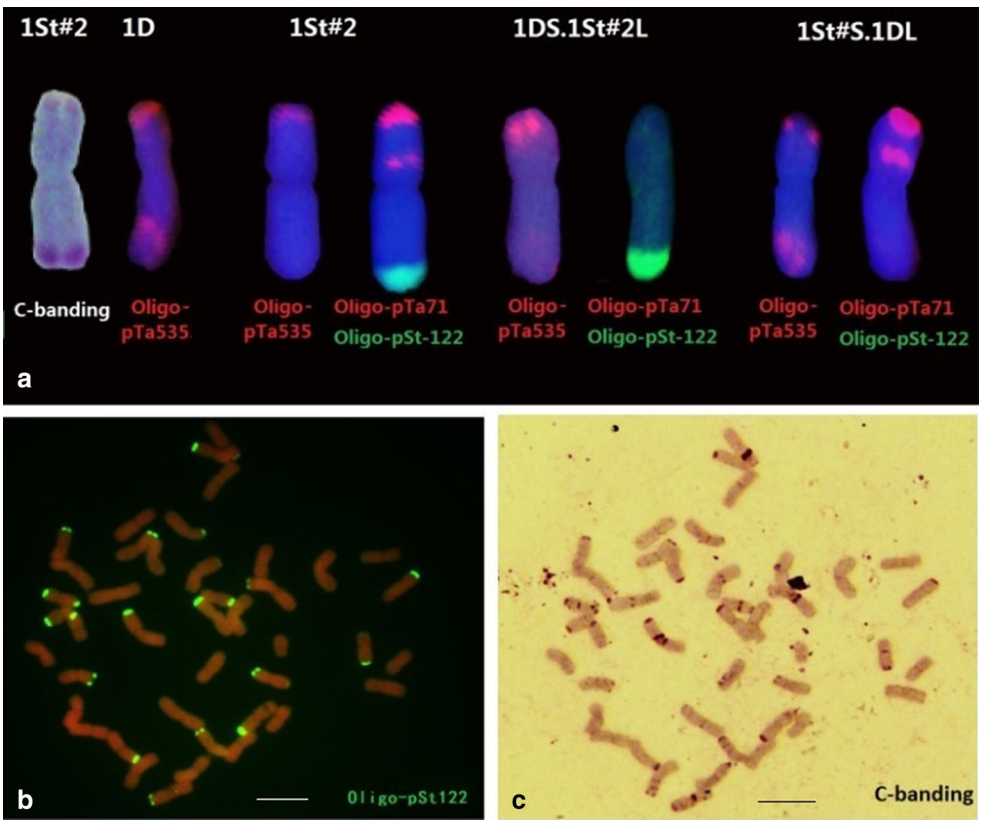

Fig. 4 The compared chromosome karyotype of the 1St#2 (**a**) and a mitotic chromosome spread of *Th. intermedium* ssp. *trichophorum* after FISH (Oligo-pSt122) (**b**) and C-banding (**c**). *Bar* shows 10 μm

hybridization signals of Oligo-pSt122 appeared to be one or both telomeric regions of each of the 12 *Thinopyrum* chromosomes of TE-3, in which the 1St chromosome with both Oligo-pTa71 and Oligo-pSt122 signals was easily recognizable. The results suggested that the FISH by Oligo-pSt122 can be used to trace specific *Th. intermedium* chromosomes in a wheat background.

Discussion

Wheat group 1 homoeologous chromosomes are one of the most studied and understood primarily because they contain major clusters of agronomically important genes. Numerous genes and gene families expressed during seed development are located in group 1 chromosomes (Peng

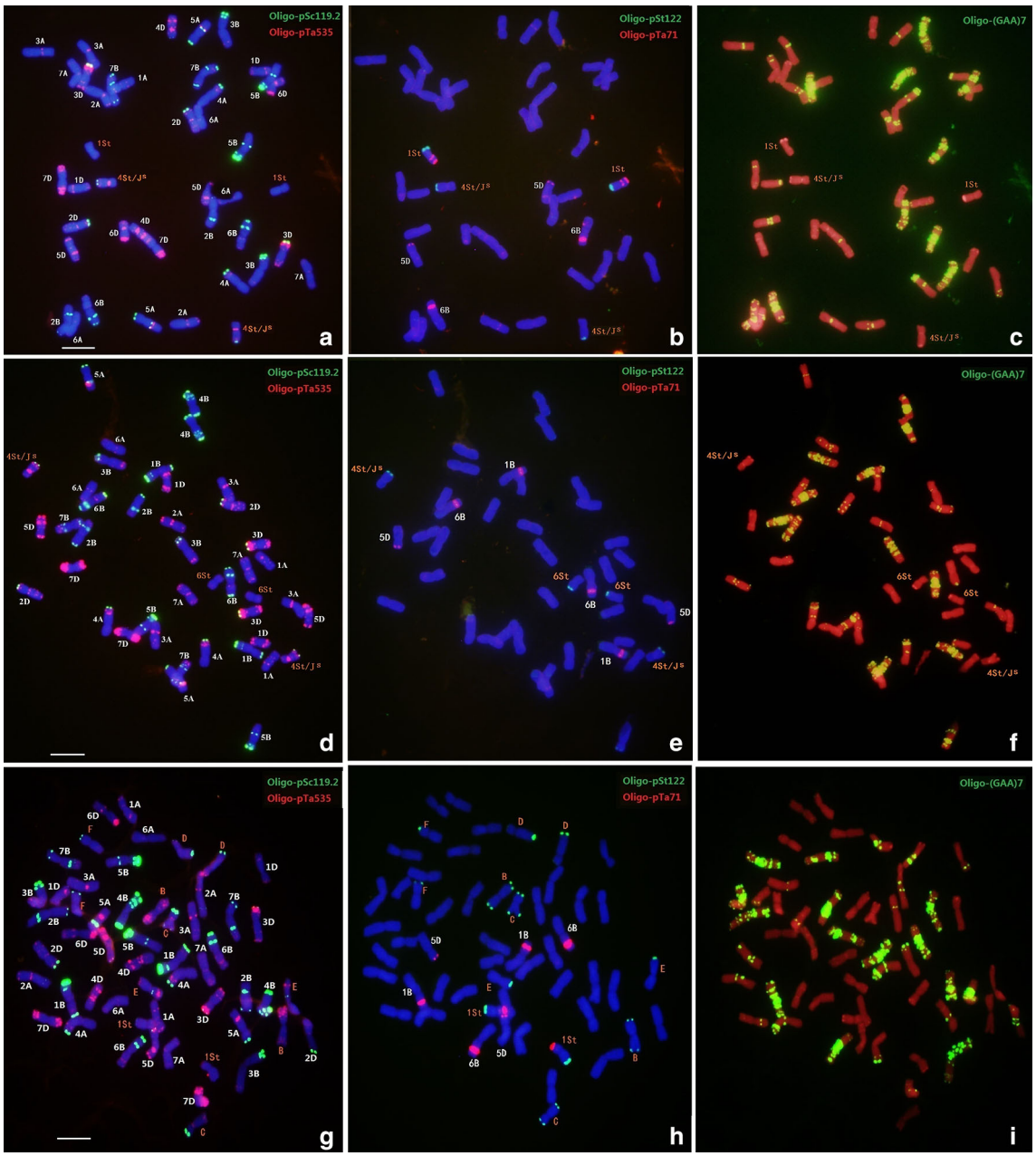

Fig. 5 FISH of wheat-*Th. intermedium* ssp. *trichophorum* derivatives X479(**a**–**c**), X482 (**d**–**f**) and TE-3 (**g**–**i**). The **a**–**f** of TE-3 are *Th. intermedium* chromosomes in addition to 1St. *Bar* shows 10 μm

et al. 2004). Triticeae homoeologous group 1 chromosomes display highly conserved synteny compared to group 1 chromosomes of wheat, and carries a number of important genes for wheat breeding. The most famous is rye (*S. cereale*) chromosome 1R, which possesses numerous resistances to biotic and abiotic stresses as well as a factor associated with increased yield potential (Rabinovich 1998; Howell et al. 2014). The previously effective resistance genes *Sr31, Yr9*, *Pm8* and *Lr26* were derived from Petkus rye, transferred to wheat as the 1BL.1RS translocation chromosome and used widely around the world commercial cultivars (Bartos 1993; Pretorius et al. 2000; Hurni et al. 2014). Furthermore, the recently cloned stem rust resistance gene *Sr50* (=*SrR*) also on chromosome 1RS but derived from Imperial rye, shows promise as a replacement for the now ineffective *Sr31* for stem rust resistance (Anugrahwati et al. 2008; Mago et al. 2015). The genes located on chromosome 1E of *Th. elongatum* added to wheat have shown a positive effect on seed storage proteins (Garg et al. 2009a) and tolerance to Fusarium head

blight (*Fusarium graminearum*) (Jauhar et al. 2009). Genes located on chromosome 1V of *Dasypyrum villosum* in both addition (De Pace et al. 2001) and translocation lines (Zhang et al. 2014) had also largely positive effects on bread-making quality. Furthermore, Garg et al. (2009b) found that seed storage proteins of *Th. intermedium* had positive effects when transferred to wheat. In this study, we successfully transferred *Th. intermedium* chromosome 1St#2 to wheat and subsequently produced substitution and translocation lines (Hu et al. 2011; Li et al. 2013, 2015a) which enabled us to determine the effect of 1St#2 chromosomes in a wheat background. Our results suggest that the *Th. intermedium* 1St#2 chromosome can easy compensate for the loss of wheat chromosome 1D and possibly increase the overall seed protein and wet gluten contents. Expression of genes coding for HMW-GS derived from *Th. intermedium* has been reported by Niu et al. (2011) working with the 1Ai addition line TA3649 and also Wang and Wang (2016) studying the 1 J or 1JL-St addition lines. Moreover, the *Th. intermedium* 1St#2 chromosome has also been found to carry novel stripe rust resistance genes, which are easily transferred into wheat (Hu et al. 2011; Li et al. 2015a). Hence, group-1 chromosomes derived from different *Th. intermedium* accessions might act as useful resources in wheat breeding programs for both qualities related characters as well as disease resistances.

Molecular markers, especially those derived from conserved coding regions, are useful for determining the homoeologous relationships of chromosomes from different grass species by comparative mapping (van Deynze et al. 1998; Heslop-Harrison 2000). These new techniques in marker development enable the efficient production of highly dense and accurate maps of related Triticeae chromosomes. PCR-based markers are more convenient in terms of manipulation and application. Our previous study developed 39 pairs of PLUG markers located on homologous groups 1 to 7 (Hu et al. 2012). The expressed sequence tag derived simple sequence repeats (EST-SSR) markers derived from conserved coding sequences of wheat have been successfully used to characterize *Th. intermedium* accessions (Wang et al. 2015). The lack of sequencing data of the complex Triticeae genomes including *Th. intermedium* currently hinders the high-throughput identification of chromosomes of these uncultivated species by both molecular markers and cytogenetic approaches. Based on the NGS technology, the SLAF-seq is a recent high-resolution strategy developed for large scale de novo discovery and genotyping of SNPs (Sun et al. 2013). Compared with previous single-end RAD sequencing, pair-end reads obtained by SLAF-seq can somewhat increase marker specificity and accuracy, which has been used for mapping the *Th. elongatum* 7E chromosome (Chen et al. 2013) and *Agropyron cristatum* (P genome) chromosomes (Han et al. 2014). In this study, we were able to develop 37 *Th. intermedium* chromosome 1St#2 markers by SLAF-seq and assign them to the long and short arms. Compared with the 1St chromosome derived from the wheat-*Th. intermedium* addition line Z3 (Hu et al. 2011), we found that the 60 % percent of SLAF markers give polymorphic amplification with chromosome 1St#2. These types of markers will enable fine mapping of traits of agronomic importance.

The abundance of repetitive sequences within the Triticeae tribe may pose problems in genome analysis and alien chromosomal manipulation in wheat (Heslop-Harrison and Schwarzacher 2011). The sequence assembly of large and complex genomes causes a great challenge when studying plants where highly repetitive sequences comprise more than 70 % of the whole genome. Wicker et al. (2009) reported differential amplification of transposable element (TE) families in the A and B genomes of wheat. Middleton et al. (2012) analyzed the constitution of TEs for several Triticeae taxa, including *Triticum aestivum* (ABD genomes), *Hordeum vulgare* (H genome), and *Secale cereale* (R genome) together with relatives of the A, B and D genome donors of wheat, *Triticum urartu* (A), *Aegilops speltoides* (S) and *Ae. tauschii* (D). Senerchia et al. (2013) suggested that the sequencing of 2.5 % of *Aegilops* genomes was sufficient to survey genome-wide copies of several abundant TEs, including characteristic families from pericentromeric regions. Our SLAF method surveyed with short reads can thus be applied to abundant TEs of any genome sequencing for identifying and quantifying the TE families within genomes. We found that the *CACTA* superfamily of the St genome occurred more frequently than on the chromosomes of the wheat genomes. The TE composition differs greatly between Triticeae species, indicating that they separated long before the divergence of the Triticeae species. In this study, the detailed knowledge of TEs within genomes is assessable at the genus level by comparing the FISH patterns of the St genome with wheat genomes (Fig. 4). The retrotransposon families were more abundant in the *Ps. spicata* St genome, suggesting that the different TEs from Triticeae followed contrasting amplification trajectories after the divergence of the species. The diverse sites of the TEs in the Triticeae chromosomes have enabled the identification of wheat and related species by in situ hybridization (Li et al. 2004; Stein 2007). Sergeeva et al. (2010) isolated the Caspar family among the most abundant of *CACTA* DNA-transposons in wheat, which significantly contributed to the formation and differentiation of subtelomeric regions in Triticeae species revealed by FISH. We noted that the Conan and Clifford family of *CACTA* TEs was most abundant in *Ps. spicata* (Fig. 3b). It may play an important role in the evolution of subtelomeric regions of *Thinopyrum* genomic divergence by the

comparative genomic sequences in combination with FISH studies.

The analysis using a similarity-based NGS read clustering approach allows de novo identification of all major types of genomic repeats (Macas et al. 2007; Novak et al. 2010). The studies reveal evidence that subtelomeric domains are among the most dynamic regions in eukaryotic genomes (Torres et al. 2011).The subtelomeric repeat sequences have been reported in numerous plant species including rye (Vershinin et al. 1995), barley (Brandes et al. 1995), *Leymus* (Wang et al. 2006), *Dasypyrum* (Li et al. 1995). However, an in-depth study of the structure and organization of subtelomeric repeats has been done in only a few species (Cheng et al. 2001; Dechyeva and Schmidt 2006). Most importantly, it remains largely unknown how these repeats emerged and evolved. The subtelomeric locations of these repeats were confirmed mostly by in situ hybridization on somatic metaphase chromosomes (Li et al. 2004). FISH of the *Leymus racemosus* chromosomes indicated that two specific LTR families were located in subtelomeric heterochromatin (Kishii et al. 1999). Cultivated rye has accumulated the pSc200 and pSc250 repeats in telomeric and subtelomeric regions, however, they are largely absent in the ancestral species *S. africanum* (Yang et al. 2009; Li et al. 2015b). Similar studies on the telomeric repeats of pHv62 in *Dasypyrum villosum* (Li et al. 1995) were found to be absent in *D. breviaristatum* chromosomes according to our recent study (Li et al. 2016). In this study, the probe Oligo-pSt122 was found to be specific to the terminal region of chromosome 1St#2L, and all of the *Th. intermedium* terminal heterochromatin banding regions (Fig. 4). We also found that Oligo-pSt122 can hybridize to the *Th. bessarabicum* and *Dasypyrum villosum* chromosomes in wheat background (Supplementary Fig. 1), which supports the earlier findings that *Th. intermedium* genomes share the similar repeats of the *Dasypyrum* genome (Liu et al. 2009; Wang et al. 2015). The heterochromatic structures at the telomeres of chromosomes are indispensable for ensuring correct chromosome pairing and segregation (Biscotti et al. 2015), and hence, we presume that the telomeric repeats may play a role in ensuring the stability of genomes of the polyploid *Thinopyrum* species.

Chromosome painting can be used to identify alien chromosomes, including chromosome number and characterizing structural aberrations in wheat-alien hybrids. Combinations of chromosome banding with in situ hybridization have been sequentially performed on wheat metaphase chromosomes (Jiang and Gill 1993). The sequential C-banding and in situ hybridization techniques applied in this or in a reverse order are used to recognize targeted chromosomal regions of wheat and rye (Cuadrado et al. 1996). Friebe et al. (1992) published C-band karyotypes of *Th. intermedium*, and pointed out that there is considerable polymorphism and structural modification in this species. Xu and Conner (1994) provided a C-band karyotype of a *Th. intermedium* ssp. *trichophorum* cv. 'Greenleaf', and showed that the chromosomes exhibited strong heterochromatin bands in their terminal regions. Cai et al. (1998) identified three pairs of wheat-*Thinopyrum* translocation chromosomes and 18 pairs of wheat chromosomes. In this study, sequential C-banding and FISH analysis allowed a precise distinction of *Thinopyrum* chromosomes and wheat-*Thinopyrum* derivatives (Fig. 4). We developed the *Th. intermedium* terminal heterochromatic probe Oligo-pSt122, which can be used to effectively trace this *Thinopyrum* specific region, in combination with the preexisting probes Oligo-pTa71, Oligo-pSc119.2 Oligo-pTa535. Meanwhile, our studies found that the several rounds of Oligo-based FISH have little effect on chromosome C-banding treatment for the metaphase chromosomes. Additional probes for ND-FISH will finally be developed to more precisely identify small translocated alien chromosome segments.

Author contribution statement Z.Y. and G.L. conceived and designed the research; G.L., H.W., S.L. and J.L. conducted the experiments; G.L., T.L. and E.Y. analyzed the data; Z.Y. wrote the manuscript. All authors read and approved the manuscript.

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