Development and Application of EST-Based Markers Specific for Chromosome Arms of Rye (*Secale cereale* L.)

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**Key Words**
Chromosome • Expressed sequence tag • Marker specificity • Rye • Wheat breeding

**Abstract**
To develop a set of molecular markers specific for the chromosome arms of rye, a total of 1,098 and 93 primer pairs derived from the expressed sequence tag (EST) sequences distributed on all 21 wheat chromosomes and 7 rye chromosomes, respectively, were initially screened on common wheat 'Chinese Spring' and rye cultivar 'Imperial'. Four hundred and fourteen EST-based markers were specific for the rye genome. Seven disomic chromosome addition lines, 10 telosomic addition lines and 1 translocation line of 'Chinese Spring-Imperial' were confirmed by genomic in situ hybridization and fluorescence in situ hybridization, and used to screen the rye-specific markers. Thirty-one of the 414 markers produced stable specific amplicons in 'Imperial', as well as individual addition lines and were assigned to 13 chromosome arms of rye except for 6RS. Six rye cultivars, wheat cultivar 'Xiaoyan 6' and accessions of 4 wheat relatives were then used to test the specificity of the 31 EST-based markers. To confirm the specificity, 4 wheat-rye derivatives of 'Xiaoyan 6 × German White', with chromosomes 1RS, 2R and 4R, were amplified by some of the EST-based markers. The results indicated that they can effectively be used to detect corresponding rye chromosomes or chromosome arms introgressed into a wheat background, and hence to accelerate the utilization of rye genes in wheat breeding.

Rye (*Secale cereale* L., RR, 2n = 2x = 14) has played an important role in wheat (*Triticum aestivum* L.) breeding as a genetic resource. Compared with other wheat relatives, rye is outstanding with regard to its biotic and abiotic tolerances, making it a valuable resource for wheat improvement. For example, the short arm of chromosome 1R carries many genes for resistance, such as powdery mildew (caused by *Blumeria graminis* f. sp. *tritici*) resistance genes *Pm8* and *Pm17* [Hsam and Zeller, 1997], stem rust (*Puccinia graminis* f. sp. *tritici*), leaf rust (*Puccinia triticina*) and stripe rust (*Puccinia striiformis* f. sp. *tritici*) resistance genes *Sr31*, *Lr26* and *Yr9* [Mago et al., 2005], and the Russian wheat aphid (*Diuraphis noxia*) resistance gene *Dn7* [Lapitan et al., 2007].

H.X. and D.Y. contributed equally to this work.
Genes of rye were introduced into wheat through various types of wheat-rye hybrid derivatives including chromosome addition lines, substitution lines and translocation lines. Following such events it is necessary to identify which chromosome arms or segments of rye were transferred to wheat. Compared with C-banding, genomic in situ hybridization (GISH), fluorescence in situ hybridization (FISH) and sodium dodecyl sulfate polyacrylamide gel electrophoresis [Li et al., 2007], DNA-based markers are a convenient and efficient method to detect alien chromosome segments in common wheat [Schneider and Molnár-Láng, 2008; Wang et al., 2009a]. There are several reports on various DNA-based markers for detecting chromosome arms of rye, such as random amplified polymorphic DNA markers OPRI9 and OPJ07 [Iqbal and Rayburn, 1995], OPC10 and OPHI20 [Ko et al., 2002]; sequence-tagged site markers NOR, 55, TEL, RIS [Koebner, 1995], IAG95 [Mohler et al., 2001], GPI, PSR960 [Nagy et al., 2003; Schneider and Molnár-Láng, 2008] and F3R3 [Katto et al., 2004]; simple sequence repeat (SSR) markers SCM9 [Saal and Wricke, 1999], RMS13 [Nagy et al., 2003] and BMAC213 [Schneider and Molnár-Láng, 2008]; intersimple sequence repeat (ISSR) markers ISSR1 and ISSR9; sequence-characterized amplified region markers SC-ISSR1 and SC-ISSR9 [Vaillancourt et al., 2008], and sequence-specific amplification polymorphism markers S10, S20 and S17 [Nagy and Lelly, 2003]. However, these markers are mostly specific for the entire rye genome or to chromosome 1RS, and with some markers, there are problems with stability and reproducibility. Thus, it is necessary to develop a set of stable DNA-based markers specific for individual chromosome arms of rye to facilitate detection in wheat-rye hybrids and their derivatives.

Comparative genomics revealed collinear relationships between wheat and its related species. Expressed sequence tag sequences (ESTs) are especially valuable for developing molecular markers since they are derived from gene transcripts and are likely to be conserved among gene transcripts in related species [LaRota and Sorrells, 2004]. As of December 2011, more than 1,361,630 wheat ESTs were available from GenBank (http://www.ncbi.nlm.nih.gov/) and more than 16,000 ESTs have been physically mapped on the 21 wheat chromosomes [Qi et al., 2004]; this facilitates the development of EST-based markers. However, only a limited number of rye ESTs have been reported and chromosomally located [Khlestkina et al., 2004, 2005; Hackauf et al., 2009]. Compared with genomic SSR markers, the development of EST-based markers is less costly and EST markers do not have the disadvantage of genomic repetitiveness. Moreover, EST-based markers derived from conserved coding regions in common wheat show high levels of transferability to various relative species [Zhang et al., 2005, 2007]. Wang et al. [2009a] developed 2 wheat EST-based markers, STS_{WE5} and STS_{WE126} that were specific for rye chromosome 1RS, and these were used in the detection of T2BL:1RS chromosome translocations [Wang et al., 2009b]. Lee et al. [2009] developed EST-derived 2RL-specific markers for a 2BS-2RL translocation and assessed their function. Wang D et al. [2010] developed some EST-based markers specific for 6RL and 6RS of Chinese rye cultivar 'Jingzhou'.

In this study, 1,098 markers derived from ESTs from all 21 wheat chromosomes and 93 markers from ESTs on 7 rye chromosomes were designed and used to develop specific markers for the rye genome, and sets of wheat-rye chromosomes and chromosome arm addition lines. These specific EST-based markers can be used for detecting introgressions of rye chromosomes into wheat.

Materials and Methods

Plant Materials

Common wheat cultivars 'Chinese Spring' and 'Xiaoyan 6' (AABBD), 7 rye cultivars ('Imperial', 'German White', 'Guyuan', 'King', 'America', 'Jingzhou' and 'Austria' (RR)), 4 wheat relatives (Hordeum vulgare (HH), Haynaldia villosa (VV), Roegneria kamoji (SSHHY) and Agropyron cristatum (PPPPP)), triticale lines '06CT456' and '06CT461' (AABBRR) and wheat-rye T1BL:1RS translocation lines 'Lorvin 10' and 'Lorvin 13' [Rabinovich, 1998] are maintained in our laboratory. Four wheat-rye lines (WR41, WR64, WR81, and WR91), derived from 'Xiaoyan 6 × German White' were developed in our laboratory. Seven disomic addition lines of 'Chinese Spring-Imperial' (DA1R–7R), 9 ditelosomic addition lines and 1 monotelosomic addition line of 'Chinese Spring-Imperial' (DTA1RL, DTA4RL-DTA7RL, GTA3RS-DTA5RS, MTA6RS and DTA7RS), and one T2AS-2RL translocation line of 'Chinese Spring-Imperial' were kindly provided by Dr. S. Reader (John Innes Centre, Norwich, UK).

Fluorescence in situ Hybridization

FISH was carried out as described by Wang et al. [2009b]. Seeds were germinated on wet filter papers in Petri dishes at 23°C. Root tips 1–2 cm long were excised and pretreated in ice-water for 24 h, then fixed in freshly prepared absolute ethanol-acetic acid (3:1) solution for 2 d. Root tips were stained in 1% acetocarmine for 2 h and squashed in a drop of 45% acetic acid on glass slides under a coverslip. After the coverslips had been freed in liquid nitrogen, they were removed and the slides were air-dried and stored at –20°C for future use. Multicolor FISH was carried out with 2 highly repeated DNA sequences, pAsl [Rayburn and Gill, 1986] labeled with biotin-16-dUTP (Roche, Germany) and

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The PCR profile included: 1 cycle at 94 °C for 5 min, followed by 38 cycles at 94 °C for 60 s, 50–60 °C (depending on the specific systems, Perkin-Elmer, USA) in 10-
mol of MgCl₂, 1 U of Taq DNA polymerase, and 1

Genomic in situ Hybridization

After FISH, slides were washed and air-dried for GISH analysis as described by Wang QX et al. [2010]. Total genomic DNA of rye was sonicated into 500–800-bp fragments and labeled with biotin-16-dUTP (Roche, Germany) by nick translation and used as probe. Total genomic DNA of 'Chinese Spring' wheat was sheared and used as blocking DNA and mixed with the DNA probe in a ratio of 30:1. The wheat chromosomes were counterstained by DAPI. Cells with good hybridization signals were photographed with a DVC CCD digital camera (Nikon Corporation, Tokyo, Japan).

Marker Analysis

In this study, 93 rye EST accessions [Khlestkina et al., 2005; Hackauf et al., 2009] from all 7 rye chromosomes were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/) and used to design EST-based primer pairs by using the software Primer Premier 5.0 (Premier Biosoft International, Palo Alto, Calif., USA). To develop a set of EST-based markers specific for the chromosome arms of rye 1R–7R, the 93 rye EST-based primer pairs from all 7 rye chromosomes and 1,098 wheat EST-based primer pairs randomly distributed on all 21 wheat chromosomes (http://www.wheat.pw.usda.gov/index.shtml) were used in this study. All EST-based primer pairs were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co. Ltd., Shanghai, China.

Genomic DNA was extracted from young seedling tissues following the procedure described by Ma et al. [1994]. PCRs were performed in a GeneAmp9700 PCR System (PE Applied Biosystems, Perkin-Elmer, USA) in 10-µl volumes containing 30 ng of template DNA, 2 pmol of each primer, 2 nmol of each dNTP, 15 nmol of MgCl₂, 1 U of Taq DNA polymerase, and 1× PCR buffer. The PCR profile included: 1 cycle at 94°C for 5 min, followed by 38 cycles at 94°C for 60 s, 50–60°C (depending on the specific primers) for 60 s and 72°C for 60 s, and a final extension at 72°C for 10 min. The PCR products were separated in 8% non-denaturing polyacrylamide gels with 19:1, 25:1 or 39:1 ratios of acrylamide and bisacrylamide, and then silver-stained as described by Tixier and Sourdille [1997].

Results

Confirmation of Wheat-Rye Lines with FISH and GISH

Seven disomic addition lines of ‘Chinese Spring-Imperial’ (DA1R–DA7R), 10 telosomic addition lines of ‘Chinese Spring-Imperial’ (DTA1RL, DTA4RL–DTA7RL, DTA3RS–DTA5RS, MTA6RS and DTA7RS) and one T2AS-2RL translocation line of ‘Chinese Spring-Imperial’ were used to screen for presence of specific markers on rye chromosome arms. The chromosomal constitutions of these lines were first confirmed by combined FISH with GISH as described by Mukai et al. [1992, 1993]. Figure 1 shows the results of GISH and FISH on a 6R disomic addition line (DA6R) and a 6RS monotelosomic addition line (MTA6RS). Four ‘Xiaoyan 6 × German White’ derivatives (WR41, WR64, WR81 and WR91) that were used to test the validity of specific markers were also analyzed by GISH and FISH. The results indicated that WR64 and WR81 were both T1BL-1RS translocation lines, WR91 was a 2R (2D) substitution line and WR41 was a translocation line involving rye chromosome 4R (not shown).

Screening for Rye-Specific Markers with Wheat and Rye EST-Based Markers

Polymorphisms were firstly evaluated between ‘Chinese Spring’ and ‘Imperial’ rye; 378 of 1,098 primer pairs derived from wheat ESTs and 36 of 93 primer pairs from rye ESTs produced stable amplicons in ‘Imperial’ rye and showed polymorphisms between ‘Chinese Spring’ wheat and ‘Imperial’ rye. The polymorphic rates of the wheat and rye EST-based markers were 34 and 39%, respectively. Thus, a total of 414 EST-based markers appeared to be specific for the rye genome, accounting for 35% of the 1,191 markers.

Mapping EST-Based Markers Specific for Rye Chromosome Arms

The complete set of ‘Chinese Spring-Imperial’ disomic addition lines were used to locate the 414 markers on specific chromosomes of rye using ‘Chinese Spring’ and ‘Imperial’ as the checks. Most of the markers did not produce specific amplicons in any addition line and could not be mapped to rye chromosomes, or failed to produce just 1 specific amplicon in a particular addition line and were mapped to more than 1 chromosome of rye. Of the 414 markers, 30 produced only 1 amplicon in a particular disomic addition line and ‘Imperial’, and thus were located on the corresponding rye chromosome. Marker CGG143 produced 2 different specific amplicons in ‘Imperial’, and the 1R and 6R disomic addition lines. Figure 2 shows the amplification of CGG16 on ‘Chinese Spring’, ‘Imperial’ and the 7 disomic addition lines examined. CGG16 was specific for chromosome 5R. Of the 31 rye-specific markers, 4 (SWES999, SWES1119, SWES1128 and CGG143-1RL), 4 (CGG62, CGG8, CGG9 and SWES120), 2 (SWES228 and CGG32), 6 (KSUM62,
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Fig. 1. GISH (left) and FISH (right) analysis of wheat-rye addition lines. Wheat chromosomes (blue) were counterstained with DAPI, and the green fluorescein hybridization signals (A, C) indicated alien rye chromosomes or chromosome arms (marked by arrows in B and D). A, B 6R disomic addition line, DA6R and C, D 6RS monotelosomic addition line, MTA6RS. B, D Multicolor FISH on the same metaphase cell of DA6R and MTA6RS using pAsl (green) and pSc119.2 (red) simultaneously.


CFE218, CGG49, MAG1424, SWES180 and SWES182), 6 (MAG1242, CGG4, CGG16, CGG18, CGG19 and CGG43), 7 (CGG143-6R, CGG23, CGG59, SWES78, SWES206, SWES231 and DUPW111) and 3 (CFE228, DUPW535 and CGG26) mapped on chromosomes 1R, 2R, 3R, 4R, 5R, 6R and 7R, respectively.

Nine ditelosomic addition lines, 1 monotelosomic addition line (DTA1RL, DTA4RL–DTA7RL, DTA3RS–DTA5RS, MTA6RS and DTA7RS) and one T2AS-2RL translocation line of ‘Chinese Spring-Imperial’ were further used to map the 31 specific markers of rye chromosomes to various chromosome arms using ‘Chinese Spring’ and ‘Imperial’ as checks. SWES999 among the 4 markers specific for chromosome 1R was assigned to
chromosome 1RS, and the other 3 to chromosome 1RL. In the same way, all other markers specific for 2R–7R, except CGG143-6R, were assigned to the corresponding long or short arms of rye chromosomes for which they were specific (table 1). CGG143-6R produced a specific amplicon of about 100 bp only in 'Imperial' and DA6R, and hence could not be mapped to a specific 6R arm (fig. 3). We also showed that MAG1242, which mapped to 5RS (fig. 4), produced a specific amplicon of about 500 bp in 'Imperial', DA5R and DTA5RS, but not in 'Chinese Spring', DTA5RL or any other line. Table 1 summarizes all the results on the chromosomal locations of the 31 specific markers distributed across 13 rye chromosome arms excluding 6RS.

<table>
<thead>
<tr>
<th>Chr. arm</th>
<th>Specific marker</th>
<th>EST</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
<th>Tm, °C</th>
<th>Size, bp</th>
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<td>1RS</td>
<td>SWES999</td>
<td>CJ548455</td>
<td>ACGCTGCGGATGAAATGAGT</td>
<td>GCTGCGAATGCTGAAAGG</td>
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<td>TGGCGTCTTGACTACATT</td>
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<td>160</td>
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<td>BE587051</td>
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<td>CD899455</td>
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<tr>
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<td>BE905766</td>
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<td>CAGACGGAATGCTGATAG</td>
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<td>480</td>
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<td>160</td>
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<td>TC256531</td>
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<td>150</td>
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</table>

*This marker failed to associate with 6RL or 6RS.

### Evaluation of EST-Based Markers Specific for Rye Chromosome Arms

Wheat cultivar 'Xiaoyan 6', rye cultivars 'German White', 'Guyuan', 'King', 'Amerika', 'Jingzhou' and 'Austria' (RR), and 4 wheat relatives (Agropyron cristatum (PPPP), barley (Hordeum vulgare, HH), Haynaldia villosa (VV) and Roegneria kamoji (SSHHYY)) were used to test the specificity of the 31 EST-based rye markers using 'Chinese Spring' and 'Imperial' as checks. The markers were specific only for rye. Several markers produced specific stable amplicons in 'Imperial' but not in all 6 rye cultivars due to polymorphisms among the rye cultivars; however, most of them produced stable specific amplicons in 'Imperial' and the other 6 rye cultivars, but not in...


more than 1 species are present. Thus, these markers can be used to track the alien rye chromosomes or chromosome arms in a common wheat background or in situations where alien chromosomes from more than 1 species are present.

**Application of EST-Based Markers Specific for Rye Chromosome Arms**

The 31 rye-specific markers were used to amplify DNA from the 4 'Xiaoyan 6 × German White' derivatives (WR41, WR64, WR81, and WR91), wheat-rye T1BL·1RS translocation lines 'Lovrin 10' and 'Lovrin 13', and triticale (AABBRR) lines '06CT456' and '06CT461' using 'Xiaoyan 6' and 'German White' as checks. The 4 'Xiaoyan 6 × German White' lines were confirmed by GISH and FISH to be translocation lines involving rye chromosome 4R, T1BL·1RS translocation lines (WR64 and WR81), and a 2R (2D) substitution line, respectively. The markers produced specific amplicons in 'German White' and the 2 triticale lines. The 1RS-specific marker SWES999 produced an amplicon in 'Lovrin 10', 'Lovrin 13', WR64 and WR81; and similarly, the 2R (CGG62, CGG8, CGG9 and SWES120) and 4R (KSUM62, CFE218, CGG49, MAG1424, SWE180 and SWE182) specific markers produced specific amplicons in WR91 and WR41, respectively (not shown). Figure 5 shows amplification of the 4RS-specific marker KSUM62, which produced a specific amplicon of about 160 bp in 'Imperial', 'German White', WR41 and the 2 triticale lines, but not in the other lines, indicating that KSUM62 could be used to detect chromosome 4R. These results thus demonstrated that the specific markers could be used for tracking rye chromosomes or chromosome arms in a common wheat background.

**Discussion**

PCR-based markers are more convenient and efficient tools to identify alien chromosomes, chromosome arms or translocated segments in wheat than cytological methods such as C-banding, GISH and FISH [Wang et al., 2009a]. Since Koebner [1995] first generated PCR-based markers for the detection of rye chromatin in wheat, more PCR-based markers were developed to track introductions [Lee et al., 2009; Wang et al., 2009a]. However, these markers were either specific for the entire rye genome or just to the 1RS and 2RL chromosomes. The current availability of EST sequences deposited in a public database has facilitated the development of EST-derived markers for species related to wheat. Zhuang et al. [2008] mapped 8 SSR-specific markers derived from wheat EST sequences on rye chromosomes 1R, 4R, 5R and 7R. Prior to the present study, most rye chromosome arms lacked specific markers. Now, a total of 31 EST-based specific markers different from those used in previous studies are mapped to 13 rye chromosome arms excluding 6RS.

The EST-based marker CGG143 produced a specific amplicon on 6R, but could not be mapped on either 6RS or 6RL (fig. 3 and table I). However, SCM304 [Hackauf et al., 2009], which was derived from the same EST sequence as CGG143, was mapped to 6RS with a set of ditelosomic addition lines of 'Huixianhong-Jingzhou' (wheat-rye) [Wang D et al., 2010]. The identification of the 6RS addition line (MTA6RS) of 'Chinese Spring-Imperial' used in this study was confirmed by GISH and FISH (fig. 3), which showed that it was a true monotelosomic addition line. As many polyploids undergo extensive and rapid genomic alterations [Adams and Wendel, 2005], the 6RS loci Xcggl43 and Xscm304 were presumably eliminated or altered in the 6RS monotelosomic addition line of 'Chinese Spring-Imperial' used in this study.

Among the 31 specific markers for rye chromosome arms, 17 and 14 of them were derived from wheat and rye ESTs, respectively. Due to high transferability between the wheat and rye genomes [Zhuang et al., 2008], wheat ESTs were used by others to develop 1RS- and 2RL-specific markers [Lee et al., 2009; Wang et al., 2009a]. In this study, 17 specific markers derived from wheat ESTs were identified on 8 rye chromosome arms besides 1RS and 2RL. In addition, 14 specific markers from rye EST sequences [Khlestkina et al., 2005; Hackauf et al., 2009] were mapped to 2RS and the 7 long chromosome arms of rye (table I). Up to now, the development of rye-specific markers from rye ESTs was rarely reported due to the accuracy of rye EST sequences which were relatively short and difficult to compare with the available sequence data [Lee et al., 2009; Wang D et al., 2010]. Irrespective of their origin (wheat or rye), these specific markers will be helpful in the analysis of genetic similarity, comparative mapping between wheat and rye, and isolation of functional genes.

Only after alien chromosome or chromosomal segments will have been transferred into wheat and precisely identified, they can be efficiently utilized. Obviously, rye-specific markers can contribute to the transfer from rye to wheat, although less specific markers for rye chromatin were used in the past. While this study aimed at the development of a set of specific markers for rye chromosome arms, further work is required to position the specific markers within the arms to enable their use in reducing the size of alien segments.
A well-saturated molecular linkage map can be used for studying genetic diversity, analyzing high numbers of genotypes and gene tagging. In this study, 1,098 and 93 EST-based markers derived from wheat and rye ESTs, respectively, were screened for polymorphisms between wheat and rye, and more than 378 and 36 markers produced the polymorphic amplicons in wheat and rye. These markers will be an important resource for constructing high-density molecular linkage maps of wheat and rye. Conversely, a high-density molecular linkage map of wheat or rye will also enable the development of rye-specific markers. In addition, the rye-specific EST-based markers developed in this study will be useful in functional assessments of the genes on rye chromosomes [Lee et al., 2009].

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