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ORIGINAL PAPER

Development of simple sequence repeat markers specific for the *Lr34* resistance region of wheat using sequence information from rice and *Aegilops tauschii*

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Abstract Hexaploid wheat (*Triticum aestivum* L.) originated about 8,000 years ago from the hybridization of tetraploid wheat with diploid *Aegilops tauschii* Coss. containing the D-genome. Thus, the bread wheat D-genome is evolutionary young and shows a low degree of polymorphism in the bread wheat gene pool. To increase marker density around the durable leaf rust resistance gene *Lr34* located on chromosome 7DS, we used molecular information from the orthologous region in rice. Wheat expressed sequence tags (wESTs) were identified by homology with the rice genes in the interval of interest, but were monomorphic in the 'Arina' × 'Forno' mapping population. To derive new polymorphic markers, bacterial artificial chromosome (BAC) clones representing a total physical size of ~1 Mb and belonging to four contigs were isolated from *Ae. tauschii* by hybridization screening with wheat ESTs. Several BAC clones were low-pass sequenced, resulting in a total of ~560 kb of sequence. Ten microsatellite sequences were found, and three of them were polymorphic in our population and were genetically mapped close to *Lr34*. Comparative analysis of marker order revealed a large inversion between the rice genome and the wheat D-genome. The SWM10 microsatellite is closely linked to *Lr34* and has the same allele in the three independent sources of *Lr34*: 'Frontana', 'Chinese Spring', and 'Forno', as well in most of the genotypes containing *Lr34*. Therefore, SWM10 is a highly useful marker to assist selection for *Lr34* in breeding programs worldwide.

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Introduction

Bread wheat (*Triticum aestivum* L., genome formula AABBDD) is a crop with one of the largest known genomes and a low gene density. Genetically, wheat is an allohexaploid species that evolved by hybridization of three closely related wild grasses: *Triticum urartu* (A genome), a relative of *Aegilops speltoides* (B genome), and *Aegilops tauschii* (D genome) (McFadden and Sears 1946). Among the three wheat genomes, the D-genome was the last to be acquired by polyploid wheat, about 8,000 years ago. Although the hybridization with *Ae. tauschii* seems to be polyphyletic, the D-genome has a low degree of polymorphism within the cultivated germplasm (reviewed by Ogbonnaya et al. 2005).

In wheat, simple sequence repeat markers (SSRs) are a powerful tool for genetic mapping (Röder et al. 1998; Pestsova et al. 2000), diversity analysis (Huang et al. 2002) and marker assisted selection (Dubcovsky 2004). Wheat SSRs have been mostly developed on a genome-wide scale (Röder et al. 1998; Gupta et al. 2002; Song et al. 2005). Consensus mapping of publicly available microsatellites has provided an overview of the saturation reached in the hexaploid wheat genome (Somers et al. 2004). However, with a total genome size of 16,000 Mbp and a content in repetitive DNA estimated to be ~80%, there is little chance that a randomly developed marker tags a small and specific genomic region of interest. In the past, the development of microsatellite markers for specific regions of the wheat genome was not possible because of technical

limitations and lack of genomic resources. More recently, some technical advances have allowed to partially overcome these problems (reviewed by Keller et al. 2005). Arrayed bacterial artificial chromosome (BAC) libraries have been developed for wheat diploid ancestors and their close relatives. Specifically, a BAC library from *Ae. tauschii* was developed from the accession 'AL8/78' which has a genome that is closely related to the wheat D-genome. The library consists of more than 300,000 BAC clones and has a 8.5× genome coverage. Around 200,000 clones have been fingerprinted and arranged into ~12,000 contigs (Luo et al. 2003; http://wheatdb.ucdavis.edu:8080/wheatdb/). This library provides an excellent tool to access large portions of anonymous intergenic sequences from the D-genome. In addition, pooled PCR-screenable BAC libraries have been constructed from genomic DNA of hexaploid wheat (Nilmalgoda et al. 2003).

Since the sequence of the rice genome has been released (assembly 4 of TIGR), a thorough comparative analysis of the rice and wheat genomes has demonstrated that, based on genome synteny, it is possible to predict gene order in wheat using the rice sequence as template (Sorrells et al. 2003). However, numerous exceptions to collinearity have also been found between rice and the family of the Triticeae, complicating approaches relying on comparative genomics (Bennetzen and Ramakrishna 2002; Brunner et al. 2003; Guyot et al. 2004).

The combined use of new molecular tools in wheat and rice has a high and unexploited potential for the development of microsatellite markers tagging small chromosomal regions in bread wheat. The isolation of microsatellite markers from BAC libraries for targeted genomic regions was described first in soybean by Cregan et al. (1999) and later in several other crops. Recently it was also reported in wheat by Shen et al. (2005) using an arrayed BAC library of cv. 'Chinese Spring', and the markers developed were tagging loci from all the three genomes. The close similarity of the bread wheat D-genome to the genome of *Ae. tauschii* provides a unique opportunity to use genomic information derived from *Ae. tauschii* for marker development in wheat. High transferability of SSRs from *Ae. tauschii* to wheat was demonstrated by Guyomarc'h et al. (2002).

One of the most relevant genes in wheat disease resistance breeding is *Lr34*, located on chromosome 7DS (Suenaga et al. 2003; Schnurbusch et al. 2004a, 2004b). *Lr34* confers adult plant, durable resistance to leaf rust in bread wheat, and it was originally detected in the spring wheat material of CIMMYT (Singh 1992). In the Swiss winter wheat cv. 'Forno', we identified a quantitative trait locus (QTL) for leaf rust resistance, *QLrP.sfr-7DS*, which had a very similar mapping location and phenotype as *Lr34* (Schnurbusch et al. 2004a). Therefore, although no records are available to link the pedigree of 'Forno' to the spring wheat germplasm at CIMMYT, *QLrP.sfr-7DS* and *Lr34* are possibly the same locus. *Lr34* is associated with the morphological character 'leaf tip necrosis' (LTN, Singh 1992) and the stripe rust resistance gene *Yr18* (McIntosh 1992). The *Lr34* locus has provided durable resistance to both leaf rust (causal fungus: *Puccinia triticina* Eriks.), and stripe rust (*Puccinia striiformis* Westend. *f. sp. tritici*, Ma and Singh 1996), making *Lr34* a unique resource for breeding and a model for understanding the molecular basis of partial horizontal resistance. Previous work in the 7DS chromosomal region demonstrated close genetic linkage of *Lr34* with SSRs GWM130, GWM295, GWM1220, and ESTs CDO475, BF473324, and BE493812 (Suenaga et al. 2003; Schnurbusch et al. 2004b; Spielmeyer et al. 2005). However, there is still no diagnostic marker available.

The aim of this study was to develop new microsatellite markers specific for the chromosomal region of *Lr34*, based on orthologous regions in rice and *Ae. tauschii*. In addition, we wanted to analyze the newly developed markers for allelic diversity in a set of wheat lines with or without *Lr34* and to test their diagnostic value for marker-assisted selection.

Materials and methods

Plant material

Two *Lr34* recombinant populations were used for genetic mapping: 240 'Arina' × 'Forno' $F_{5:7}$ recombinant inbred lines (Paillard et al. 2003) and a set of 600 F2 plants derived from a cross between 'Arina' and an 'Arina-*Lr34*' introgression line (BC2F4, with 'Arina' as the recurrent parent). This introgression line was verified to be homozygous for the 'Forno' *Lr34* segment by phenotypic evaluation and with the *Lr34* flanking markers *Xsfr.BE493812/PstI*, and *Xswm5* (developed within this study).

An allele diversity study was performed on a set of lines from different breeding programs around the world for which information was available on *Lr34*. This set contains five 'Chinese Spring' chromosome-substitution lines, having the chromosome 7D of 'Chinese Spring' (which has *Lr34*, Dyck 1991) replaced by chromosome 7D of lines which do not have *Lr34* ('Cheyenne', 'Hope', 'Red Egyptian', 'Timstein', and 'Thatcher'); the line 'Lalbahadur'

(without *Lr34*), and the corresponding 7D substitution line 'Lalbahadur-Parula 7D' ('Parula' has *Lr34*, Singh 1992); the near isogenic lines 'Jupateco 73', and 'Jupateco 73R' (with *Lr34*, Singh 1992); 'Avocet' and 'Avocet R' (with *Lr34*); 'Thatcher *Lr16*' and 'Thatcher *Lr16* + *Lr34*'.

Development of probes from the *Lr34* orthologous region in rice

The rice BAC clones orthologous to the wheat '*Lr34* orthologous region' defined by the two flanking markers *Xsfr.BE493812* and *Xsfr.BF473324* were identified by Schnurbusch et al. (2004b). The nucleotide sequences of genes predicted by RICEGAAS (Sakata et al. 2002) in the rice BAC clones AP000399, AP003708, AP003487, AP003767, and AP003632 were compared with BLASTn to Triticeae and oat ESTs. A set of 24 ESTs of wheat, barley, and oat was identified with a similarity threshold value of 10^{-15} and was hybridized to wheat Southern blots to test for copy number. Sixteen probes had a low-copy number, whereas eight of them exhibited a high-copy hybridization pattern. Four of these clones were converted into low-copy probes by subcloning an intron segment of the corresponding gene by PCR. The position of the introns on the wheat ESTs was predicted aligning the coding sequence of wheat to the rice genomic DNA with the software SIM4 (http://gamay.univ-perp.fr/analyse_seq/sim4). Sequence tag site (STS) primers were designed on the EST exons flanking one of the introns as follows: STS 40 was designed on CD453029 (for. 5′-ggtgttttaagtgttttgcatgg-3′; rev. 5′-cttcaacatgactggaggacg-3′), STS 41 on BJ210832 (for. 5′-gaaagcttggaagaggcag-3′; rev. 5′-cccggttgtgccactgg-3′), STS 62 on BE516643 (for. 5′-gacatcgtgctgccggag-3′; rev. 5′-ccatccactcagagataatgacatcaa-3′), and STS 77 primers are from BJ222628 (for. 5′-ggaccgcatcttcttcagcgt-3′; rev. 5′-atcttcttcttcttcttcatctatgcacc-3′). All the probes with a low-copy hybridization pattern were first used to screen parental blots for polymorphisms, and were then hybridized to the *Ae. tauschii* BAC library. Parental blots included genomic DNA of the wheat cultivars 'Forno', 'Arina', 'Chinese Spring', and the Chinese Spring deletion line '7DS-4' (Endo and Gill 1996). DNA was digested with 27 endonucleases: *Alu*I, *Ava*II, *Dde*I, *Dpn*II, *Hae*III, *Msp*I, *Taq*I, *Bgl*I, *Nco*I, *Aci*I, *Dpn*I, *Hind*III, *Mse*I, *Sal*I, *Nde*I, *Kpn*I, *Cla*I, *Sac*II, *Bam*HI, *Dra*I, *Eco*RI, *Bgl*II, *Hind*III, *Xba*I, *Eco*RV, *Apa*I, and *Sac*I. Digested DNA was blotted on membranes and hybridized with ^{32}P-labeled probes to detect polymorphisms according to standard methods (Sambrook and Russell 2001).

Bacterial artifical chromosomes library screening, sequence analysis and genetic mapping

Screening of the *Ae. tauschii* BAC library was performed by hybridization as described by Wicker et al. (2003) using the low-copy probes listed in Table 1. Screening of the Glenlea BAC library was done by PCR according to Nilmalgoda et al. (2003) using the PCR markers STS 77, GWM1220, and GWM295. Subcloning of BAC DNA was done as described by Stein et al. (2000). Low-pass sequencing of BAC clones was performed with ABI®3730 to identify genes and SSRs. Genes were detected with the BLASTn algorithm (Altschul et al. 1997). SSRs were identified using the SPUTNIK Perl Script (Abajian 2003). PCR primers were designed manually; primers were tested for annealing temperature and self-complementarity with the software FastPCR (Kalendar 2006). Microsatellite markers were PCR amplified from wheat genomic DNA and visualized with the LiCor® DNA Sequencer 4200. The markers polymorphic between 'Arina' and 'Forno' were mapped in the 'Arina' × 'Forno' population, consisting of 240 single seed descent lines (Paillard et al. 2003). The linkage map was constructed using MAPMAKER 3.0b for MS-DOS (Lander et al. 1987). The new DNA markers on chromosome 7DS were integrated into the previously published 7DS genetic map (Schnurbusch et al. 2004b) using the 'try' command and the new marker sequence was confirmed with the 'ripple' command. Linkage groups were drawn with the software MapChart (Voorrips 2002). Physical mapping and bin assignment was conducted using the deletion line 7DS-4 (breakpoint 0.61) derived from cv. 'Chinese Spring', in which 39% of the short arm of chromosome 7D is missing (Endo and Gill 1996).

Results

Polymorphism analysis of wheat ESTs derived from the '*Lr34* orthologous region' of rice and identification of *Aegilops tauschii* BAC contigs

The two EST markers BE493812 and BF473324 flanking the QTL *QLrP.sfr-7DS* had been previously located on rice chromosome 6S, where they define a physical contig of ~300 kb (Schnurbusch et al. 2004b). To increase marker density around the *Lr34* locus, we identified with a BLASTn search the wheat ESTs, which are homologous to the open reading frames predicted in the rice orthologous region.

Table 1 List of wheat probes detected with a BLAST search at the rice '*Lr34* orthologous region'

Probe	Accession	Position in rice (kb)	Copy number	Number of identified *Aegilops tauschii* contigs	Analyzed BACs from *Aegilops tauschii*
WHE1275_C07_E13	BE493812	AP003708 (50–54)	Low	1	
WHR21F21	BJ281290	AP003708 (160–164)	High	Not used	
WHE1452_F03_K06 STS 40	CD453029	AP00039 (13–15)	High Low for STS 40	1	
WH36C19 STS 41	BJ210832	AP000399 (16–19)	High Low for STS 41	1	RI033N19
WHE1114_C04_E08	BE443044	AP000399 (21–23)	Low	1	RI033N19
WHF22A08	BJ252160	AP000399 (48–53)	Low	–	
WH19L04	BJ220373	AP000399 (88–91)	Low	3	HD007J19
WHE0802_D01_G02	BE517741	AP000399 (93–98)	Low	1	HD007J19
WHE618_B12_C24 STS 62	BE516643	AP000399 (106–109)	High Low for STS 62	2	HD007J19
WHE4020_H05_P10	CA500527	AP000399 (134–137)	Low	1	RI005C8[a] TaBAC1466J2
WHDL14M17 STS 77	BJ222628	AP003487 (14–20)	High Low for STS 77	1	RI005C8[a] TaBAC1466J2
WHE0418_F09_K18	BE406581	AP003487 (42–50)	Low	1	
WHE0923_B09_D17	BF473324	AP003487 (88–93)	Low	1	BB045B13
CDO475	CDO475	AP003487 (79–86)	Low	1	BB045B13
WHF1M10	BJ251589, 3×	AP003487 (56–57), (94–95), (98–99)	Low	3	
FGAS053185	CV758803	AP003767 (13–14)	Low	2	HD099L21
FGAS016080	CK163453	AP003767 (14–15)	Low	1	HD099L21
BAGS32F04	BJ463527	AP003767 (36–38)	Low	1	
HVU507094	AJ507094	AP003767 (44–49)	High	Not used	
EBRO01_SQ003_A08	BI779107	AP003767 (58–64)	High	Not used	
BAH11M12	AV835586	AP003767 (64–68)	Low	1	HD099L21
TALR1130G03F	BG904265	AP003767 (68–70)	High	Not used	
HA23F03	BU981349	AP003767 (95–99)	High	Not used	
FGAS017807	CK206224	AP003632 (58–64)	Low	1	

Only the probes with a low-copy pattern were used to screen the *Aegilops tauschii* BAC library

[a] RI005C8 is the biggest BAC clone of *Aegilops tauschii* identified by hybridization with probes STS 77 and EST CA500527. The STS 77 was also used as a PCR marker to screen the bread wheat BAC library of cv. 'Glenlea' and yielded TaBAC1466J2. Because these two BAC clones are derived from the same orthologous locus of chromosome 7 in *Aegilops tauschii* and chromosome 7D in wheat cv. 'Glenlea', the BAC clone of 'Glenlea' was preferred to the one of *Aegilops tauschii* for sequencing

The wheat ESTs were then hybridized to wheat genomic DNA to test for copy number and polymorphism, but no polymorphism was detected by any probe on the wheat D-genome in the cross 'Arina' × 'Forno'. To access potentially more polymorphic intergenic regions 19 low-copy wheat ESTs and EST-derived

probes (Table 1) were used to screen a BAC library of *Ae. tauschii* by hybridization. Seventy BAC clones were initially identified and 59 of them were found to be organized into 11 BAC contigs (Luo et al. 2003; http://wheatdb.ucdavis.edu:8080/wheatdb/). Five BAC contigs contained clusters of two to three ESTs. The corresponding genes in rice were also physically close to each other, with less than 10 kb separating them (Fig. 1). This suggests that these genes are collinear between rice and *Ae. tauschii*, although we cannot infer their relative physical distances in *Ae. tauschii* because a full BAC sequencing was not performed. One of the BAC contigs contained one cluster of genes as well as a single gene, which are conserved between rice and *Ae. tauschii* and are separated by a region of ~300 kb in *Ae. tauschii*. In rice they are separated by 110 kb. This BAC contig of *Ae. tauschii* is estimated to be ~1.2 Mbp (BAC contig containing the BAC clone HD099L21, Fig. 1).

For some low-copy sequences we found gene redundancy in the *Ae. tauschii* genome. Five probes identified two or three BAC contigs, possibly derived from different genomic regions of *Ae. tauschii*. To increase the probability of selecting *Ae. tauschii* BAC clones from the chromosomal region of interest, we selected BAC clones independently identified by at least two probes. These were BAC clones BB045B13, HD099L21, RI033N19, and HD007J19 (Fig. 1).

Selection of BAC clones from chromosome 7D of the hexaploid wheat cv. 'Glenlea'

The BAC library of the hexaploid wheat cv. 'Glenlea' has a 3.5× genome coverage, an average insert size of 85 kb and is PCR screenable (Nilmalgoda et al. 2003). Since PCR markers are more specific than restriction fragment length polymorphism (RFLP) markers, they often allow tagging only one locus of the three orthologous genomes. Therefore, the microsatellite markers GWM1220, GWM295, and the sequence tagged site marker STS 77 were used to isolate BAC clones from the BAC library of 'Glenlea'. GWM1220 and GWM295 were described in previous studies to be linked to *Lr34* (Suenaga et al. 2003; Schnurbusch et al. 2004a, 2004b; Spielmeyer et al. 2005), and we could assign them to the bin 7DS-4 by deletion mapping (data not shown). The STS 77 is an intron sequence of the EST BJ222628 (Table 1). This STS amplifies two bands of similar size, with the smaller assigned to bin 7DS-4. Only one BAC clone, TaBAC1466J2 with a size of ~85 kb, was found to contain the lower band of STS 77. Because TaBAC1466J2 was from bread wheat, it was preferred for sequencing to the corresponding BAC clone from *Ae. tauschii* (Table 1). Six BAC clones were identified with GWM1220 and three with GWM295. For these two markers, the BAC clones with the largest inserts were, respectively, TaBAC940L4 and TaBAC470M18.

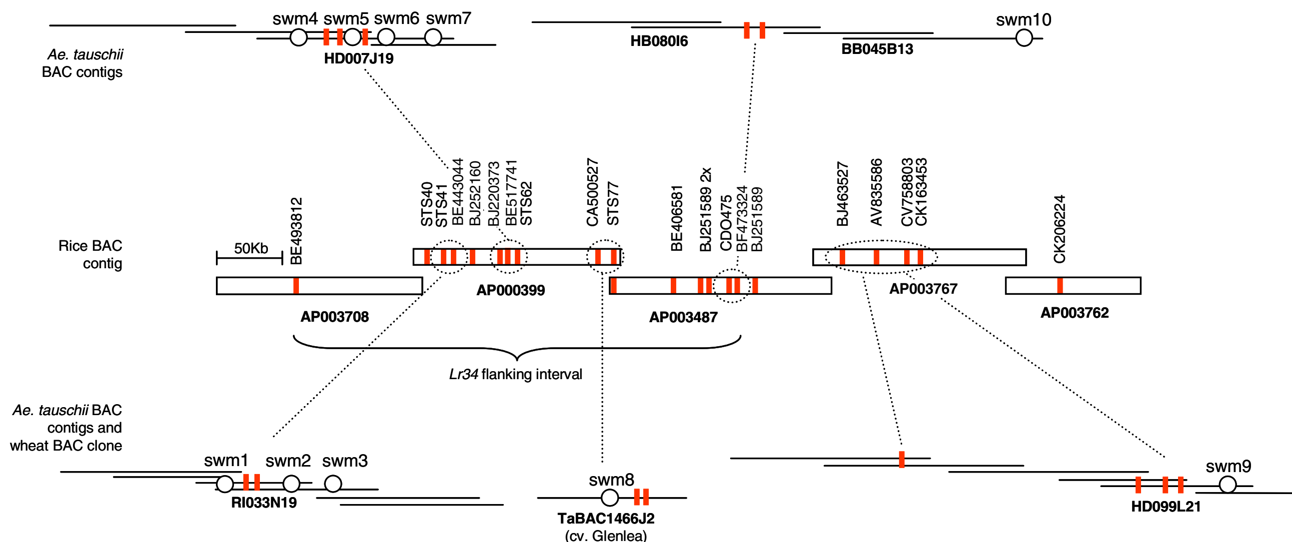

Fig. 1 Identification of *Aegilops tauschii* BAC contigs and wheat BAC clones from the targeted '*Lr34* orthologous region' of rice chromosome 6S. Only BACs from which new SSR were derived are shown. The SSRs detected are indicated with a *white circle*. Low-copy wheat, barley, and oat EST and STS probes were used to screen the BAC library of *Aegilops tauschii*. Probes are represented as *gray boxes*. Conserved gene clusters are framed by a *dashed circle*. The rice '*Lr34* orthologous region', delimited by ESTs BE493812 and BF473324, is marked with a *curly bracket*. TaBAC1466J2 from bread wheat cv. 'Glenlea' was preferred to the *Aegilops tauschii* homolog to maximize SSR transferability. The relative sizes of the BAC contigs of *Aegilops tauschii* are not drawn to scale

Identification of microsatellite sequences in BAC clones of *Aegilops tauschii* and bread wheat cv. 'Glenlea'

Three BAC clones of the 'Glenlea' D genome (TaBAC1466J2, TaBAC470M18, and TaBAC940L4) and four from *Ae. tauschii* (HD007J19, RI033N19, BB045B13, and HD099L21) were sub-cloned and low-pass sequenced to identify SSRs and coding sequences. From each BAC clone a shotgun library was derived and sequenced.

Four SSRs were detected in BAC clone HD007J19, three in BAC RI033N19, only one microsatellite was found in the BACs HD099L21, BB045B13, and TaBAC1466J2 (Table 2). In TaBAC470M18 and TaBAC940L4 no additional SSRs were detected besides GWM295 and GWM1220, which had been used for screening. A BLASTn analysis of the sequences flanking the simple sequence repeats revealed that these microsatellites were not located in known coding regions or repetitive elements. The size of the repeats ranged from 18 to 134 bp (Table 3). The PCR primers were designed in the 5′ and 3′ regions flanking the repeat and were tested on genomic DNA of the lines 'Arina', 'Forno', 'Chinese Spring', and its deletion derivative '7DS-4'. SWM1, SWM5, SWM8, SWM9, and SWM10 produced bands specific for 7DS-4. SWM6 amplified two fragments, with one of them mapping to 7DS-4. SWM3, SWM4, and SWM7 were neither polymorphic, nor was it possible to physically assign them to a specific chromosome with the nullitetrasomic lines of Chinese Spring (Sears 1966). Therefore, they were not considered for further analysis. Amplification of SWM2 was successful in the *Ae. tauschii* accession 'AL8/78', but it did not amplify a fragment in any wheat line (data not shown). This SSR was probably lost during the evolution of the wheat D-genome or mutations in the primer sequences occurred.

Genetic mapping of microsatellites reveals a large-scale inversion between rice and wheat orthologous regions

All the newly developed microsatellites were tested for polymorphism between the parental lines for mapping in the 'Arina' × 'Forno' population (Paillard et al. 2003). SWM1, SWM5, SWM6, and SWM10 were polymorphic between the parents. SWM6 was assigned to the 7A linkage group. SWM1, SWM5 and SWM10 mapped in the 7D chromosome, closely linked to *Lr34*. However, in the wheat genetic map of chromosome 7D, the three SSR markers SWM1, SWM5 and SWM10 do not map inside the *Lr34* flanking interval, but outside and in a position more proximal to BF473324 (Fig. 2). Based on the assumption of wheat–rice collinearity, SWM1 should have been the marker mapping closest to EST BE493812, but in wheat it is the marker, which maps most distant to it (18 cM). Similarly, SWM10 should have been the SSR with the largest distance to EST BE493812, but it is the closest (8.9 cM). SWM5 maintains its mapping position between SWM1 and SWM10, and it maps 13.6 cM proximal to BE493812. Thus, the genetic mapping in wheat suggests that the marker order in wheat is conserved, but in an inverted pattern compared to rice. This indicates the presence of a large inversion between rice and wheat '*Lr34* orthologous regions'. This hypothesis was further supported by sequencing the wheat BAC clone TaBAC470M18, which contains the SSR GWM295. This BAC clone was found to contain a gene encoding an early noduline-like sequence (CD904551). In the wheat genetic map, GWM295 maps between SWM5 and SWM1, within the inverted region. As expected according to the observed inversion, an orthologous early noduline-like gene was located in rice between orthologous sequences to the BAC clones RI033N9 and HD007J19. The genetic map was partially verified in a population of 600

Table 2 Sequencing and molecular characterization of BAC clones from bread wheat cv. 'Glenlea' and *Aegilops tauschii* identified with markers from the '*Lr34* orthologous region' of rice

BAC name—origin	Estimated BAC size (kb)	Total contigs length (and number of contigs)	SSRs	Contigs with repetitive DNA	Predicted genes	Predicted genes collinear in rice
RI033N19—*Aegilops tauschii*	150	83 kb (56)	3	32	–	–
HD007J19—*Aegilops tauschii*	190	160 kb (88)	4	55	4	1
TaBAC1466J2—*Triticum aestivum*	85	68 kb (25)	1	14	2	2
HD099L21—*Aegilops tauschii*	140	84 kb (46)	1	21	7	4
BB045B13—*Aegilops tauschii*	140	51 kb (85)	1	42	3	1
TaBAC940L4—*Triticum aestivum*	120	42 kb (64)	1 (GWM1220)	15	4	1
TaBAC470M18—*Triticum aestivum*	110	71 kb (35)	1 (GWM295)	16	3	3

The last column indicates the number of wheat predicted genes, which were conserved in the orthologous rice region (see also Table 5)

Table 3 Ten microsatellite repeats were identified in the *Aegilops tauschii* and wheat BAC clones of the rice '*Lr34* orthologous region'

Probes used	BAC clones	*Lr34* SSR (7DS-4 deletion mapping)[a]	Motif	Forward primer (5′–3′)	Reverse primer (5′–3′)	Alleles detected in 17 lines
BE443044 STS 41	RI033N19	**SWM1 (y)**	$(tc)_{10}$	ACT CCC GAT ACA ATT CTT CCG CT	GAG ATC ATT GTA TTG AAG ATC AAA CG	6
BE443044 STS 41	RI033N19	SWM2 (n)	$(atag)_{7}$	ATG GAA TTC AAG AAT CGA TCT ACA C	GCG CTG TAA GCG AGA CAT AGG A	–
BE443044 STS 41	RI033N19	SWM3 (n)	$(cgc)_{6}$	TTC CTG AAC GAG CCT CTC C	CGG ACG ACT TGT TGT ATC ATA AGG	3
BJ220373 BE517741 STS 62	HD007J19	SWM4 (n)	$(ta)_{13}$	AGA TGC AGA TCT AAT GGT CAG AGA C	ACA ATA GCC CAC TTC TCT GCT C	1
BJ220373 BE517741 STS 62	HD007J19	**SWM5 (y)**	$(ta)_{32}/(tg)_{35}$	CCA GTA GCA TAA GCA TAA TAC AAC AC	ATA GAT TGT AAC ATT GCT TGA TTG C	9
BJ220373 BE517741 STS 62	HD007J19	**SWM6 (y)**	$(ac)_{11}$	CTT ACA CGG ACC ATG TGC AGA GG	AGG GAG TGG ATG AAC AAA GTG TG	7
BJ220373 BE517741 STS 62	HD007J19	SWM7 (n)	$(at)_{17}$	GTC GCA ATG TCA GAG AGG AAT CAG	GAG TGC ATT ACA CCA AGC TGT CTA G	1
STS 77	TaBAC1466J2	**SWM8 (y)**	$(ga)_{25}$	GCT CTT GAA CTT AGT CTC ATC AAG G	CTC TCC CGC TGC AGT GTC TC	3
CK163453 CV758803 AV835586	HD099L21	**SWM9 (y)**	$(gtt)_{14}$	GTC ATT CTG CCA ATG CAT GAT CC	GGT ACA GCC GAC ATA GGT CAT C	6
BF473324	BB045B13[b]	**SWM10[b](y)**	$(ca)_{25}$	GCC TAC TTT GAC GGC ATA TGG	CCA TCT TGA CAT ACT TTG GCC TTC C	5

Six of them amplified at least one fragment physically assigned to the bin 7DS-4 (in bold), where *Lr34* is located

[a] *y* Present in 'Chinese Spring' but absent in the 7DS-4 deletion line, *n* not specific for the 7DS-4 deletion line

[b] SWM10 was not identified from the BAC clone containing BF473324 but from a BAC clone of the same BAC contig

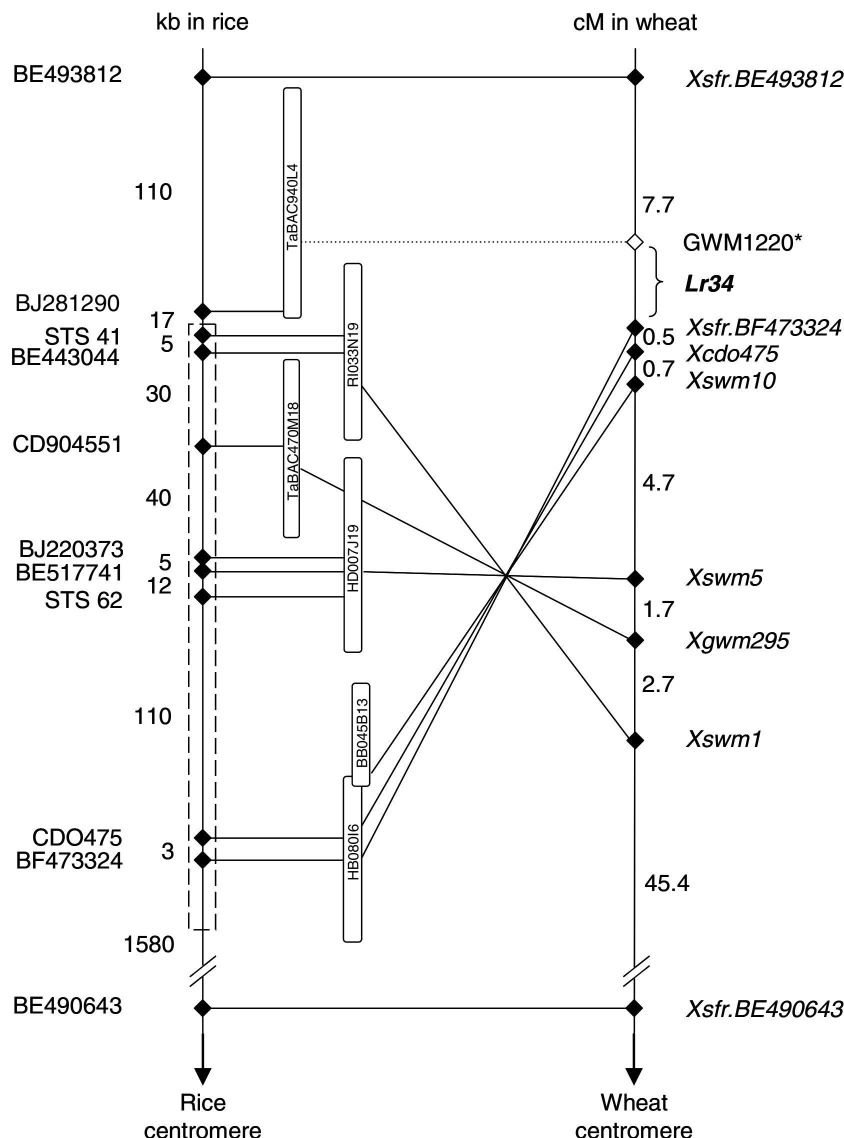

Fig. 2 Genetic mapping of polymorphic microsatellites derived from BAC clones of the wheat cv. 'Glenlea' (TaBACs) and the *wild grass Aegilops tauschii* allowed to correlate the genetic map of wheat chromosome 7D with the rice physical map of chromosome 6S. The inversion detected by mapping new SSR markers is indicated by a *dashed box*. The BAC clones of wheat and *Aegilops tauschii* are not drawn to scale. Only BAC clones HB086I16 and BB045B13 belong to the same contig, however, they do not overlap. *Microsatellite GWM1220 was not polymorphic between 'Arina' and 'Forno'. Its mapping position is inferred from published work (Schnurbusch et al. 2004b; Spielmeyer et al. 2005)

'Arina' × 'Arina-*Lr34*' F2 plants (data not shown). Thus, due to the described inversion between wheat and rice, the new microsatellite markers mapped more proximal than expected assuming wheat-rice collinearity, and the microsatellite SWM10 is the closest to the *Lr34* flanking interval that was defined by ESTs BE493812 and BF473324 by Schnurbusch et al. 2004b.

Allele diversity study in wheat lines with and without *Lr34*

An allele diversity study was performed on a set of lines with and without *Lr34* using the newly developed SWM–SSR markers, as well as the two previously published SSR markers from the *Lr34* region GWM130 and GWM1220 (Suenaga et al. 2003; Schnurbusch et al. 2004b; Spielmeyer et al. 2005). The aim of this analysis was to check the degree of polymorphism at the new SSR loci in the wheat germplasm and to assess if any of the newly developed markers was diagnostic for the presence of *Lr34*. To assess the degree of polymorphism we used a set of lines extensively used to characterize *Lr34* (Table 4, first group). All microsatellites produced an amplification product in the lines except for SWM2, which amplified a fragment only in the genomic DNA of the *Ae. tauschii* accession 'AL8/78'. SWM4 and SWM7 were monomorphic, SWM3 and SWM8 produced three alleles; SWM1, SWM5, SWM6, SWM9, and SWM10 had a higher degree of polymorphism, ranging from three to nine alleles, comparable to GWM1220 and GWM130 (six and seven alleles, respectively). It is noteworthy that the SWM5 allele in the introgression line'Arina-*Lr34*' is different from both the donor parent 'Forno' and the recurrent parent 'Arina'

Table 4 Diversity analysis of bread wheat cultivars with the SSR marker SWM10

Line	*Lr34* (yes–no)	SWM10 allele
Model lines where *Lr34* was extensively characterized		
Arina	n	b
Forno	y	a
Arina *Lr34*	y	a
Chinese Spring (CS)	y	a
CS 7D_Cheyenne	n	c
CS 7D_Hope	n	b
CS 7D_Red Egyptian	n	b
CS 7D_Timstein	n	b
CS 7D_Thatcher	n	b
Lalbahadur	n	c
Lalbahadur 7D_Parula	y	a
Jupateco 73S	n	d
Jupateco 73R	y	a
Avocet S	n	b
Avocet R	y	a
Thatcher *Lr16*	n	b
Thatcher *Lr16 Lr34*	y	a
Elite lines without *Lr34*		
Altgold	n	b
Apollo	n	b
Ares	n	b
Basalt	n	b
Bobwhite	n	b
Boval	n	c
Can 3842	n	b
Champlein	n	b
Derenburger	n	b
Disponent	n	b
Frisal	n	b
Galaxie	n	d
Granada	n	c
Greif	n	b
Hoeser	n	b
Hubel	n	b
Inia 66	n	d
Kanzler	n	b
Kavkaz	**n**	**a**
Kraka	n	b
Kronjuwel	n	c
Little club	n	b
Lueg	n	b
Maris Huntsman	n	b
Merlin	n	b
Monopol	n	c
Morocco	n	b
Obelisk	n	b
Oberkulmer	n	d
Osmut	n	d
Ostro	n	d
Pavon 76	n	b
Pegassos	n	b
Probus	n	b
Rektor	n	b
Roazon	n	b
Rouquin	n	d
Siete Cerros 66	n	b
Skalavatis 56	n	b
Sperber	n	b
Weique	n	b
Elite lines with *Lr34*		
Bezostaja	y	a
BH1146	y	a
Cappelle Desprez	**y**	**b**
Cumpas	y	a
Frontana	y	a
Glenlea	**y**	**e**
Kormoran	**y**	**b**
Tamaro	y	a
Tepoca	y	a
Terenzio	y	a
Titlis	**y**	**c**
Tonichi 81	y	a
Westphal 12A	**y**	**d**
Yecora 70	y	a

The allele 'a' is shared among the three independent sources of *Lr34*: 'Forno', 'Chinese Spring' and 'Frontana'. The genotypes in bold have *Lr34*, but do not have the *SWM10a* allele or vice versa, they have the *SWM10a* allele, but not *Lr34*. The alleles 'c', 'd', and 'e' appear with less frequency in the tested genotypes

(data not shown). It seems that in a few generations of backcrossing (BC_2) and selfing (F_4) this *Lr34* line has developed a new allele at the *Xswm5* locus. Instability observed with SWM5 might depend on the size of this repeat. The part representing only the repeat of the amplified fragment of SWM5 is 134 bp long, and is very large compared to an average SSR. It consists of $(ta)_{32}$/$(tg)_{35}$ in *Ae.tauschii*, and the repeat might be very unstable, rapidly producing new allelic variants.

The microsatellite marker closest to the *Lr34* confidence interval is SWM10 (Fig. 2). Interestingly, in the set of *Lr34* model lines, the marker SWM10 showed an allele of the same size (allele 'a', 211 bp) in all the lines with *Lr34*, but different alleles in the lines without *Lr34*. Therefore, this microsatellite was tested on a broader germplasm from different wheat breeding programs to determine its diagnostic value for detection of *Lr34*. In Table 4, second and third section, additional elite genotypes are listed. Based on literature references it is also indicated whether they contain *Lr34* or not. The lines are mostly from the CIMMYT spring wheat germplasm and from the European winter wheat germplasm. To the best of our knowledge, none of the lines of the second group has *Lr34*. Among this group, the only genotype having the *SWM10a* allele is 'Kavkaz'. Sawhney and Sharma (1999) reported that 'Kavkaz' manifests good adult plant leaf rust resistance. 'Kavkaz' is derived from a cross with the winter wheat line 'Bezostaja', which has been described to have *Lr34* (Winzeler et al. 2000), therefore, the line 'Kavkaz' might also contain *Lr34*, although this has not been published so far. Among the third group of lines

(Table 4), all described to contain *Lr34*, more variability in allele size was observed. A few lines that have been described to possess *Lr34* showed different bands than the 'a' SWM10 allele. These lines were the Canadian spring wheat cv. 'Glenlea' (reported to have *Lr34* by Dyck et al. 1985) with the unique allele 'e' found in no other line, the German winter wheat line 'Westphal 12A' (Kolmer and Liu 2001), with allele 'd' and the French winter wheat line 'Cappelle Desprez' (McIntosh 1992), with allele 'b'. The German winter wheat line 'Kormoran' shows the 'b' allele as 'Cappelle Desprez' and also contains *Lr34* (McIntosh 1992). This could be explained by the presence of 'Cappelle Desprez' in the pedigree of 'Kormoran'. For the Swiss winter wheat cv. 'Titlis' (allele 'c'), there is no official report to support the presence of *Lr34*; nonetheless a strong adult leaf rust resistance and LTN lead to assume that this gene is present in 'Titlis' (M. Winzeler, personal communication).

Putative coding regions and repetitive DNA in bacterial artificial chromosomes clones of *Aegilops tauschii* and wheat cv. 'Glenlea'

The analyzed BAC clones contained 23 putative genes, based on their homology with wheat ESTs from the TIGR database (Table 5). Nineteen sequences contained coding regions for proteins with predicted function. Two of them were homologous to the EST probes used to screen the *Ae. tauschii* BAC library and two others were similar to resistance genes of the nucleotide binding site-leucine rich repeat (NBS-LRR) class. In addition to these 19 sequences with predicted function, four additional sequences had significant homology to wheat ESTs, but they did not show a hit to any characterized protein. Thus, we refer to them as 'hypothetical proteins'. The 23 predicted coding sequences were analyzed with BLASTn to identify the homologous rice genes and their localization in the rice genome. Collinearity with rice was conserved only for 11 of them, while 12 sequences, including some genes putatively encoding hypothetical proteins or resistance gene analogues of the NBS-LRR class, were not conserved in the rice orthologous region. Earlier studies have shown that in cereals, disease resistance genes of the NBS-LRR family are subject to rapid evolution and often do not maintain collinearity (Leister et al. 1998).

Physical to genetic distances at the *Lr34* locus

The three molecular markers BF473324, CDO475, and SWM10 were polymorphic on chromosome 7D in the 'Arina' × 'Forno' population, and were derived from

Table 5 A BLASTn analysis of the BAC clone sequences of wheat and *Aegilops tauschii* reveals the presence of a number of sequences with homology to wheat ESTs and rice genes

Homologous proteins	BAC	wheat ESTs	E-value	Best rice hit
Cell wall associated kinase	HD007J19	BJ220373	1.3×10^{-95}	Os02g02120
Arginine N-methyl transferase[a]	**HD007J19**	DN829072	$\mathbf{2.2 \times 10^{-106}}$	**Os06g05090**
Hypothetical protein	HD007J19	AJ716967	4.5×10^{-61}	Os03g49250
NBS-LRR disease resistance homolog	HD007J19	CN011065	7.4×10^{-88}	Os08g28540
Sulfate transporter[a]	**TaBAC1466J2**	**CA685639**	$\mathbf{6.4 \times 10^{-32}}$	**Os06g05160**
Splicing factor PRP38	**TaBAC1466J2**	**CV765610**	$\mathbf{2.2 \times 10^{-141}}$	**Os06g05150**
Hypothetical protein	**HD099L21**	CK212017	$\mathbf{5.5 \times 10^{-79}}$	**Os06g05560**
GTP binding protein	HD099L21	BE213312	4.8×10^{-15}	Os09g19980
Proline-rich protein with lipase domain	**HD099L21**	BE422772	$\mathbf{2.2 \times 10^{-67}}$	**Os06g05550**
DNA helicase homolog	HD099L21	–	–	Os10g10730
Iron-sulfur cluster assembly accessory protein	**HD099L21**	CA485358	$\mathbf{2.3 \times 10^{-77}}$	**Os06g05400**
Mitogen-activated kinase	**HD099L21**	CK214503	$\mathbf{3.1 \times 10^{-70}}$	**Os06g05520**
Putative ABC transporter	HD099L21	–	–	Os03g64200
UDP-glucoronosyl and UDP-glucosyl transferase	BB045B13	CD871872	2.4×10^{-19}	Os01g50200
Zinc carboxypeptidase	**BB045B13**	CK208877	$\mathbf{9.4 \times 10^{-40}}$	**Os06g05240**
NBS-LRR disease resistance homolog	BB045B13	CA745220	4.6×10^{-88}	Os11g10770
Hypothetical protein	TaBAC940L4	CK207765	1.9×10^{-19}	Os08g06650
Hypothetical protein	TaBAC940L4	CK170616	2.5×10^{-07}	Os01g68130
O-methyltransferase	TaBAC940L4	CA633930	4.9×10^{-64}	Os11g20090
Serine-threonine kinase	**TaBAC940L4**	**BJ281290**	$\mathbf{3 \times 10^{-39}}$	**Os06g04880**
Early nodulin	**TaBAC470M18**	**CD870012**	$\mathbf{1.3 \times 10^{-105}}$	**Os06g05010**
Early nodulin	**TaBAC470M18**	**BQ167397**	$\mathbf{2.3 \times 10^{-73}}$	**Os06g05020**
Oxidoreductase, short chain	TaBAC470M18	CV761584	1.3×10^{-59}	Os06g19590

Hits in the rice genome with conserved collinearity in the '*Lr34* orthologous region' are shown in bold. Hits not collinear are in plain text

[a] EST probes with homology to these predicted genes were used to screen the BAC library of *Aegilops tauschii*

the same *Ae. tauschii* physical contig, which spans ~700 kb (Fig. 3). This allowed anchoring and orientation of a large DNA sequence of *Ae. tauschii* BAC clones on the wheat genetic map in chromosome 7D. In this region, the relationship between physical and genetic distances was assessed based on BAC hybridization information. The ESTs BF473324 and CDO475 were located in a ~20 kb region (Fig. 3). SWM10 was separated from them by ~560 kb of DNA. Thus, overall physical to genetic relationship is ~500 kb/cM. However, this ratio varied in the region, since the 0.7 cM genetic interval defined by *Xswm10* and *Xcdo475* corresponded to 560 kb, while the adjacent genetic interval of 0.5 cM, defined by *Xcdo475* and *Xsfr.BF473324* was only ~20 kb (Fig. 3). These two ESTs correspond to genes that in wheat have maintained a similar distance as in rice. The differences in the kb/cM ratio could possibly be explained by variations in gene content of the two intervals.

Discussion

Tagging of a specific region in the D-genome of hexaploid wheat

In this work, we have tested for polymorphism a set of 24 EST markers derived from the rice '*Lr34* orthologous region' to saturate the *Lr34* containing interval in wheat. No RFLP was detected in the D-genome between the parental lines 'Arina' and 'Forno'. This outcome is consistent with the low degree of polymorphism in the D-genome of cultivated bread wheat. The SSR markers represent a good alternative for mapping because of their high level of polymorphism (Prasad et al. 2000; Singh et al. 2006). The identification of SSR markers in *Ae. tauschii* BAC clones allowed the enrichment of the *Lr34* region on 7DS with new polymorphic microsatellite markers. Eight of nine microsatellites derived from the *Ae. tauschii* genome could be transferred to the D-genome of bread wheat (SWM8 was derived from the D-genome of 'Glenlea', SWM2 was not transferable). The *Ae. tauschii* BAC clones have been successfully used as a bridge to link the physical information of the rice sequence to genetic mapping in wheat. This strategy is of general utility for the development of markers for gene tagging in the D-genome of hexaploid wheat.

Low-pass sequencing of BAC clones revealed a high density of SSR motifs. In a total of 560 kb of sequence, ten SSRs were identified, giving an average of one microsatellite every 56 kb. Pestsova et al. (2000) reported microsatellite isolation from *Ae. tauschii* phage libraries by hybridization with the dinucleotide repeats poly(GA) and poly(GT) and found a repeat every 220 kb. In our case sequence data were available, and this allowed the detection of all the possible repeat combinations with an in silico approach. This explains the fourfold higher density of SSRs found.

Most of the developed SSRs tagged specifically the 7DS-4 bin. This was surprising as we could not exclude an occasional amplification from paralogous loci in the D-genome. In a few cases, the ESTs derived from the rice *Lr34* orthologous region hybridized with BAC clones assigned by BAC fingerprinting to different BAC contigs, suggesting that they are located in paralogous regions of the D-genome. To increase the chances to tag the BACs belonging to the *Lr34* region, we selected only BAC clones, which were hybridizing with probes of

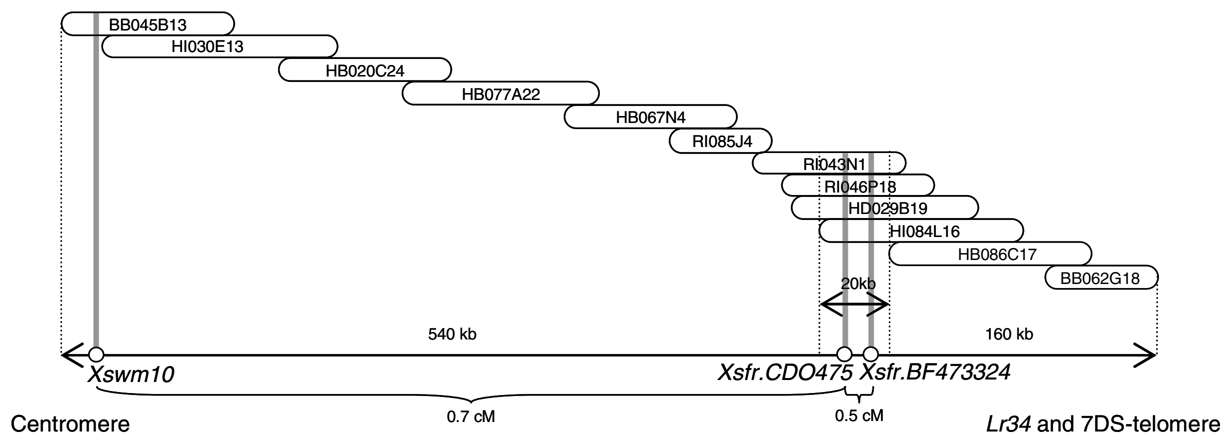

Fig. 3 Genetic to physical distances in the *Lr34* region. On the upper side a selection of BAC clones from the *Aegilops tauschii* BAC contig4981 is represented, according to the assembly of March 7th 2004 (http://wheatdb.ucdavis.edu:8080/wheatdb/). Below, *white circles* show the mapping position of three molecular markers developed from this BAC contig in the wheat 7DS chromosome. This allowed to orient the *Aegilops tauschii* BAC contig on the 7D chromosome, and to establish a relationship between physical and genetic distances at the *Lr34* region

two or more genes closely associated in rice (Table 1). Obviously, this strategy successfully avoided the erroneous targeting of paralogous loci in the D-genome. Using BACs of diploid *Ae. tauschii* also reduced the targeting of orthologous loci in the A and B genomes of bread wheat, as described by Shen et al. (2005), while developing SSR markers on a genome wide scale.

Potential and limitations of the rice genome for high-resolution analysis of the wheat genome

Rice is the only grass species with a completely known genome sequence. Comparative genetic studies revealed that within the grass family there is good collinearity and, despite major rearrangements of big chromosomal segments, the order of conserved genes inside each block is frequently maintained (Bennetzen and Ma 2003). Wheat chromosome 7D is partially collinear to the rice chromosome 6. By mapping SSR and EST markers derived from the rice '*Lr34* orthologous region', we observed a large genomic inversion including part of the *Lr34* containing interval. Small inversions affecting microcollinearity have been described earlier in Triticeae genomes (Dubcovsky et al. 2001; Isidore et al. 2005). Furthermore, in the *Ae. tauschii* and wheat BAC clone sequences, we identified a number of sequences with homology to ESTs that were not collinear to the targeted region of rice. These results are in agreement with earlier findings of frequent distortion of microcollinearity between rice and Triticeae genomes (Bennetzen and Ramakrishna 2002; Feuillet and Keller 2002; Akhunov et al. 2003). This mosaic conservation of microcollinearity between rice and Triticeae genomes is complicating the use of the rice model genome. However, as described below, the information from the rice genome can still provide very relevant information on candidate genes. For targeted SSR development, the application of rice genome information for the isolation of BAC clones/contigs of *Ae. tauschii* was extremely useful to saturate with markers a specific region of the wheat genome. A more complete physical map of wheat and/or *Ae. tauschii* and a better whole-genome knowledge at high resolution of rice/Triticeae genomic rearrangements would provide the necessary, improved tools for efficient work on the wheat genome.

Definition of a candidate region for *Lr34*

The allelic study at the *Xswm10* locus indicates that this marker is genetically close to the *Lr34* region. Microsatellite mapping revealed a large genomic inversion involving part of the *Lr34* flanking interval. According to this information, the rice genomic region syntenic to *Lr34* is now supposed to be proximal to EST marker BF473324. In this rice region, two related genes, both annotated as pectate lyase genes, were detected. Three wheat ESTs covering different parts of these rice genes were identified (data not shown). In *Arabidopsis*, the powdery mildew resistance gene *PMR6* has a pectate lyase structure and shows good similarity at the protein level with an assembly of three wheat ESTs. At the protein level, a 73% similarity was found in a sequence of 268 amino acids. In *Arabidopsis*, a mutation in the gene *PMR6* was described to confer horizontal resistance to powdery mildew (Vogel et al. 2002). Analysis of mutants revealed that *PMR6* in *Arabidopsis* alters cell wall composition and has a pleiotropic effect on leaf morphology, providing a pre-haustorial kind of resistance (Vogel et al. 2002). The *Lr34* locus also confers a pre-invasion resistance (Niks and Rubiales 2002), is effective against powdery mildew (Spielmeyer et al. 2005) and has pleiotropic effects on leaf morphology, e.g. it is cosegragating with LTN. Pectate lyases might play a central role in remodeling the structure of the cell wall. Therefore, they might exert a cell wall-based resistance by delaying the haustorial contact with the symplast that biotrophic plant pathogen need to establish. For this reason, we speculate that the wheat pectate lyase locus in 7DS could possibly be involved in the horizontal resistance conferred by *Lr34*.

Three independent sources of *Lr34* 'Frontana', 'Chinese Spring', and 'Forno' share a common allele at the *Xswm10* locus

The *SWM10a* allele is common to the three known independent sources of *Lr34*. This allele, which has a fragment size of 211 bp, is identical in the genotypes 'Frontana', 'Chinese Spring', and 'Forno'. To our knowledge, these cultivars do not share pedigree, although genetic distances of the tested genotypes are not known. The *SWM10a* allele is also common to all tested spring wheat lines developed at the CIMMYT, in which *Lr34* was derived from 'Frontana'. Nonetheless, in the European winter germplasm with *Lr34*, more variability at the *Xswm10* locus was observed. Genetic recombination, partial SSR instability or seed impurity could be involved in the observed discrepancies between presence of *Lr34* and absence of the *SWM10a* allele in some of these genotypes. Thus, although SWM10 seems not to be completely diagnostic when using a wide germplasm, it will be an extremely useful marker to assist breeding programs for *Lr34/Yr18* worldwide. Finally, the knowledge on

the orientation of the SWM10 BAC contig on the 7D chromosome by genetic mapping provides the basis for chromosome walking towards *Lr34*.

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