Front. Biol. 2010, 5(2): 187–192
DOI 10.1007/s11515-010-0036-3

RESEARCH ARTICLE

New PCR based markers allowed to identify *Secale* chromatin in wheat-*Secale africanum* introgression lines

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Abstract The genus of *Secale* has many agronomically important characters. In order to use the best of this species, markers tracking the rye chromatin incorporated into wheat must be developed. In this study, one rye genome-specific random amplified polymorphic DNA (RAPD) marker was isolated from *Secale africanum* (R^a genome). Two cloned markers, named $OPP13_{1165}$ and $OPP13_{662}$, were 1165 bp and 662 bp, respectively. Sequence analysis revealed that $OPP13_{1165}$ was highly homologous to a part of a new class of transposon-like gene called the *Revolver* family, and $OPP13_{662}$ was partially similar to LTR *gypsy*-like retrotransposon. Fluorescence *in situ* hybridization (FISH) showed only $OPP13_{1165}$ localized within the whole arms of rye except their terminal regions and no signal was detected on wheat chromosomes, while $OPP13_{662}$ had no hybridization signal detected on wheat and rye genomes. Based on these sequences, two pairs of sequence-characterized amplified region (SCAR) primers were designed, and the resulted SCAR markers were able to target both cultivated and wild *Secale* species. The FISH patterns and the two SCAR markers should be able to identify and track all wheat-rye translocation lines, especially the *S. africanum* chromatin.

Keywords *Secale africanum*, rye, molecular marker, fluorescence *in situ* hybridization (FISH), chromatin detection

1 Introduction

The genus of *Secale*, in the tribe Triticeae, has many agronomically excellent characters, such as high yield, wide adaptability and resistance to many pests and diseases, and thus represents potential to increase the genetic variability and introduce desirable genes for wheat breeding (Zeller and Hsam, 1984; Ko et al., 2002). The genus of *Secale* consists of cultivated rye (*Secale cereale* L.) and several wild species. At present, researches have mainly focused on cultivated *secale* and its introgression lines. Regarding the research of wild *Secale*, much attention has been paid to the evolutionary studies between *Secale* and other Triticeae species and there have been few reports about wild *Secale* species in wheat breeding so far (Singh and Robbelen, 1977; Liu et al., 2008). After several wheat-*S. africanum* amphiploids were produced (Jiang et al., 1992), a program for introducing the novel genes from *S. africanum* to common wheat was set up by crossing the amphiploids with cultivated wheat (Yang et al., 2001; Jia et al., 2009). It is thus necessary to develop a serial of reliable molecular markers to rapidly screen large numbers of wheat-*S. africanum* genotypes in real breeding programs.

Molecular markers based on the direct analysis of DNA sequence variation satisfy the need for markers anywhere in the genome (Iqbal and Rayburn, 1995; Liu et al., 2008). At present, some DNA sequences from the rye genome have been isolated by random amplified polymorphic DNA (RAPD) and converted to rye specific markers (Ko et al., 2002; Liu et al., 2008; Jia et al., 2009). However, most of these markers designed on the (LTR) retrotransposons of the Ty1-*copia* or Ty3-*gypsy* groups, and few of these reports involved markers coming from new transposable elements family or genes.

In this study, two sequences, including a new class of transposon-like gene called the *Revolver* family, were isolated from the genome of *S. africanum* using RAPD analysis. Based on these two sequences, two pairs of SCAR markers were produced. Experimental data suggested that these primers can be used to detect the presence of rye chromatin in wheat background. In addition, the chromosomal organization of the markers was also revealed by fluorescence *in situ* hybridization (FISH).

Received February 4, 2010; accepted February 23, 2010

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2 Materials and methods

2.1 Plant materials

S. africanum and *Secale. silvestre* were introduced from J. P Gustafson of Missouri Garden, USA. *Secale cereale* L. cv. Qingling rye, *Secale cereale* L. cv. Jingzhou rye, *Secale strictum* subsp. *anatolicum*, and *Secale vavilovii*, Chinese Spring (CS), wheat variety Mianyang11 (MY11), *Triticum aestivum* cv. Anyuepaideng, *Triticum turgidum* cv. Jianyangailanmai, hexaploid *triticale* lines Beagle and Hyslop were provided by the Key Lab of Plant Breeding and Genetics, Sichuan Agricultural University, China. *Triticum carthlicum* was obtained from Dr. Harold Bockelman, National Plant Germplasm System, USDA-ARS, Aberdeen, Idaho, USA. Dr. S. M. Reader of John Innes Centre, England, provided Chinese Spring-Imperial rye additions 1R-7R. *Triticum. teres* (AADD genomes), *Triticum aestivum* cv. Anyuepaideng-*S. africanum* amphiploid, *Triticum carthlicum-S. africanum* amphiploid and *Triticum turgidum* cv. Jianyangailanmai-*S. africanum* amphiploid were developed by Professor Hua-Ren JIANG, Sichuan Agriculture University, China. Professor Xianzhi ZHANG, Sichuan Agriculture University, China, produced triticale line Fenzhi-1. Lines L1-L20 were developed from BC1F7 of cross and backcross of MY11 to *Triticum durum-S. africanum* amphiploid.

2.2 DNA extraction and RAPD analysis

Total genomic DNA was prepared from young leaves according to Yang *et al*. (2005). The DNA concentration was determined by using a sizhumen DNA-protein photometer. RAPD amplification was performed in an Icycler thermalcycler (Bio-RAD Laboratories). Total reaction volume is 25 μL containing 10 mmol Tris-HCl (pH 8.3), 2.5 mmol $MgCl_2$, 200 μmol/L of each dNTP, 30 ng template DNA, 0.2 U *Taq* polymerase (Takara, Japan), and 400 nmol primer. The cycling parameters were 94°C for 3 min to pre-denature; followed by 40 cycles of 94°C for 1 min, 38°C for 1 min, 72°C for 2 min; and then a final extension at 72°C for 10 min. The amplification products were electrophoresed through 1.0% agarose gels in 1×Tris-acetic-EDTA (TAE) buffer and detected by staining with 10 μg/mL ethidium bromide.

2.3 Cloning and sequencing of rye RAPD products

The genome-specific RAPD products identified from PCR were excised from 1.0% agarose gels and purified by a gel extraction kit (Qiagen, Valencia, Calif.). The purified products were ligated into the pT7 Blue R-Vector using T4 ligase, and then introduced into *Escherichia coli* DH5α, by heat shock transformation. Nucleotide sequencing was performed on a polyacrylamide gel with the ABI prism 377 sequencer (Perkin Elmer) as an automated fluorescent sequencing system. The BLAST program in the GenBank database was used to search for sequence similarities with DNA (BLASTN).

2.4 Specific PCR primer design and amplification

Based on the cloned sequence of RAPD products, a pair of specific PCR primers was designed by using software Primer 5.0, and synthesized by Invitrogen Biotech, Shanghai, China. The PCR reaction, with a 25 μL volume containing 50 ng genomic DNA, 0.2 μmol/L of each primer, 200 μmol/L of each dNTP, 1×PCR buffer, 2.0 mmol/L $MgCl_2$ and 1 U *Taq* polymerase (Takara, Japan), was performed at 94°C for 3 min; then through 30 cycles of the following program: 1 min at 94°C, 1 min at 65°C, and 2 min at 72°C; with a final extension at 72°C for 10 min. The PCR products were fractionated on 1% agarose gels in 1×Tris-acetic-EDTA (TAE) buffer and detected by staining with 10 μg/mL ethidium bromide.

2.5 Chromosome preparation and FISH

Germinating seeds were placed at 4°C for 24 h to synchronize cell divisions and transferred to room temperature (25°C). After 24 h, root tips were harvested at 2–3 cm in length, and then pretreated in ice water for 24 h, before fixing in 1∶3 acetic acid-ethanol. The root tips were macerated finely on a glass slide, and then squashed by hands, followed by removal of cover slips after freezing with liquid nitrogen.

For FISH analysis, the genome respective DNA from *S. africanum* was labeled with digoxigenin-11-dUTP according to the manufacturer's instruction (Roche Diagnostics, Indianapolis, IN, USA). The insert of the plasmid was labeled with fluorescence-14-dUTP (Roche Diagnostics) by nick translation. The probes were diluted to a final concentration of 1 μg/mL in the hybridization solution and the hybridization mixture was prepared as described by Mukai (1993). The digoxigenin labeled genomic DNA signal was detected with fluorescein-conjugated antidigoxigenin antibody (Roche Diagnostics). The slide was finally mounted in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA) with (0.25 μg/L) propidium iodide for only FITC detection. Microphotographs of GISH chromosomes were taken with an Olympus BX-51 microscope.

3 Results

3.1 Isolation of *Secale* specific fragment

Total 240 RAPD primers were used to produce the *Secale* specific PCR markers, in which one primer OPP13 (5′-GGAGTGCCTC-3′) gave rise to two specific DNA bands about 1200 bp and 700 bp from all materials containing rye

chromatin, including cultivated rye (*S. cereale*), wild *Secale* and hexaploid triticale, while it could not amplify the DNA sample from the tested common wheat cultivars (Fig. 1).

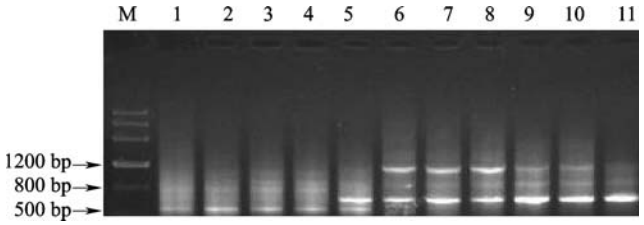

Fig. 1 The amplification result of RAPD primer OPP13 on rye and common wheat. M: Marker (DL4500); 1: CS; 2: MY11; 3: *T. aestivum*; 4: *T. carthlicum*; 5: Fenzhi-1; 6: *S. vavilovii*; 7: *S. africanum*; 8: *S. silvestre*; 9: *S. strictum.* subsp. *anatolicum*; 10: *S. cereale* L. cv. Qinling; 11: *S. cereale* L. cv. Jingzhou.

3.2 DNA fragment cloning, sequencing and analysis

The *Secale* genome-specific RAPD band from primer OPP13 was cloned and sequenced. The full lengths of the products were 1165 bp (Fig. 2A) and 662 bp (Fig. 2B), and were designated as $OPP13_{1165}$ and $OPP13_{662}$, respectively. Blastn search in NCBI GenBank revealed that $OPP13_{1165}$ has 88% sequence identity to the second intron of a new class of transposon-like gene called the *Revovler* family. tBlastx analysis revealed that the deduced amino acid sequence of $OPP13_{662}$ is significantly homologous to retrotransposon partially.

3.3 Chromosomal distribution of $OPP13_{1165}$ FISH analysis

In order to determine the chromosomal distribution of $OPP13_{1165}$, the probe $OPP13_{1165}$ was labeled to hybridize

GGAGTGCCTCGTGTGTTCTTGTGTTTGAGTAGAGCCAGAAGCCGAGTACGAGAACGAGGTTACTACCGAGGTT
GAGAACGAGGAGCCCTCTTACTATCCCATCGACGACTCAACAGGCAAGATGACCGGACCCAGATATCATTACT
ATATTTGCCTATTGCTAGAAGTCTCGCTCTTTAGCTTATTGCCTCGATGCCCATGTTTTCCTGTCAGCCTCCTAT
TGTAACCATGATCCTGTCTAACCACCCTACCTATGCAAACCATTGATTGGCTACGTAGGCTTCGCTCAGCCCCT
CTTATAGCATTGCTAGTTGCAGGAGAAGATTGAAGATTCTTGCTTCTTGTTGAAGCTTGTTGGGATATCACACT
ATATAAACTCTTAATGAAATCATCTATATATTGGTAATGGGTGGAAGGCTAAGCCTTTTGCTTGGTGATTTGTT
CCACTCATGCCGCCCTAGGAACCGTATAAACCGGTGTTATGTTCCTTGATTTTGCGTTCCTCACACGGTTGGGG
TTATGGGACCCCCTCGATAAACCGCTAAGTGCTAAGTCTTTTCCAGCAAGTCCCAACATTGGTACTATTTGCCT
AATAACTAAAAACTTGCCGAGGGAGTAATTAACCCGAGGATTAATTAATCAACCCTCCTGGGCCAGTGCTCG
ATTTGAGTGTTGGTCCAAAATGGGCGGACTGCAGGGCCACCTTGGGGGAACCCGAGGGCTGGTATTTTCCTGT
AGGCTGACCTATCCGGTCGTGGCCTGAGACTAGATACGCGCGGCTATTATCAGGGTGTCGGTACGCCGGGAG
GATTTGCTGGATTAGCCTTACCTTAGTCAGAGTATCTTGAGCACGGGATTCCGAGGATACTCGGGCCTTCCCA
CCTTGGAGTTGCGACTCCGCGGATCATGGGCTTGTCATGGGCCAAGTTGGAACACCCCTGCAGGGTTTTGAAC
TTTCGAAAGCCGTGCCCGCGGTTATGTGGCAGATGGGAATTTGTTAATATCCGGTTGTAGAAAACTTGAAACC
ATGTTCAGAACACACTACCAGCGTGAGTACCGTGACGGTCATTTTCCGAAAGGGATTCGGGAAGTGAACACG
GTGGGGGTTATGTTTGACTTGCTTAGTTAGGATCACTTCGTGATCATCTTATCGTCCGAGGCACTCC

A

GGAGTGCCTCACTGAACTCAGCCCAGGTAGTGACTTGGCCAGCAGGACGCATAGCTTCAAAGTTTTC
CCACCAAAGACTGGCAGGGCCCTCAAGATGATAGGCGGCGTAGATAACCTTGTCAGCTTCGGCCACA
CGGGCGGAGTATAGCTTGTGGGTAATACTACGTAGCCAGTCATCAGCATCCAGAGGTTCAGTGGAGT
GATGGAACTTCGGTGGACACATCTTGATAAAATCATGGATTGACACCACATCATTCCTCTGACGGAA
CCCAGTGTTCTGCTCTATGCGTTCCAGCAGACGGTTGGTCTCTCTCTTGTTCCGTTCTGCTTCAAGCAT
AACTTCCGCCAAGGAGGGTGGCTGGGGCAGATTTCCATCTCTAGCCCCACTAGCCTCACCCTGCTCCT
GAGCAGCAGGAGTGTTGCGAGTGTTGACCATCCTAGGAAAACAAAACAACGGTTTAGACAAGGATG
GCAAAAATTTGACATAGAAGTGCAGAATGTAATGGATGACACAGAATGCAGAGATGTTCATCCGTA
CGACATGGTAATATAGGAACTGCTATATATACACCAGTGGTCATACACACCATACAGAGTTTAGTAC
AAGCCAGGGCTAAAGTACAACTGCGGTGAAAGACGATACATCCCATCAGAGGCACTCC

B

Fig. 2 The sequence of $OPP13_{1165}$ (A) and $OPP13_{662}$ (B). The SCAR marker primers were synthesized just as it showed by arrows in the picture; Primers OPP13 were in the frame.

S. cereale L. cv. Qinling mitotic metaphases by FISH. The result showed that the signals distributed on proximal regions of the chromosome arms but not on the centromeric and terminal regions (Fig. 3A). $OPP13_{1165}$ was hybridized to a somatic mitotic metaphase of *T. turgidum* cv. Jianyangailanmai-*S. africanum* amphiploid. It was found that there were 14 *S. africanum* chromosomes showing strong signals dispersed across the chromosome arms, except centromeric and terminal regions (Fig. 3B). This result was the same as Tomita et al. (2008) gained, in which FISH signals also dispersed on the seven rye chromosomes.

3.4 Application of PCR marker for $OPP13_{1165}$ and $OPP13_{662}$ in wheat-*Secale africanum* introgression lines

Based on the nucleotide sequence of $OPP13_{1165}$, two pairs of SCAR markers P13L-F/R (P13L-F: 5′-TCCTGTCTAACCACCCTACC-3′, P13L-R: 5′-TCACTTCCCGAATCCCTTT-3′, length between the two primers is 862 bp) and P13S-F/R (P13S-F: 5’-GGAGTGCCTCACTGAACTCAG-3′, P13S-R: 5′-GGGATGTATCGTCTTTCACCG-3′, length between the two primers is 648 bp)(Fig. 2) were designed to test the validity of the molecular marker in *Secale*. PCR amplification result showed that the target band could be detected in *S. africanum* and other materials containing *Secale* chromatin, while the target band was absent in CS, MY11, *T. aestivum, T. carthlicum* and *T. teres* (Fig. 4A and 4B). At the same time, P13L and P13S were used to amplify *T. aestivum* cv. Anyuepaideng-*S. africanum* amphiploid, *T. carthlicum-S. africanum* amphiploid, *T. turgidum* cv. Jianyangailanmai-*S. africanum* amphiploid and their corresponding wheat parents. The results suggested that the target band amplified in all amphiploids, but was absent in their corresponding wheat parents (Fig. 4E and 4F). Meanwhile, we used the P13L and P13S to amplify BC_1F_7 derivatives derived from *Triticum durum-S. africanum* amphiploid. The amplified results were consistent with those by H20-F/R (Fig. 4C and 4D). Moreover, to understand on which chromosomes the two sequences exist, the two pairs of SCAR markers were performed on a set of Chinese Spring-Imperial addition lines. The result showed that $OPP13_{1165}$ distributed on all rye chromosomes (Fig. 4G), which is consistent with the result from FISH in Fig. 3. $OPP13_{662}$ distributed on six rye chromosomes except the fourth chromosome (Fig. 4H).

4 Discussion

Transposable elements are ancient and ubiquitous components of plant genomes. Some of them were used as molecular markers in cereal breeding or in evolution study (Tomita et al., 2008). By far, several approaches have been used for isolating transposable elements. The DNA library screen is an effective approach to isolate specific repeated sequences. Ito et al. (2004) isolated the sequence of $pHind_{258}$ like Ty3-*gypsy* retrotransposons from *Triticum monococcum*, and Francki (2001) isolated a diverged centromeric Ty1-*copia* retrotransposon family from cultivated rye cv. *Billy*. Zhang et al. (2004) identified a sequence in clone pAet6-J9 with high similarity to the Ty3-*gypsy* centromeric retrotransposon from *Aegilops tauschii*. Moreover, recovery of specific DNA fragment from restriction enzyme-digested products is also an approach for isolation of retroelement. Linares et al. (1999) isolated a repetitive sequence, pAs17, from *Avena strigosa* (As genome) through this approach, which was highly homologous to the long terminal repeat (LTR) sequences of the specific family of Ty1-*copia* retrotransposons represented by WIS2–1A and Bare. Through the deletion enrichment scheme, Tomita et al. (2008) isolated *Revovler* family, a new class of transposon-like gene. Compared with the three approaches above, PCR based techniques including the RAPD method also give rise to the easy isolation of transposable elements of repetitive sequences. pSc20H (Ko et al., 2002) and pSaO5 (Jia et al., 2009) were isolated from rye, which showed significant homology to several reported retrotransposonsl. In this study, we have isolated two rye specific transposable elements using one RAPD marker. Blastn revealed that $OPP13_{1165}$ had 88%

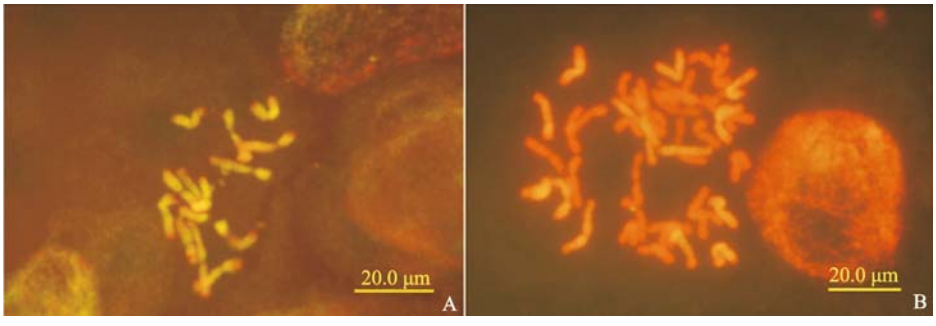

Fig. 3 Fluorescence *in situ* hybridization results using the probe $OPP13_{1165}$ on mitotic metaphases of *S. cereale* L cv. Qinling (A) and *T. turgidum* cv. Jianyangailanmai-*S. africanum* amphiploid (B).

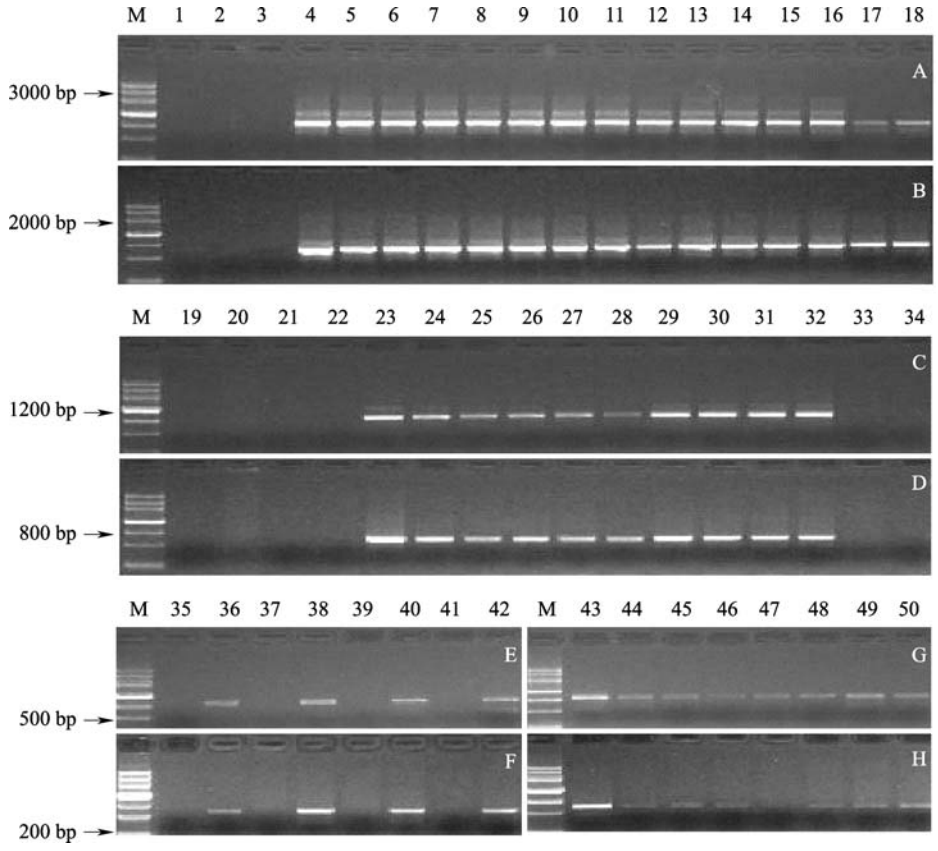

Fig. 4 The amplification result of SCAR primers P13L and P13S on wheat-*S. africanum* introgression lines. M: Marker (DL4500); 1: CS; 2: MY11; 3: *T. aestivum*; 4: Fenzhi-1 *triticale*; 5: Beagle; 6: XY285; 7: HYslop; 8: *S. africanum*; 9: *S. cereale* L. cv Qingling; 10: *S. cereale* L. subsp. *ancestrale*; 11: *S. strictum.* subsp. *anatolicum*; 12: *S. vavilovii*; 13: *S. Silvestre*; 14: *Secale cereal* cv. *kustro*; 15: *Secale strictum*; 16: *S. cereale* L. cv. Jingzhou; 17: *S. cereale subsp. cereale*; 18: *S. cereale subsp. cereale* CISE45; 19–34: *T. durum-S. africanum* amphiploid's BC1F7 derivatives; 35: *T. aestivum* cv. Anyuepaideng; 36: *T. aestivum* cv. Anyuepaideng-*S. africanum* amphiploid; 37: *T. carthlicum*; 38: *T. carthlicum-S. africanum* amphiploid; 39: *T. turgidum* cv. Jianyangailanmai; 40: *T. turgidum* cv. Jianyangailanmai-*S. africanum* amphiploid; 41: *Aegilops tauschii*; 42: *Aegilops tauschii-S. silvestre* amphiploid; 43: *S. africanum*; 44–50: CSDA1R-7R.

sequence identity to the second intron of *Revovle*-4 and $OPP13_{662}$ was similar to part of an LTR *gypsy*-like retrotransposon in *Triticum aestivum* with 80% identities. This is the first report that one RAPD marker isolated two rye specific transposable elements.

Repetitive DNA sequences have been used as an effective tool in the detection of *Secale* chromatin in wheat background. The PCR-based markers (Lapitan et al., 1988; McIntyre et al., 1990; Iqbal and Rayburn, 1995; Brunell et al., 1999) and FISH (Nkongolo et al., 1993; Alkhimova et al., 1999; Liu et al., 2008) correlated with these repetitive DNA sequences have been released. In this study, sequences $OPP13_{1165}$ and $OPP13_{662}$ have proved to be useful as a molecular marker to detect *Secale* chromatin of cultivated wild *Secale* species, hexaploid triticale, as well as different kinds of wheat-*S. africanum* amphiploids, additions and relative derivatives. Therefore, compared with previous markers, P13L and P13S markers can be used in a wide scope. FISH revealed that the signals of $OPP13_{1165}$ distribute on all *Secale* chromosomes. Therefore, we can expect that the PCR marker and FISH for the sequences $OPP13_{1165}$ can detect the *Secale* chromatin introduced in wheat background especially in the early generation of wheat-*Secale* hybridization.

Acknowledgements We thank the National Natural Science Foundation of China (Grant No. 30671288, 30871518), Program for New Century Excellent Talents in Universities (No. NCET-06-0810) and Young Scholars Foundation from the Science and Technology Committee of Sichuan Province (No. 2008-31-371) for financial support.

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