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ORIGINAL PAPER

Leymus EST linkage maps identify 4NsL–5NsL reciprocal translocation, wheat-*Leymus* chromosome introgressions, and functionally important gene loci

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Abstract Allotetraploid ($2n = 4x = 28$) *Leymus triticoides* and *Leymus cinereus* are divergent perennial grasses, which form fertile hybrids. Genetic maps with $n = 14$ linkage groups (LG) comprised with 1,583 AFLP and 67 heterologous anchor markers were previously used for mapping quantitative trait loci (QTLs) in these hybrids, and chromosomes of other *Leymus* wildryes have been transferred to wheat. However, identifications of the $x = 7$ homoeologous groups were tenuous and genetic research has been encumbered by a lack of functional, conserved gene marker sequences. Herein, we mapped 350 simple sequence repeats and 26 putative lignin biosynthesis genes from a new *Leymus* EST library and constructed one integrated consensus map with 799 markers, including 375 AFLPs and 48 heterologous markers, spanning 2,381 centiMorgans. LG1b and LG6b were reassigned as LG6b* and LG1b*, respectively, and LG4**Ns** and LG4**Xm** were inverted so that all 14 linkage groups are aligned to the $x = 7$ Triticeae chromosomes based on EST alignments to barley and other reference genomes. Amplification of 146 mapped *Leymus* ESTs representing six of the seven homoeologous groups was shown for 17 wheat-*Leymus* chromosome introgression lines. Reciprocal translocations between 4L and 5L in both *Leymus* and *Triticum monococcum* were aligned to the same regions of *Brachypodium* chromosome 1. A caffeic acid *O*-methyltransferase locus aligned to fiber QTL peaks on *Leymus* LG7a and *brown midrib* mutations of maize and sorghum. Glaucousness genes on *Leymus* and wheat chromosome 2 were aligned to the same region of *Brachypodium* chromosome 5. Markers linked to the *S* self-incompatibility gene on *Leymus* LG1a cosegregated with markers on LG2b, possibly cross-linked by gametophytic selection. Homoeologous chromosomes 1 and 2 harbor the *S* and *Z* gametophytic self-incompatibility genes of *Phalaris*, *Secale*, and *Lolium*, but the *Leymus* chromosome-2 self-incompatibility gene aligns to a different region on *Brachypodium* chromosome 5. Nevertheless, cosegregation of self-incompatibility genes on *Leymus* presents a powerful system for mapping these loci.

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Introduction

Leymus Hochst. is a genomically defined allopolyploid perennial Triticeae genus including about 30 species worldwide, which shares a similar set of two distinct subgenomes, **Ns** and **Xm**, evident by homologous chromosome pairing in pollen mother cells of inter-specific hybrids (Dewey 1970, 1972, 1984; Löve 1984). The **Ns** genome originates from *Psathyrostachys*, but the origin of the **Xm** genome has not been determined (Zhang and Dvorak 1991; Wang and Jensen 1994; Wang et al. 1994, 2006). Several *Leymus* species have been successfully hybridized with wheat and some of the resulting introgression lines display potentially useful traits including biological nitrification inhibition (Subbarao et al. 2007), resistance to *Fusarium* head blight (Chen et al. 2005; Qi et al. 2008; Wang and Chen 2008; Wang et al. 2009, 2010a), and salt tolerance (Liu et al. 2001). Biological nitrification inhibition was particularly strong in one of 11 disomic wheat-*Leymus racemosus* chromosome addition lines ($2n = 42 + 2$), *Lr*#n, which reportedly corresponds to homoeologous groups 3 and 7 (Kishii et al. 2004). However, genetic characterization of the wheat-*Leymus* chromosome addition lines has been encumbered by the lack of well-characterized genome-specific *Leymus* markers.

Basin wildrye, *Leymus cinereus* (Scribn. & Merr.) Á. Lőve, is one of the largest native grasses in western North America, growing up to 3 m tall. Creeping wildrye, *Leymus triticoides* (Buckley) Pilg., is a shorter but highly rhizomatous species specifically adapted to wet, often saline sites in this same region. Basin wildrye and creeping wildrye are both allotetraploid ($2n = 4x = 28$) and form robust, fertile hybrids. Genetic maps were constructed for two full-sib families, TTC1 and TTC2, based on segregation of pollen gametes from two interspecific hybrids, TC1 and TC2, crossed with one *L. triticoides* tester, T, which was used as the female seed parent (Wu et al. 2003). These full-sib mapping populations segregate for genes and QTLs controlling growth habit (Larson et al. 2006), forage quality (Larson and Mayland 2007), seed shattering (Larson and Kellog 2009), and many other traits such as cuticle wax that were not previously analyzed. Molecular genetic maps for the TTC1 and TTC2 families included a combined total of 1,583 AFLP markers and 67 heterologous anchor markers (markers from other grass species) with 14 linkage groups (LG) corresponding to the $n = 14$ chromosomes. Two sets of $x = 7$ homoeologous linkage groups were tentatively identified based on synteny of the heterologous anchor markers from closely related wheat (*Triticum* spp.), barley (*Hordeum vulgare*), and cereal rye (*Secale cereale*) (Wu et al. 2003; Larson et al. 2006). Synteny of genome-specific markers distinguishing the **Ns** and **Xm** subgenomes was also demonstrated for homoeologous groups 4 and 5 (Wu et al. 2003). However, the identification of the LG1b was based on only one heterologous marker, WMC336, mapped to wheat 1A and 1D and the identification of LG6b was deduced by process of elimination even though it originally did not contain any chromosome-6 anchor markers (Wu et al. 2003).

Bushman et al. (2008) sequenced and identified 11,281 expressed sequence tags (ESTs) unigene contigs from subterranean rhizome and tiller buds of *L. triticoides* × *L. cinereus* hybrids and aligned 9,389 of these unigenes to the rice genome sequence. Primers flanking 1,798 simple sequence repeats (SSRs) in these unigenes were designed and tested for polymorphism among the two *L. triticoides* × *L. cinereus* TC1 and TC2 hybrids, the *L. triticoides* T-tester plant, and one *Psathyrostachys juncea* genotype (Bushman et al. 2008). Moreover, *Leymus* EST-SSR primers that aligned to rice chromosome 2 were tested for synteny in *Leymus* (Bushman et al. 2008; Kaur et al. 2008) based on predictions of colinearity to wheat homoeologous groups 6 (La Rota and Sorrells 2004). Twenty-four of the twenty-seven markers aligned to rice chromosome 2 tested mapped to *Leymus* LG6a and LG1b (Bushman et al. 2008), which confirmed the identification of LG6a but indicated problems involving the original identifications of LG6b and LG1b by Wu et al. (2003). Thus, *Leymus* LG1b was renamed LG6b* (Bushman et al. 2008). It may also be reasonable to assume that LG6b corresponds to homoeologous group 1 and should be designated LG1b*, but there was no direct evidence to support this assertion. Thus, identifications of some linkage groups were left uncertain and the map location of some genes, including *VRN2*, the *S* self-incompatibility gene, and glaucous wax synthesis genes raise new questions that can be addressed using comparative EST maps.

Primers designed from the wheat *VRN2* gene (Yan et al. 2004) mapped to the distal long arm of *Leymus* LG5**Ns** (Larson et al. 2006). The *VRN2* orthogene maps to a region on wheat 5**A**L (Dubcovsky et al. 1998; Yan et al. 2004), which was involved in a reciprocal translocatation between 4$\mathbf{A^m}$L and 5$\mathbf{A^m}$L in the diploid *Tritcum monococcum* wheat ancestor (Devos et al. 1995; Dubcovsky et al. 1996). The *VRN2* orthogene is also present on homoeologous group 4 of winter barley (Yan et al. 2004; Karsai et al. 2005) and other Triticeae species (King et al. 1994). However, other reciprocal translocations between the distal long arms of homoeologous groups 4 and 5 have been detected in *Triticum uratu*, *Aegilops umbellulata*, and *Thinopyrum bessarabicum* (King et al. 1994) and *Secale cereale* (Devos et al. 1993). Thus, Larson et al. (2006) postulated that *Leymus* may contain a reciprocal translocation similar to *T. monococcum*, but this hypothesis was based on only one marker, *VRN*2, on LG5**Ns**.

Basin and creeping wildryes are both highly self-sterile, allogamous species (Jensen et al. 1990). Self-incompatibility is controlled by two genes, *S* and *Z*, located on homoeologous groups 1 and 2 in Poaceae grasses (Baumann et al. 2000) including *Phalaris coerulescens* (Bian et al. 2004), *Secale cereale* (Hackauf and Wehling 2005), and *Lolium* (Shinozuka et al. 2010). The *Bm2* RFLP marker, derived from a thioredoxin gene (Li et al. 1994), is closely linked to the *S* self-incompatibility gene on homoeologous group 1 of *Phalaris coerulescens* (Li et al. 1994; Langridge et al. 1999; Bian et al. 2004), *Secale cereale* (Senft and Wricke 1996; Voylokov et al. 1997), and *Lolium* (Jones et al. 2002; Shinozuka et al. 2010). Primers designed from this *Bm2* thioredoxin clone (Taylor et al. 2001; Patterson et al. 2005) amplified two distinct sets of DNA sequences designated *TRX-1* and *TRX-2* from both *L. cinereus* and *L. triticoides* (Larson et al. 2006). Gene-specific primers designed from the *Leymus TRX-1* sequences amplified 287 bp products that mapped to non-homologous loci on LG1a and LG2a in the TTC1 and TTC2 families, respectively (Larson et al. 2006). Gene-specific primers designed from the *Leymus TRX-2* sequences also amplified 287 bp products that mapped to homologous loci on LG6b in both TTC1 and TTC2 families (Larson et al. 2006), which may actually be homoeologous group 1 (LG1b*). Therefore, gene-specific primers designed from the *Leymus TRX-1* and *TRX-2* sequences amplified different 287 bp sequences that presumably map to homoeologous loci on LG1a and LG1b*, which are linked to the *S* self-incompatibility genes. Yet seemingly identical 287 bp *TRX-1* amplicons also mapped to non-homologous loci on LG2a in the TTC2 family.

Leymus wildryes also display phenotypic variation for glaucous cuticle wax coloration (Jefferson 1994; Jefferson and Kielly 1996), which has also been observed in the *Leymus* TTC1 and TTC2 families. Cuticular wax can affect plant water balance, adhesion of water and other substances to leaf surface, and susceptibility to pathogens and insects (Kunst and Samuels 2003; Jenks et al. 1994; Eigenbrode and Espelie 1995). Genetic variation for cuticular wax was negatively correlated with forage yield among half-sib families of Altai wildrye (*Leymus angustus*) (Jefferson 1994), but seed yield, seed weight, and germination were greater in the glaucous (waxy) lines of Altai wildrye (Jefferson and Kielly 1996). Alfalfa and white clovers transformed with transcription factor genes that induce cuticle wax synthesis show enhanced tolerance and physiological responses to water stress (Jiang et al. 2009, 2010). Wheat cuticle wax and glaucousness is controlled by dominant *W1* and *W2* wax synthesis genes on 2BS and 2DS, respectively, and dominant wax inhibitor genes *Iw1* and *Iw2* also located on 2BS and 2DS, respectively (Nelson et al. 1995; Goncharov et al. 1998; Tsunewaki and Ebana, 1999; Watanabe et al. 2005; Liu et al. 2007). Thus, we hypothesize that variation for glaucousness in *Leymus* may be controlled by genes orthologous to the wheat chromosome 2 *W* wax synthesis or *Iw* wax inhibitor genes.

Although EST-SSRs provide one source of useful genetic markers, many functionally important genes may not contain SSRs. At least ten known enzymes are involved in the synthesis of lignin (Humphreys and Chapple 2002; Li et al. 2008). Lignin is the second most abundant biological polymer on earth having important structural and protective functions in plants that also impacts industrial uses of cellulose for paper, livestock forage, and liquid fuel production (Jung and Ni 1998; Li et al. 2008). Mutations of the *caffeic acid O-methyltransferase* (*COMT*) and *cinnamyl alcohol dehydrogenase* (*CAD*) lignin biosynthesis genes are responsible for some *brown midrib* (*bm*) phenotypes of maize (Vignols et al. 1995) and *Sorghum* (Bout and Vermerris 2003; Saballos et al. 2009; Sattler et al. 2009). Transgenic downregulation of various lignin biosynthesis genes suggests a key role of the *COMT* gene and enhances forage digestibility in maize (He et al. 2003), tall fescue (Chen et al. 2004), perennial ryegrass (Tu et al. 2010), and switchgrass (Fu et al. 2011). Lignin gene sequences were present in the *Leymus* EST library and provide a potentially useful source of functionally important candidate gene markers even though these genes did not contain SSRs. The two strongest neutral detergent fiber (NDF) and acid detergent fiber (ADF) quantitative trait loci (QTLs) in both TTC1 and TTC2 families co-localized to the centromeric region of LG7a (Larson and Mayland 2007), which also corresponds with fiber content QTLs and lignin biosynthesis genes in perennial ryegrass (Cogan et al. 2005).

Relatively new EST libraries for perennial Triticeae grasses provide an abundant source of polymorphic SSRs (Bushman et al. 2008) and other potentially informative markers that can be aligned in silico to genome reference sequences of *Brachypodium distachyon* (The International Brachypodium Initiative 2010), rice (*Oryza sativa)* (Ouyang et al. 2007), and *Sorghum* (Paterson et al. 2009). Alignment of *Leymus* EST maps to grass genome reference sequences of cereals and model grass species will leverage extensive information from these intensively studied species to identify and manipulate genes controlling functionally important traits in these perennial grasses. Conversely, the development of *Leymus* EST will also provide new tools to identify unique genes and assimilate special adaptations from the tertiary gene pool of wheat and other Triticeae cereals. The objectives of these analyses are to: (1) construct one integrated consensus map for the TTC1 and TTC2 *Leymus* mapping families including all informative *Leymus* EST-SSR markers (Bushman et al. 2008) and conclusively identify the $x = 7$ homoeologous chromosome pairs for all $n = 14$ linkage groups of allotetraploid *Leymus* based on alignments of *Leymus* EST markers

to barley EST maps and other grass reference genomes, (2) test synteny and identification of EST linkage groups in comparisons with amplification of mapped ESTs among the wheat-*Leymus racemosus* chromosome addition lines, (3) design primers and map lignin biosynthesis ESTs and test for correspondence of the lignin genes with the LG7a fiber content QTL as postulated by Larson and Mayland (2007), (4) determine if *Leymus* contains a reciprocal translocation between homoeologous groups 4 and 5 similar to *T. monococcum* as postulated by Larson et al. (2006), and (5) reexamine the mapping and orthology of the 287 bp *TRX1* and *TRX2* amplicons that were allegedly mapped to LG1a, LG1b*, and LG2b in relationship to the location of chromosome-1 *S* and chromosome-2 *Z* self-incompatibility genes of *Phalaris*, *Secale*, and *Lolium* using a new integrated EST consensus map.

Materials and methods

Plant materials and DNA markers

The TTC1 and TTC2 mapping families were derived from one *L. triticoides* Acc:641 plant (T-tester) pollinated by two different *L. triticoides* Acc:641 × *L. cinereus* Acc:636 hybrid plants (TC1 and TC2) with 164 and 170 full-sib progeny, respectively, as described by Wu et al. (2003). The wheat-*Leymus racemosus* chromosome addition lines used in this study were described by Kishii et al. (2004) and Qi et al. (1997).

Markers tested for use in the new integrated consensus map included 1,583 AFLP markers (Wu et al. 2003), 67 heterologous anchor markers from other species such as wheat or barley (Wu et al. 2003; Larson et al. 2006), and 71 *Leymus* EST markers (Bushman et al. 2008; Kaur et al. 2008) previously mapped in the TTC1 and/or TTC2 families. In this study, all polymorphic EST-SSR primers (Bushman et al. 2008) were genotyped. A complete description of all *Leymus* EST-SSR primer sequences is available as supplementary data (Kaur et al. 2008) and also available under the Perennial Triticeae grasses project at (http://www.titan.biotec.uiuc.edu/). We also designed and tested 69 EST sequence-tagged site (STS) primer pairs in the 3′ UTRs of *Leymus* ESTs similar to known lignin biosynthesis genes.

At least ten known enzymes are involved in the synthesis of lignin including phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxlase (C4H), 4-hydroxyl-cinamyl CoA ligase (4CL), hydroxycinnamoyl CoA: shikimate hydroxycinnamoyltransferase (HCT), *p*-coumarate 3-hydroxylase (C3H), caffeoyl CoA *O*-methytransferase (CCoAOMT), cinnamoyl CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid *O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD) (Humphreys and Chapple 2002; Li et al. 2008). *Leymus* EST annotations were obtained by BLASTX or tBLASTX queries against *Arabidopsis*, *Festuca*, barley, maize, wheat, and rye protein databases with a cut-off expectation values (*E* value) of 10^{-5} as described by Bushman et al. (2008). Because the cDNAs were directionally cloned, the STS primers were designed only in reverse sequence reads from 90 EST clones that showed significant similarity to nine of the ten known lignin biosynthesis genes, excluding *C3H*, that were present in the library. Where sequences length permitted, up to three different STS primer pairs were designed in different regions of 39 EST unigenes to increase the chance of detecting size polymorphisms. Thus, 69 primer pairs were designed from 39 lignin biosynthesis unigenes including 90 unique reverse EST sequence reads (Supplemental material S1).

One new set of heterologous STS primers were also designed and tested, from the wheat *Altm1* aluminum activated malate transporter gene (Sasaki et al. 2004) GenBank accession (AB081803): *Altm1*-3379f (5′aagctgggaactctttgtcg3′) and *Altm1*-3621r (5′gccacatgtttttcgaacct3′).

Glaucous cuticle wax color evaluations

The TTC1 and TTC2 families transplanted into clonally replicated field evaluations near Hyde Park, UT in the spring of 2007 and evaluated for glaucousness (cuticle wax coloration) on 25 July 2008. Plots comprised five space-planted clones for 161 of the 164 TTC1 genotypes and 158 of the 170 TTC2 genotypes, with all plots in a randomized complete block experiment with two replications. Plants were spaced 0.5 m apart within rows and 1 m between rows. Insufficient vegetative plant material was available for three of the TTC1 genotypes and 12 of the TTC2 genotypes when the field experiments were established in 2007, so these genotypes were excluded from morphological evaluations. Each plot was scored as '1' or '0' for the presence or the absence of glaucousness.

Genotyping and genetic mapping

The EST-SSR primers were multiplexed into sets of three primer pairs, based on observed amplicons sizes from parents of the two mapping populations (Kaur et al. 2008), and amplified in 10 µl reactions prepared using 1× PCR buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 1.25 units jump start *Taq* DNA polymerase, 0.1 µmol [R110] dCTP, and 20–50 ng of DNA. PCR touch-down protocol was used with the following profile: (1) initial denaturation at 95°C for 90 s, (2) five cycles of 95°C for 1 min, 65°C for 1 min, decreasing annealing temperature 1°C/cycle, 72°C for 1 min, (3) 30 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and (4) and a final extension of 72°C for

6 min. The PCR amplicons were fractionated by capillary electrophoresis using GS500 LIZ internal size standard and ABI 3730 genetic analyzer (PE Applied Biosystems Inc., Foster City, CA, USA) and Genescan software (PE Applied Biosystems). The presence or the absence of specific PCR amplicons was scored across the parents and mapping families using Genographer version 1.6.0 (http://www.hordeum.oscs.montana.edu/genographer/).

New EST-SSR markers were initially assigned to one of 14 possible linkage groups (map nodes) in the TTC1 and/or TTC2 families (Wu et al. 2003; Larson et al. 2006) using the 'Create Groups Using a Map Node' and 'Assign Ungrouped Loci to Strongest Cross Link (SCL) Groups' functions of JoinMap 4.0 (Van Ooijen 2006). A *Strongest Cross Link* (SCL) threshold of 10 LOD was initially used to assign new (ungrouped) EST-SSR markers to the previous linkage groups. Homologous TTC1 and TTC2 linkage groups were joined using the 'Combine Groups for Map Integration' function, of JoinMap 4.0, and consensus maps for homologous linkage groups, as shown by Wu et al. (2003) were calculated by 'Regression Mapping' using only linkages with a recombination frequency smaller than 0.4, linkage LOD greater than 1.0, goodness-of-fit jump threshold of 5.0, ripples after each added locus. Thus, we used a high stringent 10 LOD threshold to place markers into linkage groups and a low stringent 1 LOD value to test many possible map orders within linkage groups. Only markers that could be mapped within the first or the second rounds of integration, within constraints of the maximum threshold for goodness-of-fit and positive map distances were included. Map diagrams and QTL graphs were developed using MapChart 2.2 (Voorrips 2002).

The *Leymus* ESTs were aligned to the *Brachypodium* genome sequence (The International Brachypodium Initiative 2010), the TIGR 6.1 rice genome annotation (Ouyang et al. 2007), *Sorghum* genome sequence (Paterson et al. 2009) and high-density barley EST SNP maps (Close et al. 2009) using the GrainGenes 2.0 (http://www.wheat.pw.usda.gov/) with a minimum BLASTN e-value 10^{-5} or smaller. Sequence-based DNA markers from other species were aligned to the same *Bracyhypodium* genome sequence using BLASTN on the Gramene website (http://www.gramene.org/).

QTL analyses of fiber content data (Larson and Mayland 2007) and glaucousness were conducted using MapQTL® 6 (Van Ooijen 2009) using the Interval Mapping procedure for the TTC1 and TTC2 families, separately and combined, using the integrated consensus map.

Results

Integrated consensus map

A subset of 303 (16.9%) out of 1,798 *Leymus* EST-SSR primers detected 245 and 291 *Leymus* EST-SSR marker

Table 1 Description of *Leymus* TTC1 and TTC2 maps including the numbers of AFLP markers, *Leymus* EST SSR markers, *Leymus* lignin biosynthesis EST STS markers, and "cross-species" heterologous STS markers

	TTC1 Map							TTC2 Map						
	AFLP	EST-SSR	Lignin EST STS	Cross-species STS	Total no. markers	Map length (cM)	cM per marker	AFLP	EST-SSR	Lignin EST STS	Cross-species STS	Total no. markers	Map length (cM)	cM per marker
LG1a	25	13	1	2	41	141.4	3.4	22	13	1	3	39	139.0	3.6
LG1b*	35	12	0	1	48	163.4	3.4	37	19	0	1	57	168.9	3.0
LG2a	30	14	2	4	50	187.0	3.7	34	16	1	1	52	186.8	3.6
LG2b	22	23	0	4	49	188.7	3.9	22	25	1	1	49	211.5	4.3
LG3a	31	21	1	5	58	188.6	3.3	31	23	2	4	60	211.5	3.5
LG3b	24	15	0	3	42	154.8	3.7	25	19	0	4	48	169.1	3.5
LG4**Ns**	15	20	0	4	39	186.1	4.8	15	27	0	3	45	153.8	3.4
LG4**Xm**	18	17	0	7	42	130.5	3.1	18	19	0	3	40	127.0	3.2
LG5**Ns**	23	20	3	2	48	194.0	4.0	22	21	2	1	46	192.7	4.2
LG5**Xm**	22	14	2	2	40	144.1	3.6	21	22	3	3	46	168.2	3.7
LG6a	20	14	1	0	35	175.7	5.0	23	17	1	1	42	182.3	4.3
LG6b*	21	11	1	0	33	149.5	4.5	19	16	2	1	38	154.2	4.1
LG7a	13	29	4	2	48	190.9	4.0	19	27	3	2	51	157.8	3.1
LG7b	21	22	3	1	47	150.4	3.2	21	27	5	1	54	172.9	3.2
Overall	320	245	18	37	620	2,345.1	3.8	329	291	21	29	670	2,395.7	3.6

Map length in centiMorgans (cM) of recombination distance; and average numbers of markers per cM

Table 2 Description of *Leymus* TTC integrated consensus maps including the numbers of AFLP loci, *Leymus* EST-SSR loci, *Leymus* Lignin biosynthesis EST-STS loci, and "cross-species" heterologous STS loci

	Markers common to both TTC1 and TTC2 maps					TTC Consensus Maps (including markers present in TTC1 or TTC2 maps)						
	AFLP	EST SSR	Lignin EST STS	Cross-species STS	Total no. markers	AFLP	EST SSR	Lignin EST STS	Cross-species STS	Total no. markers	Map length (cM)	cM per marker
LG1a	19	8	1	1	29	28	18	1	4	51	135.9	2.7
LG1b*	29	11	0	1	41	43	20	0	1	64	166.9	2.6
LG2a	25	12	1	1	39	39	18	2	4	63	188.5	3
LG2b	21	14	0	1	36	23	34	1	4	62	207.2	3.3
LG3a	31	16	1	3	51	31	28	2	6	67	200.8	3
LG3b	21	14	0	2	37	28	20	0	5	53	164.7	3.2
LG4**Ns**	14	18	0	2	34	16	29	0	5	50	168.1	3.4
LG4**Xm**	18	11	0	3	32	18	25	0	7	50	127.3	2.5
LG5**Ns**	18	14	2	0	34	27	27	3	3	60	194.8	3.2
LG5**Xm**	18	9	2	1	30	25	27	3	4	59	160.5	2.7
LG6a	17	9	1	0	27	26	22	1	1	50	179.2	3.6
LG6b*	14	9	1	0	24	26	18	2	1	47	153.6	3.3
LG7a	9	23	3	2	37	23	33	4	2	62	171.7	2.8
LG7b	20	18	1	1	40	22	31	7	1	61	161.8	2.7
Overall	274	186	13	18	491	375	350	26	48	799	2,381	3.0

Map length in centiMorgans (cM) of recombination distance, and average numbers of markers per cM

loci in the TTC1 and TTC2 families, respectively (Table 1), which were merged to create 350 marker loci in the consensus map (Table 2). A subset of 186 (53.1%) of these 350 marker loci showed segregation in both families and map to homologous loci, which were joined into 186 marker loci in the consensus map (Fig. 1; Table 2; Supplemental material S2). Another 59 markers were specific to TTC1 family and another 105 were specific to TTC2 family, which merged to create another 164 marker loci in the consensus map. Alignments of these *Leymus* EST-SSR markers to the *Brachypodium,* rice, and *Sorghum* genome reference sequences and high-density barley EST linkage maps (Table 3, Supplemental material S3), confirmed identification of the $x = 7$ homoeologous linkage linkage groups, except that the original LG6b and LG1b were renamed LG1b* and LG6b*, respectively, and LG4**Ns** and LG4**Xm** were invereted. A subset of 45 (14.9%) of the 303 informative EST-SSR primer pairs detected multiple marker loci including 43 primer pairs that detected two loci and two primer pairs that detected three marker loci. A total of 40 (89%) out of the 45 primers, which detected multiple loci, mapped to homoeologous linkage groups. One primer pair detected loci on LG1a and LG1b, three on LG2a and LG2b, six on LG3a and LG3b, five on LG4**Ns** and LG4**Xm**, seven on LG5**Ns** and LG5**Xm**, seven on LG6a and LG6b, and 11 on LG7a and LG7b.

A total of 29 (42%) of the 69 EST-STS primer pairs designed from 39 putative *Leymus* lignin biosynthesis unigenes displayed polymorphism among the parents, but six of these were redundant with other primers designed from the same unigene contig (Supplemental material S1). Thus, primers designed from 23 (59%) of the 39 putative lignin unigenes detected 26 loci, including at least one locus for eight of the ten known lignin biosynthesis enzymes (Table 4). Three primer pairs for two putative *CL* unigenes failed to detect polymorphism for this lignin biosynthesis gene and the *C3H* gene was not present in the EST library. Nevertheless, the overall success rate of mapping EST-STS primers designed from the 3′ UTRs was actually higher than EST-SSR primers. Two primer pairs for one putative *C4H* unigene detected one marker locus. Fourteen primer pairs for eight *CAD* unigenes detected three marker loci for three unigenes. The *Leymus CAD-2*, *CAD-3*, and *CAD-8* (Supplemental material S1) unigenes showed significant similarity to the *bmr6* brown midrib gene (FJ554573) and the *CAD-8* unigene aligns to the *bmr6* locus on the *Sorghum* genome sequence. However, the *CAD-8* unigene was not mapped in the *Leymus* TTC1 or TTC2 families. The *CAD-1*, *CAD-2*, and *CAD-3* map to homoeologous regions of LG5**Ns** and LG5**Xm** (Supplemental material S2) and align to the same locus in *Sorghum*. However, the *CAD-3* unigene aligns about 6 kb away from the *CAD-1*/*CAD-2* alignments in rice and all three mapped *CAD* unigenes align to different loci on *Brachypodium* chromosome 4. Five primer pairs for three *CCoAOMT* unigenes detected two marker loci for two unigenes. The two *CCoAOMT* unigenes are more than 50 cM apart on LG7b (Supplemental material S2) but align to the same *Sorghum* locus and two separate, but nearly

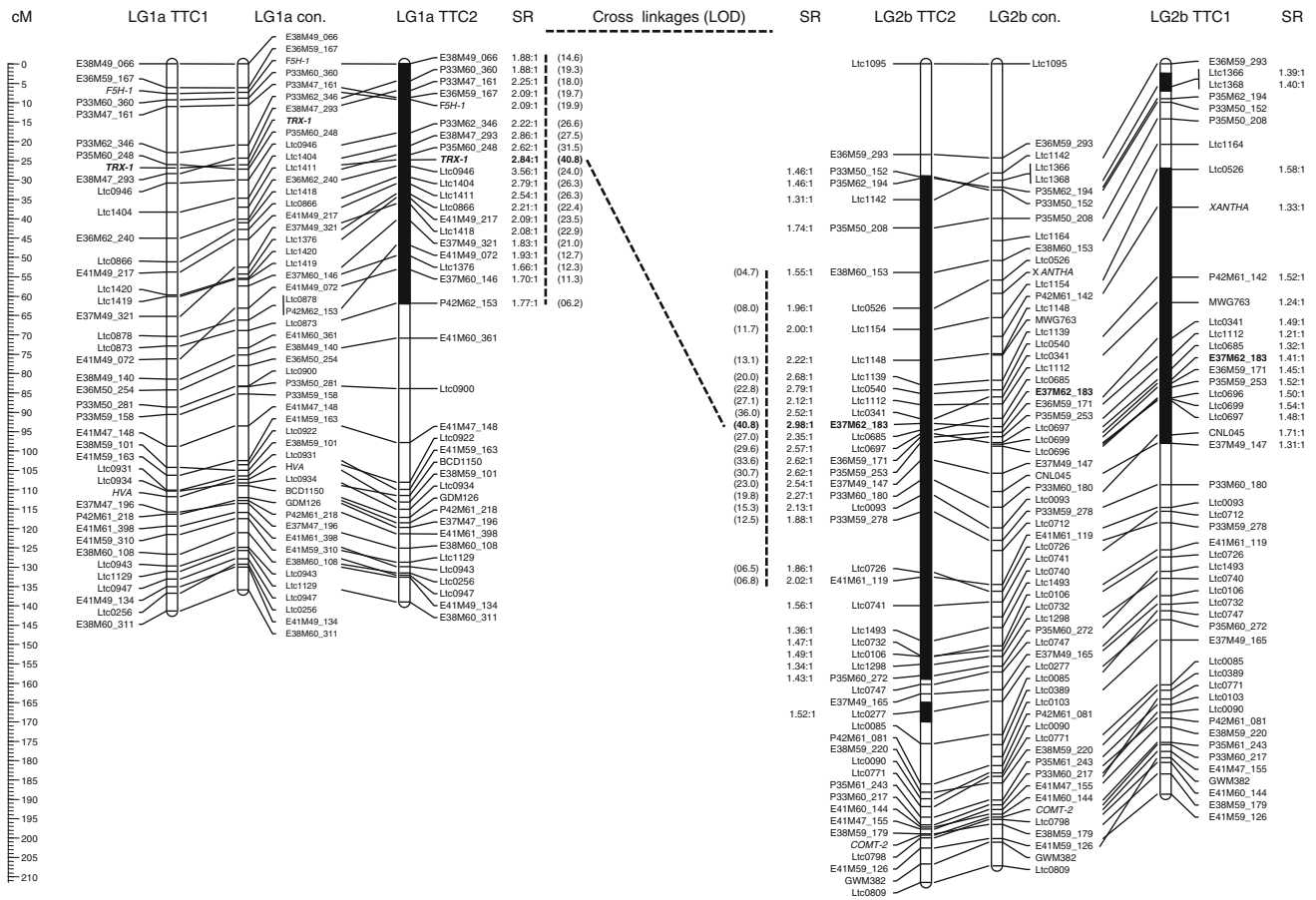

Fig. 1 The *Leymus* consensus maps demonstrate cross-linkages between LG1a and LG2b in the TTC2 family. The segregation ratio (SR) of *L. cinereus* to *L. triticoides* parental alleles is shown for DNA markers that showed significant deviation from the 1:1 expected ratio (*dark shaded* chromosome regions). Markers that show significant cross-linkages between different linkage groups, in the TTC2 family, are outlined by *dashed lines* with the corresponding LOD significance values shown in parentheses. The TRX-1 (LG1a) and E37M62_183 (LG2a) markers (*bold text*) co-segregated in the TTC2 family. Linkage maps are drawn to the same scale, in centiMorgans (cM), shown in the *left side* of figure

adjacent sequences in *Brachypodium* (12 kb apart) and rice (11 kb apart). Sixteen primers for nine *CCR* unigenes detected five loci for five unigenes, but the *CCR-2* and *CCR-4* loci align to the same orthologous in *Brachypodium*, rice, and *Sorghum* and map to homoeologous regions of LG7a and LG7b. The *CCR-1* and *CCR-9* unigenes mapped about 50 cM apart on LG5**Ns** and align to different loci in *Brachypodium*, rice, and *Sorghum*. Seven primer pairs for three *COMT* unigenes detected three marker loci for two unigenes, including two homoeologous *COMT-1* loci on LG7a and LG7b. The *COMT-1* unigene aligns to the same locus on *Sorghum* chromosome 7 as *Sorghum bmr12* (AY217766) and maize *bm3* (X80669). Transgene sequences used to downregulate COMT gene expression and lignin content in perennial ryegrass (AF033538), tall fescue (AF153824), switchgrass (HQ645965), and maize (*Sorghum* AF387790) also align to this same orthologous on *Sorghum* chromosome 7. The *COMT-1* unigene contig also contained more EST clones than any other lignin biosynthesis gene tested (Table 4). Six primer pairs for three *F5H* unigenes detected four marker loci for three unigenes, including two homoeologous *F5H-2* loci on LG7a and LG7b. Ten primers for six *HCT* unigenes detected four loci for four unigenes. Three of these *HCT* markers loci are located on homoeologous group 7 and the *HCT-2* and *HCT-6* markers map within a few cM of each other on LG7b and align to loci about 37 kb apart in *Brachypodium*, but have more dispersed alignments in rice (1.5 Mb apart) and *Sorghum* (unlinked). The *HCT-1* marker aligns to a region of LG7a that is not homoeologous with the *HCT* loci on LG7b and aligns to different regions of *Brachypodium,* rice, and *Sorghum*. Six primer pairs for four *PAL* unigenes detected four marker loci for three unigenes, including two homoeologous *PAL-1* marker loci on LG6a and LG6b. The *PAL-4* maps less than 0.6 cM from the *PAL-1* locus on LG6b and these two unigenes align to adjacent sequences in *Brachypodium* (11 kb apart), rice (11 kb apart), and *Sorghum* (13 kb apart). Thus, we

Table 3 Alignments of *Leymus* EST-SSR and lignin EST-STS marker loci to the five *Brachypodium distachyon* (*Bd*) chromosome sequences, the 12 *Oryza sativa* (*Os*) chromosome sequences, the 10 *Sorghum bicolor* (*Sb*) chromosome sequences, and seven high-density *Hordeum vulgare* (Hv) EST linkage groups

	LG 1a	LG 1b*	LG 2a	LG 2b	LG 3a	LG 3b	LG 4**Ns**	LG 4**Xm**	LG 5**Ns**	LG 5**Xm**	LG 6a	LG 6b*	LG 7a	LG 7b	**Sum**
*Bd*1	1	2	**4**	**8**	2	1	**21**	**16**	**9**	**5**	1	2	**14**	**15**	**101**
*Bd*2	**11**	**9**	0	4	**25**	**17**	1	1	2	0	0	0	0	0	**70**
*Bd*3	**6**	**7**	2	3	2	0	1	2	2	0	**20**	**16**	**16**	**18**	**95**
*Bd*4	1	1	0	1	1	0	**6**	**6**	**15**	**23**	2	2	1	0	**59**
*Bd*5	0	0	**12**	**16**	0	0	0	0	0	0	0	0	1	1	**30**
Bd	**19**	**19**	**18**	**32**	**30**	**18**	**29**	**25**	**28**	**28**	**23**	**20**	**32**	**34**	**355**
*Os*1	1	1	0	1	**21**	**15**	0	1	1	0	0	0	1	1	**43**
*Os*2	0	1	2	3	0	0	1	0	0	0	**14**	**13**	0	0	**34**
*Os*3	0	0	1	1	1	2	**17**	**11**	**7**	**3**	0	1	1	1	**46**
*Os*4	0	0	**9**	**11**	0	0	0	0	0	0	0	0	0	1	**21**
*Os*5	**8**	**6**	0	0	3	0	0	0	2	0	0	0	0	0	**19**
*Os*6	0	0	0	0	0	0	1	0	0	0	3	0	**11**	**11**	**26**
*Os*7	1	1	**1**	**6**	1	0	0	2	0	0	0	0	0	0	**12**
*Os*8	0	1	0	0	1	0	0	2	0	1	0	0	**14**	**17**	**36**
*Os*9	0	0	0	0	0	0	2	0	**10**	**14**	0	0	1	2	**29**
*Os*10	**6**	**7**	1	2	1	0	0	1	1	0	0	0	0	0	**19**
*Os*11	0	0	0	1	0	0	**2**	**4**	5	4	2	2	0	2	**22**
*Os*12	0	1	0	0	0	0	1	1	**4**	**6**	0	0	0	0	**13**
Os	**16**	**18**	**14**	**25**	**28**	**17**	**24**	**24**	**28**	**28**	**19**	**16**	**28**	**35**	**320**
*Sb*1	**3**	**6**	1	0	2	1	**18**	**11**	**7**	**4**	0	1	1	1	**56**
*Sb*2	1	1	2	4	0	0	3	1	**8**	**12**	0	0	1	0	**33**
*Sb*3	1	1	0	2	**20**	**13**	0	2	1	0	0	0	0	0	**40**
*Sb*4	0	1	2	2	0	0	2	0	0	0	**17**	**13**	1	0	**38**
*Sb*5	0	0	0	0	0	0	2	4	0	3	1	2	0	0	**12**
*Sb*6	0	1	**7**	**12**	1	0	0	0	1	0	0	0	1	1	**24**
*Sb*7	0	0	0	2	2	0	0	2	3	1	0	0	**14**	**14**	**38**
*Sb*8	0	0	0	2	0	0	2	2	**5**	**4**	1	0	1	2	**19**
*Sb*9	**8**	**8**	0	0	3	0	0	0	1	0	0	0	0	0	**20**
*Sb*10	0	0	0	0	0	0	0	0	2	1	1	0	**9**	**13**	**26**
Sb	**13**	**18**	**12**	**24**	**28**	**14**	**27**	**22**	**28**	**25**	**20**	**16**	**28**	**31**	**306**
Hv1	**2**	**5**	0	0	1	0	0	0	0	0	0	0	0	0	**8**
Hv2	0	0	**5**	**8**	0	0	0	0	0	0	0	0	0	1	**14**
Hv3	0	0	0	0	**9**	**2**	0	0	0	0	0	0	0	0	**11**
Hv4	0	0	0	0	0	0	**3**	**4**	**3**[a]	0	0	1	1	1	**13**
Hv5	0	0	0	0	0	0	**4**[a]	0	**3**	**7**	0	1	0	0	**15**
Hv6	0	0	0	0	0	0	1	0	0	0	**7**	**4**	0	0	**12**
Hv7	0	0	0	0	1	0	0	0	0	0	0	0	**5**	**9**	**15**
Hv	**2**	**5**	**5**	**8**	**11**	**2**	**8**	**4**	**6**	**7**	**7**	**6**	**6**	**11**	**88**

Possible homoeologous chromosome regions are indicated by bold underlined text

[a] Reciprocal translocation

detected 26 markers loci for 23 lignin unigenes including two homoeologous loci each for *COMT-1*, *FSH-2*, and *PAL-1* (Table 4). Moreover, genetic and physically linked clusters of related unigene sequences were also detected for the *CAD*, *CCoAOMT*, *HCT*, and *PAL* lignin biosynthesis genes (Table 4). The map locations and alignments of the *Leymus* lignin biosynthesis unigene sequences to *Brachypodium,* rice, and *Sorghum* genome reference sequences (Table 4) were consistent with patterns of synteny among other *Leymus* ESTs (Table 3).

Table 4 List of putative lignin biosynthesis EST-STS unigene contigs; corresponding unigene alignments to chromosomes in *Brachypodium distachyon* (*Bd*), *Oryza sativa* (*Os*), and *Sorghum bicolor* (*Sb*); and genetic map loci detected in the *Leymus* TTC1 or TTC2 mapping families

Unigene Marker Name	Keck EST or EST contig ID	Reverse ESTs	*Bd*	*Os*	*Sb*	TTC1 linkage group	TTC Consenus group	TTC2 Linkage group
C4H	BG01_2.2568.C3.Contig3426	1	**2**	**1**	**3**		3a	3a
CAD-1	BG01_2.2032.C3.Contig2826	2	**4**	**9**	**2**	5**Ns**	5**Ns**	
CAD-2	BG01_2.2032.C2.Contig2825	3	**4**	**9**	**2**		5**Xm**	5**Xm**
CAD-3	BG01_2.2032.C1.Contig2824	2	**4**	**9**	**2**	5**Xm**	5**Xm**	5**Xm**
CCoAOMT-1	BG01_2.1676.C1.Contig2386	8	**3**	**8**	**7**		7b	7b
CCoAOMT-2	BG01_2.2104.C1.Contig2904	1	**3**	**8**	**7**		7b	7b
CCR-1	BG01_2.464.C3.Contig781	2	3	**9**	10	5**Ns**	5**Ns**	5**Ns**
CCR-2	BG01035B2B03.r1	1	**1**	9	**10**	7a	7a	
CCR-4	BG01_2.464.C1.Contig779	2	**1**	9	**10**	7b	7b	7b
CCR-6	BG01_2.954.C2.Contig1447	3	**2**	**1**	**3**	3a	3a	3a
CCR-9	BG01_2.3424.C1.Contig4348	3	3	–	**2**	5**Ns**	5**Ns**	5**Ns**
COMT-1	BG01_2.1606.C2.Contig2291	12	**3**	**8**	**7**	7a/7b	7a/7b	7a
COMT-2	BG01_2.3981.C1.Contig4926	1	2	8	7		2b	2b
F5H-1	BG01024A1B05.r1	1	**3**	**10**	**1**	1a	1a	1a
F5H-2	BG01039A1H03.r1	1	1	–	1	7a	7a/7b	7a/7b
F5H-3	BG01_2.3666.C1.Contig4601	1	3	10	1	2a	2a	2a
HCT-1	BG01_2.2456.C1.Contig3307	6	**3**	9	**7**	7a	7a	7a
HCT-2	BG01039B1D09.r1	1	**1**	**6**	4	7b	7b	
HCT-4	BG01_2.2456.C2.Contig3308	2	**4**	**9**	**2**	5**Xm**	5**Xm**	5**Xm**
HCT-6	BG01007B1G10.r1	1	**1**	**6**	**10**		7b	7b
PAL-1	BG01_2.479.C2.Contig806	10	**3**	**2**	**4**	6a/6b*	6a/6b*	6a/6b*
PAL-3	BG01037A1E05.r1	1	**5**	**4**	**6**	2a	2a	
PAL-4	BG01033B2F06.r1	1	**3**	**2**	**4**		6b*	6b

Chromosome alignments that are syntenous with other mapped *Leymus* EST alignments (Table 3) are indicated by bold underline text

A total of 47 of the 67 anchor markers originally derived from heterologous RFLP probes or other heterologous gene sequences (Wu et al. 2003; Larson et al. 2006) were retained in the integrated consensus map. Many of the heterologous RFLP probes and STS primers were difficult to genotype. Thus, heterologous anchor markers that had relatively large amounts of missing data were eliminated from the integrated consensus map. One new heterologous anchor marker, designed from the wheat *Altm1* gene, was also mapped to the long arm of LG4**Xm**. The wheat *Altm1* aluminum tolerance gene also maps to the long arm of wheat homoeologous group 4. Thus, a total of 48 anchor markers originally derived from heterologous sequences are present in the integrated consensus map (Table 2). However, the *TRX-1* marker that originally mapped to LG1a in the TTC1 family and LG2b in the TTC2 family (Larson et al. 2006), merged to the same locus on LG1a of the consensus map (Fig. 1). Further investigation revealed that the *TRX-1* marker cosegregated and mapped with the E37M62_183 marker on LG2b in the TTC2 family (LOD = 41), but also showed strong linkage (LOD > 30) to P35M60_248 and other markers on LG1a in the TTC2 family (Fig. 1a). In fact, relatively strong cross-linkages were detected between many markers on LG1a and LG2b in the TTC2 family, all of which centered on the *TRX-1* LG1a and E37M62_183 LG2b markers. Although *TRX-1* marker shows very strong linkage to both LG2b to LG1a in the TTC2 family, it was reassigned from LG2b to LG1a based on integration with other homologous markers from the TTC1 family (Fig. 1a). However, the cosegregation of physically independent *TRX-1* (LG1a) and E37M62_183 (LG2b) loci indicates strong gametophytic selection in favor of species-phased allele combinations. Significant segregation distortion, in favor of the *L. cinereus* pollen alleles from the TC1 and TC2 hybrids, was also associated with the E37M62_183 marker in both families and the *TRX-1* marker in the TTC2 family (Fig. 1). The *TRX-2* marker maps to LG1b*, which was previously misidentified as LG6b (Fig. 2). Therefore, despite the previous confusion, it appears that both *TRX-1* and *TRX-2* marker loci map to homoeologous group 1, in both TTC1 and TTC2 families.

Overlapping sets of 320 and 329 AFLP markers were retained from the TTC1 and TTC2 families, respectively (Table 1), to help fill gaps between *Leymus* ESTs and other

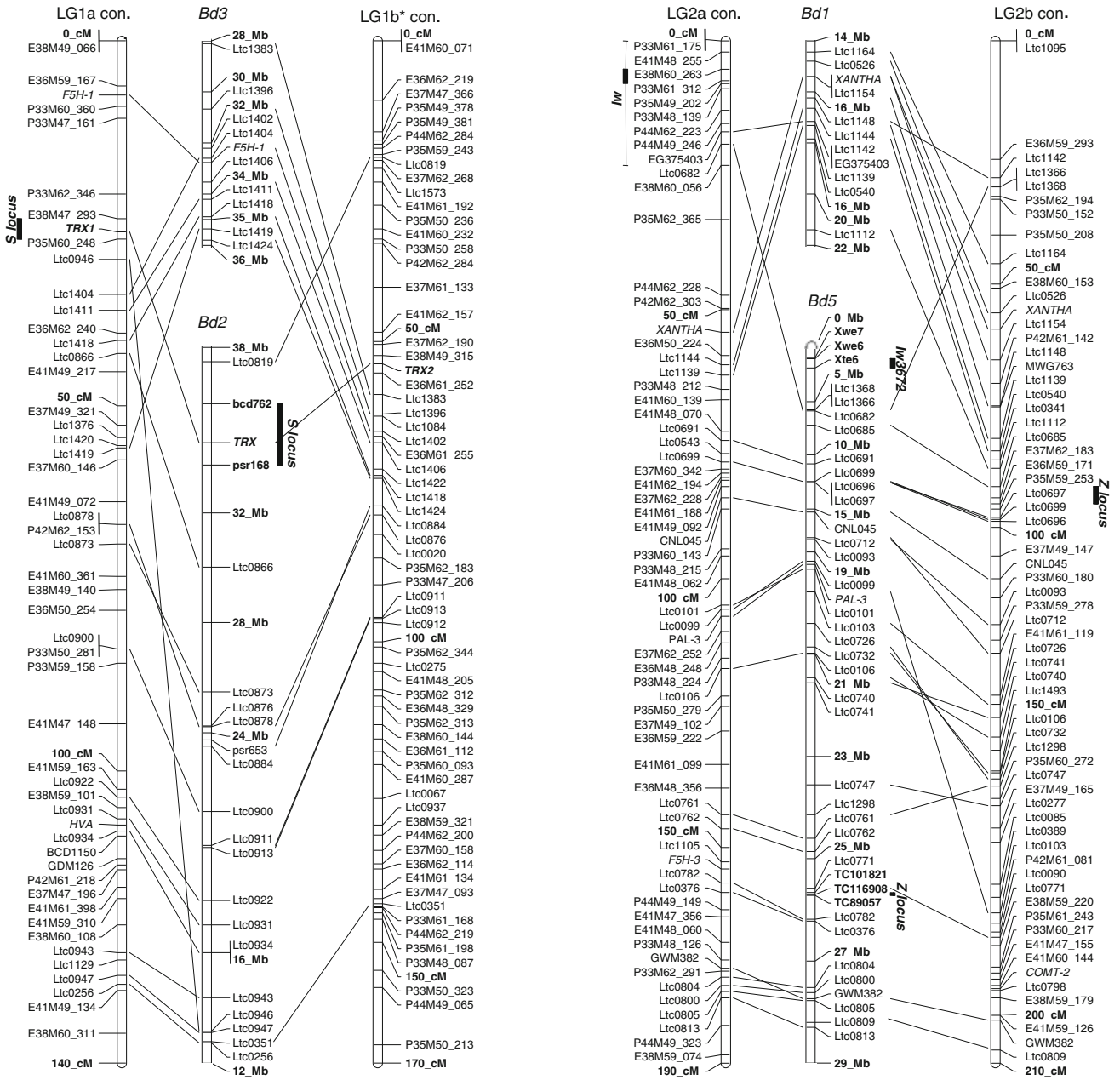

Fig. 2 Comparative mapping of genes controlling self-incompatibility and cuticle wax synthesis. The *Leymus* linkage groups (LG), scaled in centiMorgans (cM), are aligned to *Brachypodium distachyon* (*Bd*) chromosome sequences scaled in megabases (Mb). The map location of cosegregating regions of *Leymus* LG1a and LG2b consensus maps are compared to alignments of *S* and *Z* self-incompatibility gene markers from *Secale* and *Lolium*. Dominant inhibitors of cuticle wax (*Iw*) on the short arm of *Leymus* LG2a are compared with alignments of DNA markers for the dominant gene inhibitor of cuticle wax (*Iw3672*) from wheat 2S

anchor markers of the integrated consensus map. These AFLP markers merged into 375 AFLP loci in the consensus map (Table 2). Preference was given to AFLP markers that were informative in both families. Thus, a relatively large subset of 274 (73%) out of these 375 AFLPs were segregating in both families and map to homologous loci, which were joined in the integrated consensus map (Fig. 1; Table 2; Supplemental material S2). However, 46 and 55 family-specific AFLP markers were also retained in the TTC1 and TTC2 maps, respectively, to help fill remaining gaps as best as possible (Table 2).

In summary, the integrated consensus map contains a total of 799 markers including 375 AFLP markers, 350 *Leymus* EST-SSR markers, 26 *Leymus* ligning biosynthesis

EST-STS markers, and 48 heterologous anchor markers spanning 14 linkage groups and 2,381 cM. A relatively large subset of 491 (61.5%) out of these 799 markers detected homologous loci in the TTC1 and TTC2 families, which were merged in the integrated consensus map (Supplemental material S2). Most of the markers that detected homologous loci in the TTC1 and TTC2 families showed collinear map order (Supplemental material S2). However, some relatively small differences in map orders of homologous loci were detected between the TTC1 and TTC2 maps, which may be caused by data ambiguity rather than real genetic differences since the parents and pedigrees of the two families are closely related and are very similar. The average space between markers is 3.0 cM (Table 2). The three largest gaps are about 25 cM on distal short arm of LG2b, 16 cM on the distal long arm of LG3a, and 15 cM on LG2a (Fig. 1; Supplemental material S2).

Comparative mapping of self-incompatibility genes

Strong cross-linkages (cosegregation) between the *TRX-1* marker on LG1a and the E37M62_183 marker on LG2b (Fig. 1) suggested possible interactions of the *S* and *Z* gametophytic self-incompatibility genes present on homoeologous groups 1 and 2 of *Phalaris, Lolium,* and *Secale*. Thus, we investigated alignments of the *TRX-1*, E37M62_183, and other markers on LG1a and LG2b of *Leymus* with the *S* and *Z* self-incompatibility gene markers of *Phalaris, Lolium,* and *Secale* using the *Brachypodium* genome sequence as a reference (Fig. 2).

The *Leymus* LG1a and LG1b linkage groups align to *Brachypodium* chromosomes 2 and 3 (Table 3) and the *TRX-1* and *TRX-2* gene markers align specifically to *Brachypodium* chromosome 2 (Fig. 2). In addition to the *TRX* (*bm2*) alignment, we found several other markers (BCD762 and PSR168) that are closely linked to the *S* self-incompatibility genes of *Lolium*, *Phalaris,* and *Secale* (Bian et al. 2004; Shinozuka et al. 2010) that could also be aligned to the *Brachypodium* genome (Fig. 2). Other markers linked to the *S* self-incompatibility genes of *Lolium*, *Phalaris,* and *Secale* lack available sequence information or do not have significant match to *Brachypodium*. Thus, the *Leymus TRX-1* and *TRX-2* marker loci may be syntenic with the *S* self-incompatibility orthogenes on LG1a and LG1b.

The *Leymus* LG2a and LG2b linkage groups align to *Brachypodium* chromosomes 1 and 5 (Table 3) and *Leymus* LG2b EST markers flanking E37M62_183 (Ltc0685 and Ltc699) align specifically to *Brachypodium* chromosome 2. The TC101821, TC116908, and TC89057 DNA markers are closely linked to the *Z* self-incompatibility genes of *Secale* (Hackauf and Wehling 2005) and *Lolium* (Shinozuka et al. 2010), but these markers align to a different region of *Brachypodium* chromosome 2. Nevertheless, we designate this gamete compatibility gene as *Z* in recognition of its interaction with the *Leymus S* locus (Fig. 2).

Cuticle wax and glaucous plant color variation

The TC1 hybrid parent genotype has waxy cuticle and glaucous plant coloration, whereas the TC2 hybrid and T-tester parental genotypes both have non-waxy cuticle with clear green plant coloration. Both TTC1 and TTC2 full-sib families showed phenotypic variation for glaucous color variation and initial scoring of the cuticle wax synthesis showed relatively high correlation coefficients of 0.86 and 0.98 between replications of the TTC1 and TTC2 field plots, respectively. Plots that were not scored the same were reexamined and we subsequently deduced that the glaucous phenotype was always present if at least one plot was initially scored as glaucous.

The TTC1 progeny of the glaucous TC1 and green T-tester parental genotypes segregated with 74 green and 84 glaucous phenotypes, which closely fits the expected 1:1 segregation ratio for mating of one homozygous recessive parent and one heterozygous parent in a simple one-gene model with dominant and recessive alleles. However, we did not detect any significant QTL effects for this trait because the TTC1 map was constructed based on segregation of markers from the highly heterozygous TC1 parent but the glaucous TC1 hybrid parent was evidently homozygous recessive for this glaucousness gene. Apparently, segregation of glaucousness in the TTC1 family resulted from heterozygosity of a glaucousness inhibitor gene in green T-tester parent, but we do not have a map for this parent.

The TTC2 progeny of the green TC2 and green T-tester parental genotypes segregated with 117 green and 44 glaucous genotypes, which closely fits a 3:1 expected segregation ratio for mating of heterozygous parents that both carry recessive alleles for glaucous plant coloration in a one-gene model with dominant (green) and recessive (glaucous) alleles. Significant QTL effects with LOD significance values in excess of 13 were detected with three AFLP markers between map coordinates of 5.35 and 7.93 cM on the distal short arm of LG2a (Fig. 2) when the glaucous phenotype was treated as a quantitative trait. However, we could not simply map this trait as a morphological gene because we do not have a map to control for dominant wax inhibitor alleles coming from the T-tester parent. Nevertheless, the *Leymus* TTC2 wax inhibitor QTL peak on LG2a aligns to the same region of *Brachypodium* chromosome 5 as the wheat *Iw3672* dominant inhibitor of wax synthesis (Fig. 2). The wheat *Iw3672* gene was

mapped to a 1.4 cM interval between *Xte6* and *Xwe6* loci, and also closely linked to the *Xwe7* locus on the distal region of wheat chromosome 2DS (Liu et al. 2007). Marker sequences for the wheat *Xte6*, *Xwe6* loci, and *Xwe7* loci are located in the same physical map order between 940,056 and 1,909,897 bp coordinates on the short arm of *Brachypodium* chromosome 5 (Fig. 2).

Correspondence test of LG7a fiber content QTL and lignin biosynthesis EST loci

The single best QTL marker for NDF and ADF trait data (Larson and Mayland 2007) in the combined analysis of TTC1 and TTC2 families, using the new integrated consensus map (Supplementary material S2), was the LG7b *COMT-1* locus. For the NDF trait, the *COMT-1* marker had LOD value of 3.5 with 9.5% variance explained in the TTC1 family and a LOD value of 2.0 with 5.3% variance explained in the TTC2 family, producing a combined LOD value of 5.5 for both families. For the ADF trait, the *COMT-1* marker had LOD value of 4.7 with 12.5% variance explained in the TTC1 family and a LOD value of 3.6 with 9.3% variance explained in the TTC2 family, producing a combined LOD value of 8.2 for both families. However, several other lignin biosynthesis EST loci were detected on LG7a and LG7b (Table 4) and the *F5H-2* markers were closely linked with *COMT-1* markers on both LG7a (2.6 cM apart) and LG7b (2.3 cM apart).

4**Ns**L–5**Ns**L reciprocal translocation

The distal long arm of wheat and barley chromosomes 4 and 5 align to the long and short arms of *Brachypodium* chromosome 1 (*Bd*1), respectively (The International Brachypodium Initiative 2010). Thus, genetic maps of the *T. monococcum* 4$\mathbf{A^m}$L–5$\mathbf{A^m}$L reciprocal translocation (Devos et al. 1995; Dubcovsky et al. 1998; Hori et al. 2007), barley 4**H** and 5**H** (Hori et al. 2007; Sato et al. 2004), the putative *Leymus* 4**NsL–5NsL** reciprocal translocation (Larson et al. 2006), and the *Leymus* 4**Xm**–5**Xm** consensus maps were all compared to the same *Brachypodium* chromosome 1 sequence (Fig. 3) in order to compare patterns of synteny using wheat, barley, and *Leymus* marker sequences available in GrainGenes 2.0 (http://www.wheat.pw.usda.gov/), Triticeae Mapped EST DataBase (TriMEDB) (http://www.trimedb.psc.riken.jp), and the Perennial Triticeae Grasses project (http://www.titan.biotec.uiuc.edu/).

Triticum monococcum 4$\mathbf{A^m}$ and *Leymus* LG4**Ns** show similar alignments to *Brachypodium* chromosome 1, which are different from *Leymus* 4**Xm** and barley 4**H** (Fig. 3). Likewise, *T. monococcum* 5$\mathbf{A^m}$ and *Leymus* LG5**Ns** show similar alignments to different regions *Brachypodium* chromosome 1, which are different from *Leymus* 5**Xm** and barley 4**H** (Fig. 3). *Leymus* LG4**Xm** and barley 4**H** markers are largely syntenic with the long arm of *Brachypodium* chromosome 1 (Fig. 3), whereas *Leymus* LG5**Xm** and barley 5**H** markers are largely syntenic with the short arm of *Brachypodium* chromosome 1 (Fig. 3). However, alignments of *T. monococcum* 4$\mathbf{A^m}$ and 5$\mathbf{A^m}$, *Leymus* 4**Ns** and 5**Ns**, and *Brachypodium* chromosome 1 are more complex. *Leymus* LG4**Ns** and *T. monococcum* 4$\mathbf{A^m}$ markers are syntenic with the proximal long arm and distal short arm of *Brachypodium* chromosome 1 (Fig. 3). Conversely, *Leymus* LG5**Ns** and *T. monococcum* 5$\mathbf{A^m}$ markers are syntenic with the proximal short arm and distal long arm of *Brachypodium* chromosome 1 (Fig. 3). Thus, the *VRN2* orthogenes on *T. monococcum* 5$\mathbf{A^m}$, *Leymus* LG**5Ns**, and barley 4**H** are syntenic with other markers that align to the distal long arm of *Brachypodium* chromosome 1 (Fig. 3), however the *VRN2* gene simply is not present in *Brachypodium* (Higgins et al. 2010). We deduced from this that the wheat **A** and *Leymus* **Ns** subgenomes have reciprocal translocations which align to similar breakpoint intervals on the short and long arms of *Brachypodium* chromosome 1 (Fig. 3F). We also deduced that chromosomes 4 and 5 of the barley **H** and *Leymus* **Xm** subgenomes show more synteny with the ancestral *Brachypodium* chromosome 1 sequence compared to the *T. monococcum* ***A*** and *Leymus* **Ns** subgenomes.

Fig. 3 Alignments of the *Brachypodium distachyon* chromosome 1 (*Bd*1) physical sequence, scaled in megabases (Mb), to Triticeae chromosome 4 and 5 linkage maps, scaled in centiMorgans. *Dark* and *light shaded chromosome bars* correspond to Triticeae homoeologous groups 4 and 5, respectively, including both syntenic and translocated regions. Inferred translocation break intervals are indicated by cross-hatched chromosome *bars*. Triticeae linkage maps were constructed from crosses of **a** *Triticum monococcum* accessions G1777 and G2528 (Devos et al. 1995; Dubcovsky et al. 1998) designated by genome symbol $\mathbf{A^m}$(1), **b** *Hordeum vulgare* cultivar Haruna Nijo and *H. vulgare* ssp. *spontaneum* strain H602 (Sato et al. 2004) designated by genome *symbol H*. **c** *T. monococcum* strain KT3-5 × *T. boeoticum* strain KT1-1 (Hori et al. 2007) designated by genome symbol $\mathbf{A^m}$(2), **d** and **e**) *Leymus* TTC1 and TTC2 consensus maps designated by subgenome symbols **Ns** and **Xm**. Chart **f** shows an integrated comparison of genetic markers flanking syntenic and translocated regions of *T. monococcum*, *Hordeum*, and *Leymus* chromosome 4 (*dark shaded bars*) and chromosome 5 (*light shaded bars*), including the inferred translocation breakpoint intervals (*cross-hatched bars*)

Wheat-*Leymus racemosus* chromosome addition lines

A subset of 146 (48%) of the 303 mapped *Leymus* EST-SSR primer pairs tested positive in at least one of the wheat-*L. racemosus* chromosome addition lines, including a subset of 27 (60%) of the 40 *Leymus* EST-SSR primer pairs that detected homoeologous loci. Thus, at least 173 *Leymus* EST-SSR marker loci representing all 14 linkage

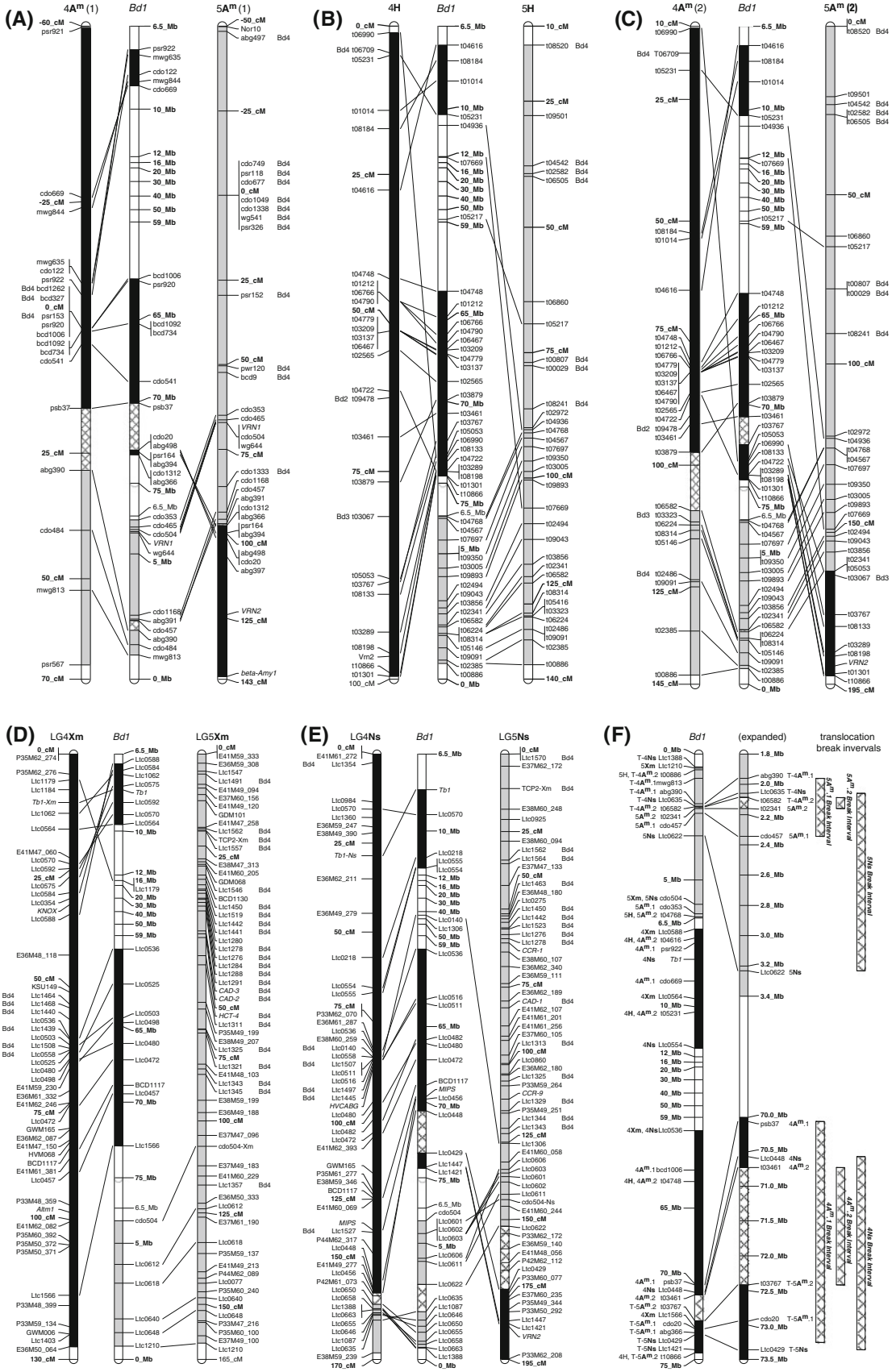
(A)
4A^m (1)
Bd1
5A^m (1)
(B)
4H
Bd1
5H
(C)
4A^m (2)
Bd1
5A^m (2)
(D)
LG4Xm
Bd1
LG5Xm
(E)
LG4Ns
Bd1
LG5Ns
(F)
Bd1
(expanded)
translocation break invervals

Table 5 Amplifications of mapped *Leymus* EST-SSR primers in 17 wheat-*Leymus* chromosome introgression lines (CIL) A–N as (Kishii et al. 2004), TA7643–TA7652 (Qi et al. 1997), *Leymus racemosus* (*Lr*), *Psathyrostachys junceae* (*Pj*), and Chinese spring wheat (CS)

Genotype (*n*)	Original chromosome identification	LG 1a	LG 1b*	LG 2a	LG 2b	LG 3a	LG 3b	LG 4**Ns**	LG 4**Xm**	LG 5**Ns**	LG 5**Xm**	LG 6a	LG 6b*	LG 7a	LG 7b
A (21 + 1)	2[a]	0	1	**6**	**8**	0	0	1[c]	0	0	0	1	1	0	0
C (21 + 1)	5[a]	0	0	1	1	0	0	1	2	**9**	**4**	0	1	0	2
E (21 + 1)		0	0	0	0	0	0	0	0	0	0	0	1	0	0
F (21 + 1)	4[a]	0	0	0	0	0	0	**4 + 4**[c]	**4**	1^3	2	1	1	0	0
H.1 (21 + 1)	3[a]	1	1	0	0	**7**	**5**	1	0	1	1	1	2	1	0
H.2 (20 + 1)	3[a]	1	0	0	0	**6**	**6**	1	0	0	1	1	1	2	1
I (21 + 1)	5[a]	0	0	0	0	0	0	1 + 1[c]	0	**6**	**9**	0	1	1	0
J (21 + 1)	3,7[a]	0	0	0	0	0	0	0	0	0	1	**7**	**6**	**16**	**11**
k (21 + 1)	6[a]	0	0	0	1	0	1	0	0	0	2	**9**	**7**	0	0
l (21 + 1)	2[a]	0	0	**7**	**6**	0	0	0	0	0	0	1	1	1	0
n (21 + 1)	3,7[a]	0	0	**3**	**15**	0	0	0	0	1	2	2	2	1	0
NAU516 (21 + 1)	2[b]	0	2	**6**	**9**	0	0	0	0	0	0	0	0	1	1
NAU551 (20 + 1)	2[b]	0	1	**5**	**9**	0	0	1	0	0	0	1	0	1	1
NAU504 (21 + 1)	5[b]	0	0	0	0	2	1	1^3	0	**10**	**14**	1	1	1	0
NAU512 (21 + 1)	6[b]	0	0	0	0	1	0	1	1	0	1	**7**	**5**	1	3
NAU524 (21 + 1)	3,7[b]	0	1	0	2	**10**	**4**	2	1	1	0	2	1	**13**	**11**
NAU502 (21 + 1)	5[b]	0	0	0	1	3	0	0	0	0	0	0	1	**13**	**11**
Lr (14)		1	4	11	19	13	10	7 + 4[c]	7	15 + 1[c]	18	14	10	21	18
Pj (7)		1	3	9	16	11	7	5 + 5[c]	6	14	18	11	9	19	16
CS (21)		0	0	0	0	1	0	0	0	0	0	0	2	0	0

Probable *Leymus* homeologous groups that might be present within each CIL are indicated using bold underline text

[a] Kishii et al. (2004)

[b] Qi et al. (1997)

[c] Markers in 4**Ns**L–5**Ns**L reciprocal translocation region

groups were potentially detected (Table 5). The wheat-*L. racemosus* chromosome addition lines all tested positive for at least eight and up to 24 *Leymus* EST-SSR marker loci representing at least one homoeologous group, most of which match the previous chromosome identification (Table 5). However, no more than two markers from homoeologous group 1 (LG1a and LG1b*) tested positive in any one wheat-*L. racemosus* chromosome addition line (Table 1). Thus, these wheat-*L. racemosus* chromosome addition lines (Table 5) did not carry *Leymus* homoeologous group 1. Disomic addition line *Lr*#J reportedly cross-hybridized with RFLP probes from groups 3L and 7L (Kishii et al. 2004) showed amplification of EST markers mapped to linkage groups 6 and 7 (Table 5). Disomic addition lines NAU502 reportedly cross hybridized with RFLP probes from group 5 (Qi et al. 1997) showed amplification of EST markers mapped to group 7 (Table 5). Group 4 disomic addition line *Lr*#F (Kishii et al. 2004), tested positive for *Leymus* ESTs from homoeologous group 4 and markers translocated from 5**Ns**L (Table 5). Disomic addition line *Lr*#n cross hybridized with two RFLP probes from 3S, one RFLP probe from 5S, and one RFLP probe from 7S (Kishii et al. 2004). Subbarao et al. (2007) reported that *Lr*#n corresponds to homoeologous groups 3 and 7. However, a strong preponderance of LG2a and LG2b *Leymus* EST markers amplified from *Lr*#n (Table 5).

Discussion

The integrated consensus map for the *Leymus* TTC1 and TTC2 families is a major advance over the previous maps (Wu et al. 2003; Larson et al. 2006) in part because it includes 376 new EST markers including functionally important lignin biosynthesis genes that have been aligned to the *Brachypodium,* rice, and *Sorghum* genome reference sequences as well as a high-density barley EST linkage map. The LG1b and LG6b maps (Wu et al. 2003; Larson et al. 2006) were reassigned LG6b* and LG1b*, respectively, and LG4**Ns** and LG4**Xm** were inverted so that all 14 linkage groups are now aligned to the $x = 7$ homoeologous groups of barley and many other cool-season grasses including some important Triticeae cereals. Moreover, these new EST markers are a relatively small subset of the

11,281 *Leymus* EST unigene library (Bushman et al. 2008), which has also been aligned to the grass genome reference sequences and provides a deep reservoir of additional markers and candidate genes for QTLs controlling growth habit (Larson et al. 2006), forage quality (Larson and Mayland 2007), seed shattering (Larson and Kellog 2009), and other important forage grass traits. Results also demonstrate that alignments of the *Leymus* EST markers to grass genome reference sequences also enable alignments of functionally important QTLs and genes controlling traits such as glaucousness (Jefferson 1994; Jefferson and Kielly 1996) and self-incompatibility (Jensen et al. 1990) of *Leymus* to corresponding genes for glaucousness in wheat (Liu et al. 2007) and *S* self-compatibility genes of *Phalaris coerulescens* (Li et al. 1994; Langridge et al. 1999; Bian et al. 2004), *Secale cereale* (Senft and Wricke 1996; Voylokov et al. 1997), and *Lolium* (Jones et al. 2002; Shinozuka et al. 2010). These markers also provide important new tools for introgression of perennial Triticeae genes into wheat (Liu et al. 2001; Chen et al. 2005; Qi et al. 2008; Wang and Chen 2008; Wang et al. 2009, 2010a, b).

Alignments of the *Leymus* EST markers from the integrated consensus map confirmed a reciprocal translocation between LG4**Ns**L and LG5**Ns**L originally suggested based on the mapping of one heterologous wheat *VRN2* marker to LG5**Ns** (Larson et al. 2006), which is located on 5**A**L of wheat (Dubcovsky et al. 1998; Yan et al. 2004) and 4**H**L of winter barley (Yan et al. 2004; Karsai et al. 2005). This translocation was also detectable in the wheat-*Leymus racemosus* chromosome addition line F (Table 5), suggesting that this translocation is not unique to North American *Leymus*. The LG4**Ns**L and LG5**Ns**L translocations are syntenic with genome-specific markers on 4**Ns** and 5**Ns**, which show high sequence similarity to *Psathyrostachys juncea* (Wu et al. 2003). Thus, we deduce that LG4**Ns**L and LG5**Ns**L translocation is specific to the **Ns** subgenome of *Leymus* and we hypothesize that this translocation may have occurred in **Ns** ancestral lineage of *Leymus*. Results also showed that breakpoint intervals of reciprocal 4L–5L translocations of *Leymus* wildryes and diploid wheat (*T. monococcum*) are overlapping based on alignments of *Leymus* and wheat markers to *Brachypodium* chromosome 1. Although *Leymus* groups 4 and 5 all align to *Brachypodium* chromosome 1, the 4**Xm** and 5**Xm** show more synteny and colinearity with *Brachypodium* chromosome 1 compared with the 4**Ns** and 5**Ns**. The *Leymus* LG4**Xm** and LG5**Xm** arrangement is also conserved in barley (Table 3), wheat **A** and **B** subgenomes, and other cool-season grasses (King et al. 1994). However, the *Leymus* LG4**Xm** and LG5**Xm** rearrangement is also shared by diploid wheat *T. monococcum* (Devos et al. 1995; Dubcovsky et al. 1996), *Secale cereale* (Devos et al. 1993), as well as other grasses (King et al. 1994). It is not clear whether the later arrangements were derived from a common ancestor or represent convergent similarities, but the alignments of *Leymus* and *T. monococcum* breakpoint intervals indicates they may derive from a common ancestor.

Larson and Mayland (2007) postulated that ADF and NDF QTLs in the centromeric region of LG7a may correspond to a cluster of lignin biosynthesis genes in perennial ryegrass (Cogan et al. 2005). Indeed, one potentially important outcome of this study is the identification of a LG7a *COMT* lignin biosynthesis gene marker, *COMT-1*, which coincided with QTL peaks for both ADF and NDF in both TTC1 and TTC2 families. This was the most common lignin biosynthesis EST found in the *Leymus* EST library and it aligns to the same locus on *Sorghum bmr12* (Bout and Vermerris 2003) and maize *bm3* (Vignols et al. 1995) *brown midrib* mutations and the same transgene sequences used to downregulate COMT gene expression and lignin content in perennial ryegrass (Tu et al. 2010), tall fescue (Chen et al. 2004), switchgrass (Fu et al. 2011), and maize (He et al. 2003). Although basin wildrye, creeping wildrye, and the wildrye hybrids have excellent biomass accumulation potential, salt tolerance, and adaptation to cold growing regions the forage quality of these grasses is a major limitation. Thus, this gene is an important target (Jung and Ni 1998; Humphreys and Chapple 2002; Li et al. 2008) for *Leymus* improvement.

The biological nitrification inhibition trait was particularly strong in the *Lr*#n ($2n = 42 + 2$) wheat-*Leymus racemosus* disomic chromosome addition lines, which reportedly corresponds to homoeologous groups 3 and 7 (Kishii et al. 2004; Subbarao et al. 2007). However, this conclusion was based on cross-hybridization of only four RFLP markers from groups 3S, 5S, and 7S (Kishii et al. 2004). However, new results show that LG2a and LG2b EST markers were preferentially amplified from *Lr*#n and provide proof that *Leymus* LG2a and LG2b EST markers belong to Triticeae group 2 based on alignments with barley chromosome 2 (Close et al. 2009), *Brachypodium* 1 and 5 (The International Brachypodium Initiative 2010), and rice 4 and 7 (La Rota and Sorrells 2004). Thus, we conclude that the *Lr*#n line analyzed in this study carries *Leymus* homoeologous group 2, specifically perhaps LG2b.

Cosegregation of the LG1b *TRX-1* and LG2b E37M61_183 AFLP marker in the TTC2 family was presumably caused by selection of pollen gametes from the TC2 hybrid that were compatible to with the *L. triticoides* (T) tester female seed parent of the TTC2 family. In theory, cosegregation of unlinked loci could have been caused by epistatic interaction of gametocidal genes that affect pollen development, pollen compatibility with the T-tester, or post-fertilization stages of seed development or germination. However, the *TRX-1* marker is closely linked to the

S gametophytic self-incompatibility orthogene of *Phalaris coerulescens* (Li et al. 1994; Langridge et al. 1999; Bian et al. 2004), *Secale cereale* (Senft and Wricke 1996; Voylokov et al. 1997), and *Lolium* (Jones et al. 2002; Shinozuka et al. 2010). Thus, we conclude that cosegregation of the LG1b *TRX-1* and LG2b E37M61_183 AFLP markers was caused by interaction of *S* and *Z* self-incompatibility genes between the TC2 hybrid that and *L. triticoides* T-tester parents of the TTC2 family. Interestingly, this gametophytic selection strictly favored coupling of *S* and *Z L. cinereus* alleles or coupling of *S* and *Z L. triticoides* alleles, with segregration distortion in favor of *L. cinereus* pollen alleles with the *L. triticoides* female parent. Although the LG2b *Z* self-incompatibility gene of *Leymus* did not align with Z self-incompatibility genes of *Secale* (Hackauf and Wehling 2005) and *Lolium* (Shinozuka et al. 2010), other self-incompatibility *Z* genes have also been reported in *Secale* (Melz et al. 1990; Voylokov et al. 1997) and *Phalaris* (Hayman and Richter 1992). Both *L. cinereus* and *L. triticoides* are highly self-incompatible; however, a few seeds are occasionally found when plants are selfed using pollen exclosures (Jensen et al. 1990). Wu et al. (2003) also identified six non-hybrid seeds in both TTC1 and TTC2 families, which were subsequently removed from the mapping populations. Thus, it seems a bit surprising that the LG1a *TRX-1* locus strictly cosegregated with the LG2b E37M62_183 locus given that the self-incompatibility occasionally breaks down (Jensen et al. 1990; Wu et al. 2003). Nevertheless, the fidelity of gametophytic selection of LG1b and LG2b self-incompatibility genes in the cross of the TC2 hybrid pollen parent and the *L. triticoides* (T) tester female seed parent could be used as powerful system for map-based cloning of these genes because it is a relatively simple to generate progeny and test for recombination between LG1a and LG2b DNA markers in seedlings of these two parents, without any phenotypic testing.

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