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ORIGINAL PAPER

Physical analysis of the complex rye (*Secale cereale* L.) *Alt4* aluminium (aluminum) tolerance locus using a whole-genome BAC library of rye cv. Blanco

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Abstract Rye is a diploid crop species with many outstanding qualities, and is important as a source of new traits for wheat and triticale improvement. Rye is highly tolerant of aluminum (Al) toxicity, and possesses a complex structure at the *Alt4* Al tolerance locus not found at the corresponding locus in wheat. Here we describe a BAC library of rye cv. Blanco, representing a valuable resource for rye molecular genetic studies, and assess the library's suitability for investigating Al tolerance genes. The library provides 6 × genome coverage of the 8.1 Gb rye genome, has an average insert size of 131 kb, and contains only ~2% of empty or organelle-derived clones. Genetic analysis attributed the Al tolerance of Blanco to the *Alt4* locus on the short arm of chromosome 7R, and revealed the presence of multiple allelic variants (haplotypes) of the *Alt4* locus in the BAC library. BAC clones containing *ALMT1* gene clusters from several *Alt4* haplotypes were identified, and will provide useful starting points for exploring the basis for the structural variability and functional specialization of *ALMT1* genes at this locus.

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Introduction

Cereal rye (*Secale cereale* L.) is a distinctive crop species possessing traits of both practical and scientific interest. It is grown principally in northern and eastern Europe, and has a world production of around 15.7 million tons (2007; http://faostat.fao.org/). In terms of the total world cereal harvest of 2,342 million tons, rye is not a major crop. However, it performs relatively well on sandy, infertile, or acid soils, and can tolerate extreme low temperatures during overwintering. Among the cereals, rye is one of the most tolerant of aluminum (Al) toxicity, which is a widespread problem on acid soils (Mugwira et al. 1976; Kim et al. 2001). Consequently, rye can often be grown on marginal agricultural land considered to be too poor for other crops (Madej 1996). Unlike the other cultivated members of the Triticeae tribe, wheat (*Triticum aestivum* L. and *T. turgidum* ssp. *durum* (Desf.) MacKey) and barley (*Hordeum vulgare* L.), rye is an outbreeding species, exhibits substantial inbreeding depression, possesses a genetic system of self-incompatibility (Hackauf and Wehling 2005), and shows a comparatively high frequency of polymorphism at the DNA sequence level (Varshney et al. 2007; Collins et al. 2008). Hybrid rye breeding methodologies based on cytoplasmic male sterility and fertility restorer genes were adopted in Germany in the 1970s and have led to significant yield increases (Tomerius et al. 2008). On better soils, hybrid rye varieties can yield as highly as wheat.

Rye is a parent of triticale (×*Triticosecale* Wittmack), a hybrid of rye and wheat (Oettler 2005) that is cultivated on a similar scale to rye (http://faostat.fao.org/). Furthermore, translocated rye chromosome segments have been utilized extensively in wheat breeding (Rabinovich 1998; Graybosch 2001). Rye translocations that have been used in

wheat contain genes for resistance to powdery mildew and rust fungal pathogens, and insect pests (Graybosch 2001), and have been demonstrated to confer increased yield, wide adaptation and traits associated with drought tolerance, including increased root mass (Moreno-Sevilla et al. 1995; Villareal et al. 1998; Graybosch 2001; Ehdaie et al. 2003; Kim et al. 2004; Hoffmann 2008). Therefore, rye is not only important as a crop in its own right, but also holds significance as a repository of variation that can be used in wheat and triticale improvement.

Loci controlling Al tolerance have been mapped in rye by linkage analysis, on 6RS and 7RS chromosome arms (*Alt1* and *Alt4* loci, respectively) (Gallego et al. 1998; Matos et al. 2005; Miftahudin et al. 2005; Collins et al. 2008), and studies using cytogenetic stocks have indicated the presence of Al tolerance genes on other rye chromosomes (Aniol and Gustafson 1984; Aniol 2004). We previously reported that the rye *Alt4* locus contains a cluster of up to five genes homologous to the wheat *aluminum-activated malate transporter-1* (*ALMT1*) gene (Collins et al. 2008). At the corresponding Al tolerance locus in wheat (Alt_{BH}), there is only one *ALMT1* gene copy. This wheat gene confers Al tolerance by encoding a protein that facilitates secretion of malate from the root tip, resulting in neutralization of the phytotoxic Al^{3+} cation (Sasaki et al. 2004). At the rye *Alt4* locus, two of the five *ALMT1* gene copies present in the tolerant mapping parent M39A-1-6 are responsible for providing the Al tolerance (Collins et al. 2008).

Libraries of large genomic DNA fragments (e.g., made using bacterial artificial chromosome, or BAC, vectors) are required for making physical maps of whole genomes or specific chromosome regions to facilitate sequencing, genome structure–function studies, and positional gene cloning. However, it was only recently that any BAC resources have been made available for rye. Šimková et al. (2008) described a rye chromosome arm 1RS specific BAC library, and we briefly reported the construction of whole-genome BAC library of an Al tolerant selection of the rye cultivar Blanco (Shi et al. 2007). Owing to the large genome size of rye (1C = 8.1 Gb; Bennett and Smith 1976), the creation of such a whole-genome rye BAC library required a considerable investment of effort and resources.

In the current study, we investigate the source of the Al tolerance of the Blanco selection used in BAC library construction, and show that it can be attributed to the *Alt4* locus. The library is described in more detail, and its utility demonstrated by isolating clones containing single-copy *Alt4*-linked genes, and by performing a physical analysis of the *ALMT1* gene complex. The Blanco BAC library is presented as a valuable resource for molecular genetic studies of rye.

Materials and methods

Plant material

Blanco is a white-seeded, self-compatible, spring type, day length insensitive Brazilian rye cultivar that is crossable to wheat. Seed of Blanco rye were obtained from Dr. A. Baier, Passo Fundo, Brazil, via Dr. F. J. Zillinsky of CIMMYT, Mexico, and Dr. R. J. Metzger, USDA-ARS, Corvallis, Oregon. From this material, an 'aluminum-tolerant Blanco' line was generated by the USDA-ARS at the University of Missouri by successive selections for tolerance at 70 ppm (2.6 mM) Al and intercrossing of the tolerant individuals for over four generations, at which point all plants were uniformly tolerant of 70 ppm Al (Gustafson and Ross 1990). This selection was used for BAC library construction. Seed of this selection is available on request from the USDA-Sears Germplasm Collection, 206 Curtis Hall, University of Missouri, Columbia, Missouri 65211, USA.

The Al intolerant rye inbred M77A-1 was obtained from Dr. G. Scoles, University of Saskatchewan, Canada. M77A-1 was pollinated by a single plant of the Al tolerant Blanco selection. Seed harvested from five F_1 plants were combined to obtain a bulk F_2 population for use in the genetic analysis. The 'tolerant' and 'intolerant' bulk DNAs derived from a M39A-1-6 × M77A-1 cross were those previously described by Collins et al. (2008). The original cross was made by Miftahudin et al. (2005).

Genetic markers

The chromosome locations of genes and markers are illustrated in Fig. 1. The *B11*, *B26*, *B6*, and *MATE* CAPS markers and their locations have been described (Miftahudin et al. 2005; Collins et al. 2008).

Al tolerance screening

Al tolerance was assessed using the Eriochrome cyanine R hydroponic solution culture method (Aniol 1983; Collins et al. 2008), which assays the degree of root re-growth 2 days after relief from Al stress (150 μM $AlCl_3$; pH 4.0). In this assay, intolerant genotypes show less re-growth due Al toxicity damage to the root tip.

BAC library construction

The procedures used to prepare high-molecular weight DNA was based on those described by Zhang et al. (1995). Plants were grown in a greenhouse at 22–23°C with a 12 h day/night light cycle for 1 month. Leaves were ground in liquid nitrogen and the powder combined with homogenization buffer (0.01 M Trizma base, 0.08 M KCl,

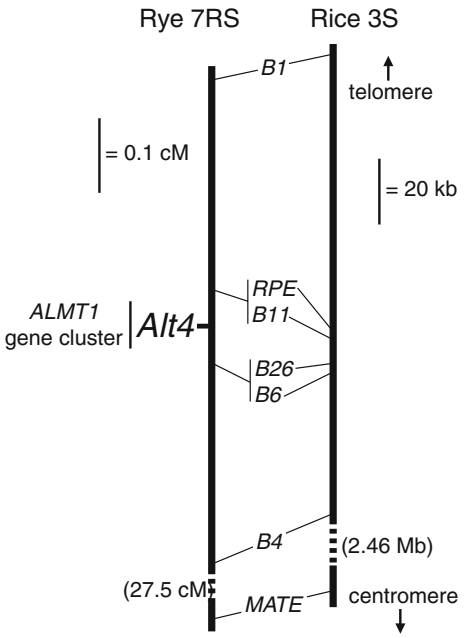

Fig. 1 Chromosome locations of markers and genes, as previously described by Collins et al. (2008). The genetic map of the *Alt4* region in rye and the sequence of the corresponding section of the rice genome are represented. There is no *ALMT1* homolog present in the corresponding interval on rice chromosome 3

0.01 M EDTA, 1 mM spermidine, 1 mM spermine, 0.15% β-mercaptoethanol, pH 9.5). The slurry was mixed with an equal volume of pre-warmed 1% low melting point (LMP) agarose and cast into plugs using plug molds (Bio-Rad). The agarose plugs were then incubated in 5–10 volumes of lysis buffer (0.5 M EDTA, 1% sodium lauryl sarcosine, 0.1–0.5 mg/ml proteinase K, pH 9.0–9.3) at 50°C for 24–36 h, before being washed twice, first with 0.5 M EDTA (pH 9.0) and then with 0.5 M EDTA (pH 8.0). Plugs prepared in this way could be stored in 0.05 M EDTA (pH 8.0) at 4°C for up to 1 year without noticeable DNA degradation.

Prior to digestion, the plugs were washed with 10–20 volumes of ice-cold 1 × TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) containing 0.1 mM phenylmethyl sulfonyl fluoride (PMSF). Each plug was cut into 16 pieces which were transferred into two 1.5-ml Eppendorf tubes, each containing 200 µl 1 × *Hin*dIII digestion buffer. Tubes were placed on ice and the buffer replaced at 1-h intervals. After the second change, 10 u *Hin*dIII restriction enzyme was added, and partial digestion carried out for 20 min at 37°C before adding 14 µl of 0.5 M EDTA (pH 8.0) stop solution. The agarose pieces were inserted into the wells of a 1% agarose gel and electrophoresis performed using a Bio-Rad CHEF Mapper XA pulse-field gel electrophoresis (PFGE) system in 1 × TAE buffer (pH 8.3), for 18 h at 11°C and 6 V/cm, using a 5–60 s pulse time and 120° field angle, and then for 4 h with a 5–5 s pulse time. The 100–350 kb gel fraction was excised and run on a second 1% agarose gel for another 18 h using a 3–5 s pulse time. The 150–350 kb gel fraction was excised and the DNA eluted for 60 min at 4°C in a Bio-Rad electro-eluter (Model 422), applying 10 mA per tube.

Size-fractionated DNA was ligated to *Hin*dIII-linearized dephosphorylated Cloning-Ready pIndigoBAC-5 vector DNA (Epicenter), using a 1:10 insert:vector molar ratio, for 16 h at 16°C. Ligations were used in transformation of ElectroMAX *E. coli* DH10B competent cells (Invitrogen), using a Bio-Rad Gene Pulser Xcell and a cuvette with a 1 mm gap (Bio-Rad) with settings 1600 V, 25 µF and 200 Ω. Transformed cells were incubated at 37°C for 1 h in 1 ml LB, plated on to LB agar medium containing 12.5 µg/ml chloramphenicol and grown overnight at 37°C.

Colonies were picked into wells of 384-well plates containing 70 µl LB freezing medium (36 mM K_2HPO_4, 13.2 mM KH_2PO_4, 1.7 mM $(NH_4)_2SO_4$, 4.4% v/v glycerol), using a VersArray Colony Picker and Array System robot (Bio-Rad). Plates were incubated overnight at 37°C and used to make four library copies. Libraries were stored at −80°C. The original library and a copy were kept at ACPFG, and the other copies were sent to IPK Gatersleben, University of Missouri, Columbia, and to the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing. The ACPFG copy was used to print filters for hybridization screening. Clones were arrayed in duplicate on to each 22 cm × 22 cm positively charged nylon membrane (Amersham Hybond-N^+ from GE Healthcare, or Performa II from Genetix) using a Qpix2 robot (Genetix).

Evaluation of insert size

Individual BAC clones were grown overnight at 37°C with vigorous shaking in 5–10 ml LB containing 12.5 µg/ml chloramphenicol, and the cultures used to prepare BAC DNA by alkaline lysis. DNA of each clone (5 µl) was digested with 0.2 u *Not*I restriction enzyme and subjected to PFGE in 1% agarose gels and 1 × TAE buffer, for 18 h at 11°C and 6 V/cm, using a 1–40 s pulse time and 120° field angle. Fragments were photographed under UV light after ethidium bromide staining, and insert sizes estimated by comparison to Lambda Ladder PFG Marker (New England BioLabs).

Screening with cereal probes

Probes were generated from the chloroplast *PsbA* and *RbcL* genes and mitochondrial *AtpA* and *CoI* genes. These corresponded to nucleotide positions 1,264 to 2,257 of X13327.1 (*PsbA*), 73 to 1,070 of AY836181 (*RbcL*), 663 to 1,682 of X99020.1 (*AtpA*), and 236 to 1,247 of Y00417.1 (*CoI*). For each organelle, a mixture of the two representative probes was used to screen a single filter representing 4,608 clones.

Probes were generated from the *Alt4*-linked nuclear genes *B1*, *B11*, *B26*, *B6*, and *B4* by PCR with primer pairs B1-1 plus B1-2, B11-1 plus B11-2, B26-4 plus B26-5, B6-1 plus B6-4, and B4-1 plus B4-6, respectively. These primers have been described previously (Collins et al. 2008), except for B6-4 (5′-CTGCACAGGATGGCCAGCGCCATC-3′) and B4-6 (5′-GCAGATCACACAAGTATATGGATTC-3′). Fragments were PCR-amplified from DNA of rye inbred line M39A-1-6, cloned in to *E. coli*, and used to prepare probe templates, using methods outlined by Collins et al. (2008). The *ALMT* cDNA probe derived from the rye *ALMT1-M39.1* gene has been described (Collins et al. 2008). Production of [α-^{32}P]-dCTP labeled probes by random priming, and hybridization and autoradiography, were performed using standard methods.

Fingerprinting, Southern and CAPS marker analysis

*Hin*dIII fingerprinting was performed by digesting ~2 μg of BAC clone DNA and separating the fragments by electrophoresis on 1% agarose gels in 1 × TAE buffer overnight at 35 V. Gels were photographed under UV light after ethidium bromide staining and fragment sizes were estimated by comparison to molecular weight markers. BAC overlaps and overhangs were determined based on the occurrence of shared and unique restriction fragments, respectively.

Southern analysis was performed using standard procedures, on filters containing approximately 3 μg of rye genomic DNA, and 0.06 to 500 ng of BAC DNA, per lane. The smaller quantity of BAC DNA per lane produced BAC and genomic bands of approximately equal intensity with the same exposure. Otherwise, filter sections containing BAC DNA produced much stronger signals and needed to be exposed separately from filter sections containing genomic DNA lanes. The restriction enzyme used (*Dra*I) is unaffected by methylation in plant or bacterial DNA, allowing direct comparison between the genomic and BAC clone-derived Southern bands.

CAPS markers were scored in BAC clones as in rye genomic DNA samples, except that only ~10 ng of DNA was used as template in the PCR reactions.

Results

Genetic basis of Al tolerance in Al tolerant Blanco

The Al intolerant inbred line M77A-1 was pollinated by a single plant from the Al tolerant Blanco selection and five F_1 plants allowed self-pollination to create a bulk M77A-1 × Blanco F_2 population. To investigate the genetic basis of Al tolerance in this Blanco selection, 53 of the F_2 plants were assessed for Al tolerance and for *B11*, *B26*, and *B6* CAPS markers which closely flank the *Alt4* Al tolerance locus (Fig. 1). The F_2 plants could be clearly categorized as tolerant or intolerant, as they either showed ≥18 mm (average 30.7 ± 1.5 mm) or ≤2.5 mm (average 1.8 ± 0.1 mm) root regrowth following relief from Al stress (Table 1). In the F_2 plants, each of the *B26* and *B6* markers showed two different CAPS marker variants derived from the Blanco parent, indicating that this parent plant carried two different variants of the 7RS chromosome segment carrying the *Alt4* locus (designated as the A and A′ haplotypes; Table 1; Fig. 2). While the *B6* marker showed the same band size in M77A-1 as in one of the Blanco haplotypes, the *B11* and

Table 1 Genetic analysis of Al tolerance in a M77A-1 × Blanco F_2 population

Genotype[a]	CAPS marker[b]			Root re-growth (mm; mean ± SEM)	Al tolerant or intolerant	Number[c]
	B11	*B26*	*B6*			
F_2 AA	U1	C1	C	36 ± 9.0	Tolerant	2
F_2 A′A′	U1	C2	U	23 ± 1.9	Tolerant	5
F_2 BB	U2	C3	U	1.8 ± 0.1	Intolerant	18
F_2 AB	U1 + U2	C1 + C3	C + U	33 ± 2.2	Tolerant	14
F_2 A′B	U1 + U2	C2 + C3	U	30 ± 2.3	Tolerant	14
Blanco[d]	U1 + U3	C1 + C2	C + U	28 ± 1.0	Tolerant	10
M77A-1	U2	C3	U	1.7 ± 0.8	Intolerant	6
M39A-1-6	U1	C1	C	n.d.	Tolerant	n.d.

[a] M77A-1 × Blanco F_2 genotypes, or Blanco, M77A-1 and M39A-1-6 parental genotypes

[b] CAPS marker variants based on whether amplicons were cut (C) or uncut (U), the sizes of uncut amplicons (U1, U2, and U3) or band patterns of cut amplicons (C1, C2, and C3). CAPS markers are illustrated in Fig. 2

[c] The sample of marker-genotyped M77A-1 × Blanco F_2s was biased to include more intolerant plants in order to more stringently test for linkage to Al tolerance genes, which are typically dominant. *n.d.* not determined

[d] Blanco DNA sample used to make the BAC library

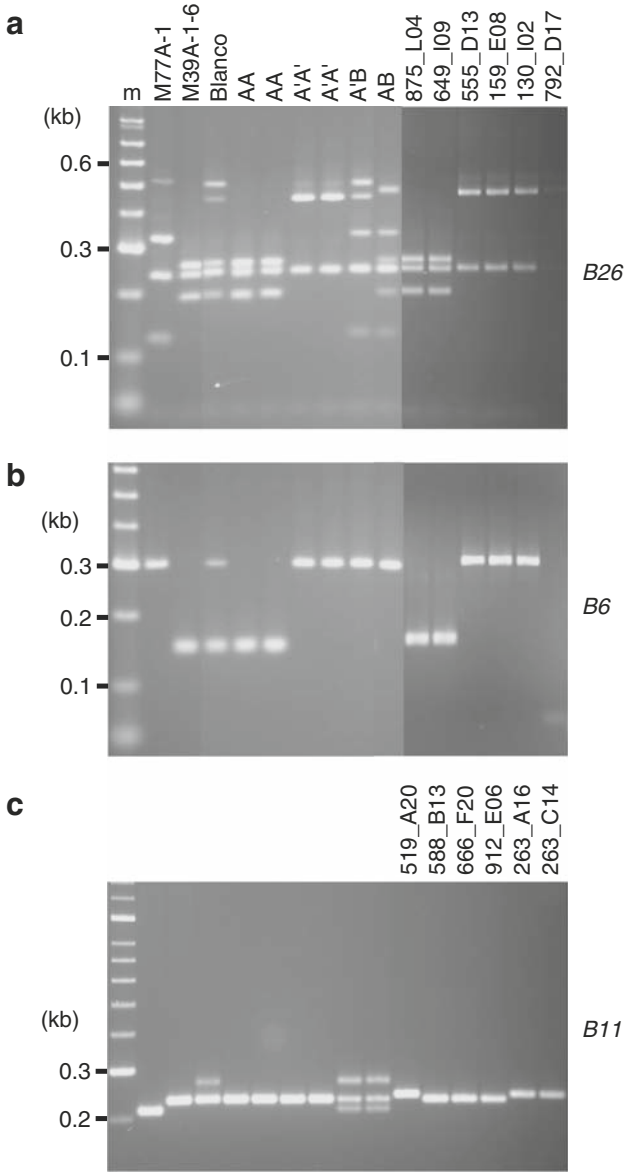

Fig. 2 *Alt4*-linked CAPS markers *B26* (**a**), *B6* (**b**), and *B11* (**c**) scored in rye lines and BAC clones. Unless indicated otherwise, templates for **b** and **c** were the same as those in **a**. Included are examples of M77A-1 × Blanco F_2 plants carrying different combinations of *Alt4* locus haplotypes (A and A′ from Blanco and B from M77A-1). The Blanco lane represents the same DNA sample as the one used to make the BAC library. Clone 792_D17 lacks the *B6* gene and the *B26* copy containing the *B26* CAPS marker. **c** Insertion–deletion differences were used to distinguish the undigested *B11* marker amplicons. The *B11* amplicon from clones 519_A20, 263_A16, and 263_C14 (*Alt4* A″ haplotype) differs in size to any amplified from the selected F_2 plants. The slowest migrating *B11* products unique to the Blanco, A′B and AB samples represent heteroduplexes of the *B11* amplicons which differ by insertion–deletions, in accordance with the phenomenon described by Ayliffe et al. (1994). The same phenomenon also explains the absence of a visible A″ *B11* amplicon in the Blanco sample

B26 markers were able to distinguish both Blanco *Alt4* haplotypes from the M77A-1 *Alt4* haplotype. Based on the *B11* and *B26* markers, all of the 18 intolerant F_2 plants were shown to be homozygous for the M77A-1 *Alt4* haplotype, while all 35 tolerant F_2 plants were found to carry at least one of the Blanco-derived *Alt4* alleles. Therefore, control of Al tolerance from the Blanco parent plant could be attributed entirely to the *Alt4* locus, with tolerance being conferred by both Blanco *Alt4* haplotypes in a dominant fashion. *Alt4*-encoded Al tolerance has been shown to be dominant in other rye populations (Fontecha et al. 2007; Collins et al. 2008).

The barley *HvMATE* (=*HvAACT1*) Al tolerance gene (Furukawa et al. 2007; Wang et al. 2007) has a rye ortholog that has been mapped 27.8 cM from *Alt4* (Collins et al. 2008; Fig. 1). A CAPS marker based on the rye *MATE* gene recombined with Al tolerance in the M77A-1 × Blanco F_2 population (not shown). Therefore, the *MATE* gene was not the basis for the Al tolerance segregating in this cross.

M77A-1 × Blanco F_2 plants carrying various combinations of *Alt4* locus haplotypes (A or A′ from Blanco and B from M77A-1) were selected using the CAPS markers and subjected to Southern blot analysis with an *ALMT1* cDNA probe, revealing complex hybridization patterns (Fig. 3a). Two hybridizing fragments appeared to be derived from a locus unlinked to *Alt4* (Fig. 3a, open triangles). In the eight examined F_2 plants, five bands from Blanco showed complete linkage to *Alt4* and were unique to the A haplotype (Fig. 3a, solid triangles). Therefore, the *ALMT1* gene clusters from the two Blanco *Alt4* haplotypes were shown to be structurally different.

The rye cv. Blanco BAC library

The rye cv. Blanco library comprised 373,632 BAC clones stored in 973 384-well plates. Analysis of 273 randomly selected clones (Fig. 4a) revealed insert sizes up to 330 kb and averaging 131 kb. Examples of insert fragments are illustrated in Fig. 4b. Approximately 1% of clones contained no insert. Hybridization screens with chloroplast and mitochondria probes revealed a low frequency of clones derived from these organelles (0.98% and 0.02%, respectively). Based on a genome size of 8.1 Gb for rye (Bennett and Smith 1976), the coverage of the library is estimated to be around 6×.

Screening with single locus probes

Probes derived from genes *B1*, *B11*, *B26*, *B6*, and *B4*, which are closely linked to *Alt4* (Fig. 1), were used to screen the BAC library. The positive clones (Table 2) were subjected to Southern hybridization analysis with *Dra*I, alongside genomic DNA of M77A-1 × Blanco F_2 plants homozygous for the A or A′ *Alt4* haplotypes (ESM Fig. 1). BAC clones showed one or two *B26* bands (Suppl Fig. 1c). The region covered by the *B26* probe was amplified and

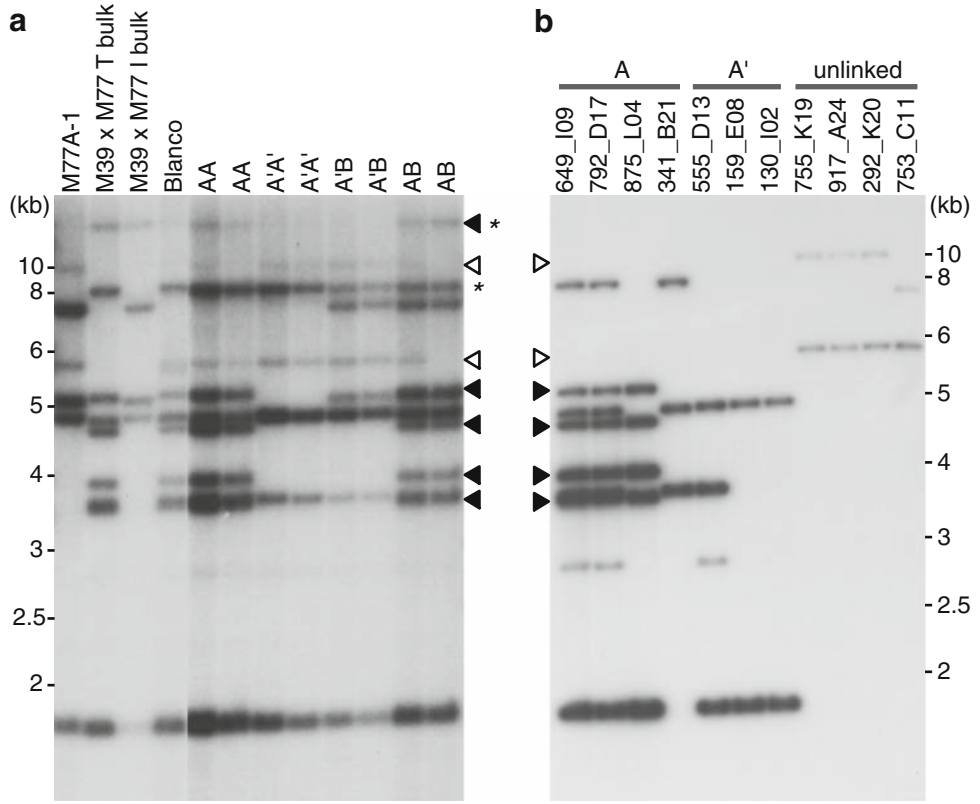

Fig. 3 Southern blot analysis of rye genomic DNAs (**a**) and Blanco BAC clones (**b**) using an *ALMT1* cDNA probe (restriction enzyme: *Dra*I). **a** Individuals homozygous for *Alt4* Al tolerance (T) and intolerance (I) alleles were marker-selected from an M39A-1-6 × M77A-1 F_3-derived F_4 segregating family and the genomic DNAs combined to make bulks. The Blanco lane represents the same DNA sample as the one used to make the BAC library. Shown are examples of marker-selected M77A-1 × Blanco F_2 plants carrying different combinations of Blanco (A and A′) and M77A-1 (B) haplotypes of *Alt4*. Asterisks indicate >10 kb and ~8 kb bands absent from BAC clones assigned to A and A′ haplotypes, respectively. **b** clones were assigned to the A or A′ haplotype of *Alt4* or the *Alt4*-unlinked locus. Fragments that are unique to the A haplotype or the locus unlinked to *Alt4* are indicated by closed and open triangles, respectively (**a** and **b**)

sequenced from plants containing the A and A′ haplotypes (FJ539080 and FJ539081). Neither sequence contained a *Dra*I recognition site. Hence, rye contains two physically linked (<100 kb) *B26*-related sequences. Clone 792_D17 contained only one of these sequences (ESM Fig. 1c) and was negative for *B26* CAPS marker amplification (Fig. 2a), indicating that the copy missing from this clone was the one containing the CAPS primer sites. The other four probes detected only one Southern band in one or both haplotypes (ESM Fig. 1). Thus, *B1*, *B11*, *B26*, *B6*, and *B4* represent single-copy or single-locus sequences in Blanco rye. These probes respectively detected 5, 6, 6, 5, and 2 clones (Table 2), consistent with the estimated 6× genome coverage of the library.

B1, *B4*, *B6*, and *B11* positive BAC clones showed RFLP differences (ESM Fig. 1), consistent with the presence of multiple allelic forms of these genes in the BAC library. CAPS (Fig. 2c) and RFLP analysis (ESM Fig. 1b) revealed the presence of a third Blanco *B11* variant in clones 519_A20, 263_A16, and 263_C14 which was not present in the Blanco parent plant of the F_2 population. This additional Blanco *Alt4* locus haplotype was designated A″.

Physical analysis of Blanco *ALMT1* gene clusters

A total of 11 positive BAC clones were obtained by screening the library with an *ALMT1* cDNA probe (Table 2). Four of the clones were from the locus unlinked to *Alt4*, as they contained one or both of the restriction fragments assigned to this locus (Fig. 3). Among the remaining seven positive clones, 6 had homology to both *B26* and *B6*, or to *B26* alone (Table 2), demonstrating close physical linkage between these genes and the *ALMT1* gene cluster at *Alt4*. The deduced gene order *ALMT1–B26–B6* corresponds to the gene order previously determined by genetic mapping in rye and predicted by comparison to rice (Fig. 1).

Based on the *B26* and *B6* CAPS and RFLP analysis, *ALMT1* and *B6* Southern banding patterns, *Hin*dIII digestion profiles of clones, and clone insert sizes determined by *Not*I digestion and PFGE, *ALMT1*-positive BAC clones were arranged into putative contigs (Fig. 5). The data were consistent with the clones belonging to two contigs for the *Alt4* locus, corresponding to the A and A′ haplotypes, and one contig for the *Alt4*-unlinked locus. However, we cannot rule out the possibility that some of these clones belong to

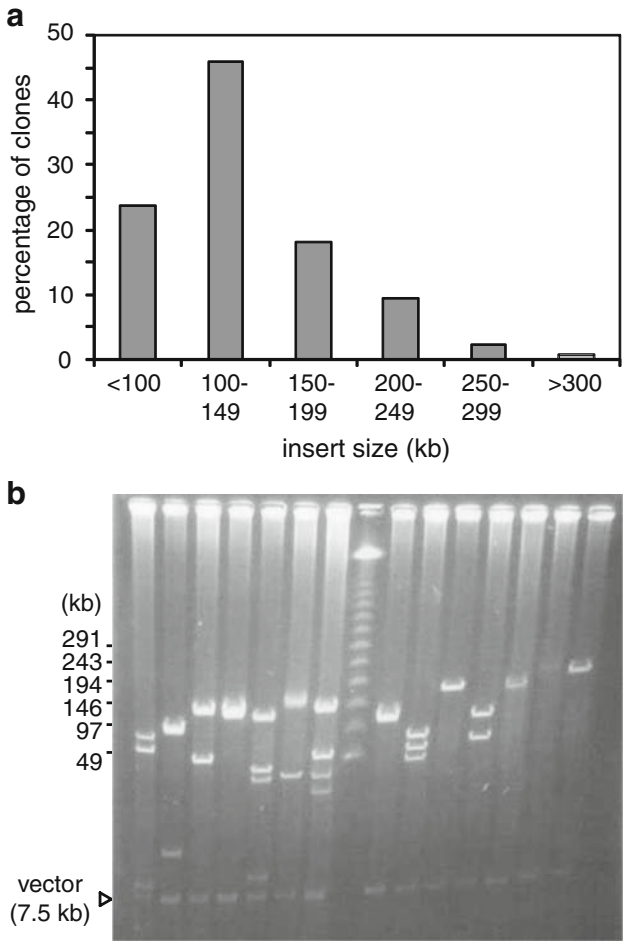

Fig. 4 Insert size distribution of 273 randomly selected clones from the Blanco BAC library (**a**) and *Not*I digests of 14 clones separated by PFGE (**b**). *Not*I cuts the vector at either side of the *Hin*dIII cloning site

Table 2 BAC clones positive for gene derived markers

BAC clone	Gene or gene cluster					
	B1	*B11*	*ALMT1*	*B26*	*B6*	*B4*
130_I02	–	–	+	+	+	–
159_E08	–	–	+	+	+	–
234_H09	–	–	–	–	–	+
263_A16	–	+	–	–	–	–
263_C14	–	+	–	–	–	–
292_K20	–	–	+	–	–	–
341_B21	–	–	+	–	–	–
353_J16	–	–	–	–	–	+
505_O04	+	–	–	–	–	–
519_A20	–	+	–	–	–	–
555_D13	–	–	+	+	+	–
588_B13	–	+	–	–	–	–
601_E09	+	–	–	–	–	–
649_I09	–	–	+	+	+	–
666_F20	–	+	–	–	–	–
753_C11	–	–	+	–	–	–
755_K19	–	–	+	–	–	–
792_D17	–	–	+	+	–	–
834_P21	+	–	–	–	–	–
875_L04	–	–	+	+	+	–
878_O23	+	–	–	–	–	–
909_H22	+	–	–	–	–	–
912_E06	–	+	–	–	–	–
917_A24	–	–	+	–	–	–
Total	5	6	11	6	5	2

additional haplotypes (e.g., A″) which could not be distinguished using the available information. One >10 kb *ALMT1* Southern band appeared to be linked to the A haplotype and was missing from all the BAC clones assigned to the A haplotype, while an ~8 kb band present in all M77A-1 × Blanco F_2 plants was missing from the BAC clones assigned to the A′ haplotype. With the possible exceptions of these bands (asterisks in Fig. 3), BAC contigs showed the complete complement of bands from the respective haplotypes (Fig. 3).

Discussion

The Blanco BAC library described here provides ~6× genome coverage of the rye genome. The genome of diploid rye ($2n = 2x = 14$; 1C = 8.1 Gb; Bennett and Smith 1976) is around 50% larger than those of the other important diploid Triticeae species, such as barley and the diploid relatives of cultivated polyploid wheats, *T. urartu*, *Aegilops speltoides*, and *Ae. tauschii* (1C = 5.4, 4.9, 5.1, and 4.9 Gb, respectively; Bennett and Smith 1976). Consequently, the 373,632 clone rye library contains more clones than barley BAC libraries of comparable genome coverage (1.7–3.1 × 10^5 clones; Yu et al. 2000; Isidore et al. 2005; Saisho et al. 2007). Coverage of the large rye genome was partly facilitated by maintaining a large insert size, achieved through use of a second round of DNA size selection. At 131 kb, the average insert size of the Blanco BAC library is larger than that of most other libraries described for diploid Triticeae species (Lijavetzky et al. 1999; Moullet et al. 1999; Akhunov et al. 2005; Saisho et al. 2007; Yu et al. 2000), but is equal to or smaller than that of most libraries of polyploid wheat species (Cenci et al. 2003; Ling and Chen 2005; Shen et al. 2005).

While Al tolerance in rye is known to be controlled by several loci, our analysis of the M77A-1 × Blanco F_2 population attributed the Al tolerance of Blanco completely to the *Alt4* locus on 7RS, due to the 100% association observed between tolerance and the *Alt4* markers. The presence of partial self-incompatibility in this population was

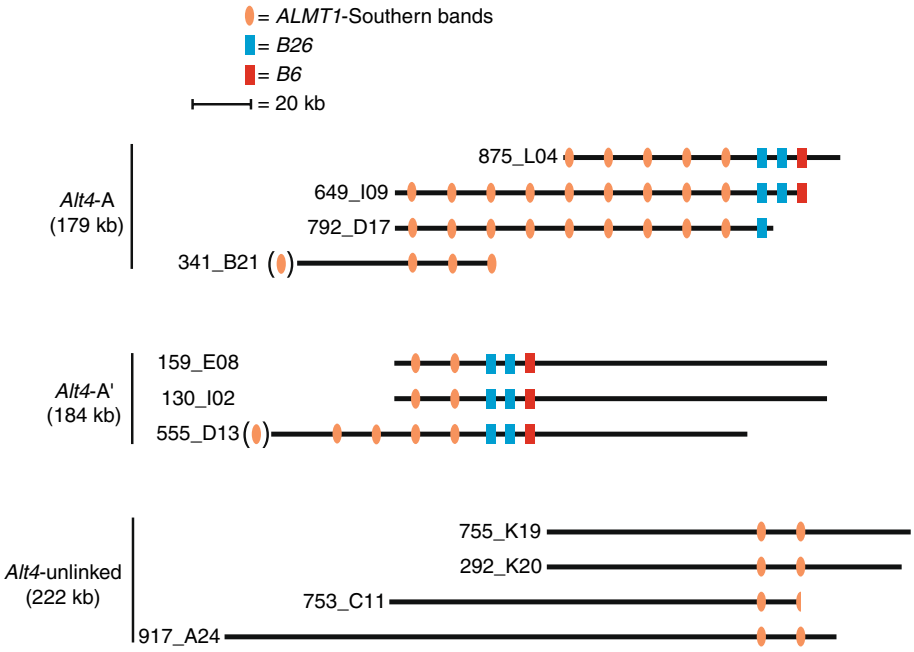

Fig. 5 Blanco BAC contigs for the A and A′ *Alt4* locus haplotypes and *Alt4*-unlinked locus. *ALMT1* symbols are based on numbers of *ALMT1* hybridizing *Dra*I bands visible on a Southern blot. Those in brackets represent fragments likely to belong to the A or A′ haplotype which are absent from the respective sets of BAC clones (asterisks in Fig. 3a). The *ALMT1* half-symbol in clone 753_C11 represents a novel-sized Southern band which may have arisen by the clone junction being located in or near a probe-binding site

not investigated, and may have potentially distorted the segregation of some chromosome regions. However, it seems unlikely that self-incompatibility prevented the detection of any additional Al tolerance genes present in Blanco, as Blanco is highly self-fertile, and tight linkage (e.g., <5 cM) to a self-incompatibility locus would have been required to completely prevent the inheritance of an Al tolerance gene. Aniol (2004) studied the basis of Al tolerance in Blanco by screening two sets of wheat–rye addition lines made using Blanco rye and either Chinese Spring or BH1146 wheat. In the Chinese Spring set, only the 3R addition line was tolerant, whereas in the BH1146 set, the 1R, 3R, 5R, 6R, and 7R chromosomes all provided some Al tolerance (2R addition was not tested). On the basis of those findings, it would appear that the Blanco selection analyzed by Aniol (2004) (and hence the BAC library) may contain Al tolerance genes at loci other than *Alt4*. Aniol (2004) suggested that the contrasting results obtained for the two addition line sets were probably due to the higher Al tolerance of the BH1146 wheat background relative to that of Chinese Spring, i.e., only the more tolerant wheat background enabled relatively minor rye tolerance genes to elevate the tolerance above the threshold level required to make the plants 'tolerant' as defined by the assay (some level of root regrowth following exposure to 590 µM Al). By a similar reasoning, it is possible that other Al tolerance genes in Blanco may be detectable using crosses to rye lines even more intolerant than M77A-1, and/or by testing segregating families at lower concentrations of Al. Use of only one Blanco plant as a parent in our genetic analysis may have prevented detection of additional tolerance genes present in the Al tolerant Blanco selection. An *Alt4*-unlinked locus of unknown genome location containing *ALMT1*-homologous sequences was detected here (Fig. 3) and in the previous study (Collins et al. 2008), and may represent a candidate for another Al tolerance gene in Blanco.

In contrast to the Al tolerant rye line M39A-1-6 which contains five *ALMT1* homologs at the *Alt4* locus, the Al intolerant line M77A-1 contains only two *ALMT1* gene copies at the corresponding position (Collins et al. 2008). During high resolution mapping, two novel Al tolerance conferring *ALMT1* gene clusters containing three or four *ALMT1* genes were generated by recombination between M39A-1-6 and M77A-1 haplotypes. Sequence identities between *ALMT1* gene family members were found in distinct stretches, providing further evidence for recombination-generated diversity at the *Alt4* locus (Collins et al. 2008). Thus, the *Alt4* locus of rye appears to be a highly variable and dynamic locus. Consistent with this notion, the Blanco A and A′ haplotypes could both be distinguished from the M39A-1-6 and M77A-1 haplotypes on the basis of *ALMT1* Southern hybridization pattern and/or sequencing of flanking genes (Fig. 3, and data not shown; see accessions FJ539076–FJ539083, EU146252, EU146272, EU146262, and EU146264). While the available data only allowed us to assign *ALMT1*-containing BAC clones to two distinct *Alt4* haplotypes, the detection of three Blanco variants of the nearby *B11* gene (Fig. 2c and ESM Fig. 1b) indicates that there is likely to be at least three variants of the *ALMT1* gene cluster represented in the Blanco BAC library. Sequencing of the BAC clones will establish how many

distinct *ALMT1* clusters are represented in the Blanco BAC library.

The Blanco selection used for BAC library construction was made by repeated rounds of selection for high levels of Al tolerance, and intercrossing of the most tolerant individuals. The fact that this selection was not entirely inbred accounts for the fact that it contained multiple *Alt4* haplotypes. This heterogeneity of the Blanco library will enable recovery of multiple allelic variants of genes and gene complexes, thereby facilitating the study of the molecular basis of some natural allelic variation in rye. The library should also find utility in positional cloning. While genotypes within individual cereal species mostly show high (although not complete) conservation of low-copy sequences, repetitive sequences are generally poorly conserved (Song and Messing 2003; Scherrer et al. 2005; Wang and Dooner 2006). Therefore, the heterozygosity may occasionally interfere with the use of the Blanco library in chromosome walking via low copy sequences. In contrast, the heterozygosity would clearly make the Blanco library unsuitable for producing a whole-genome physical map of rye, because construction of such maps relies on high-throughput fingerprinting methods based on both low- and high-copy sequences (Luo et al. 2003). For production of a whole-genome physical map and a homogeneous sequence of the rye genome, a BAC library made from a completely homozygous line (e.g., a rye doubled-haploid line; Tenhola-Roininen et al. 2006) would be required.

The 124 kb clone 792_D17 showed 9 of the 10 *ALMT1* Southern bands from the A haplotype, while the 157 kb clone 555_D13 showed four of the five *ALMT1* Southern bands from the A′ haplotype (Figs. 3 and 5). Therefore, it is reasonable to expect that the complete *ALMT1* gene cluster from either haplotype would be no larger than 200 kb. The 135 kb clone 649_I09 from the A haplotype showed the same *ALMT1* hybridization pattern as 792_D17 (Fig. 3b) but also contains two *B26* gene copies and one *B6* gene copy (Fig. 5). The Blanco A haplotype most likely contains five *ALMT1* genes, as this is the number present in the M39A-1-6 haplotype (Collins et al. 2008), which shows a similar *ALMT1* hybridization pattern (Fig. 3). If it is assumed that 649_I09 contains four *ALMT1* gene family members, this clone suggests a gene density in this region of around one gene every 19 kb, which is around tenfold greater than the value of one gene every 198 kb estimated as the genome-wide average for rye (calculated by assuming that rye contains the same number of genes as rice, i.e., ~41,000; TIGR rice genome annotation, release 5 http://rice.plantbiology.msu.edu/). This is consistent with the fact that in large cereal genomes, such as wheat, genes tend to be clustered (Keller and Feuillet 2000). The high gene density in the region may also be attributable to processes that generated the gene duplications at this locus.

The complex structure of the rye *Alt4* locus contrasts to the single-copy *ALMT1* gene found at the corresponding Al tolerance locus of wheat. Members of the *ALMT1* gene differ in their expression levels, intron splicing patterns, and their ability to confer Al tolerance (Collins et al. 2008), and the *ALMT1* gene cluster clearly exhibits high levels of structural variability. Unlike the wheat *ALMT1* gene, the rye *ALMT1* genes show induced transcription upon exposure to Al stress (Fontecha et al. 2007; Collins et al. 2008). The BAC clones containing near-complete *ALMT1* gene clusters from several *Alt4* locus haplotypes will facilitate the sequencing of these variants to explore the basis for this structural and functional variation, and should provide a convenient source of *ALMT1* coding and promoter sequences for structure–function studies of the individual genes. In conclusion, the whole-genome Blanco rye BAC library represents a valuable resource for molecular genetic studies in rye, a distinctive crop species possessing qualities of scientific and agricultural interest.

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References

Akhunov ED, Akhunova AR, Dvořák J (2005) BAC libraries of *Triticum urartu*, *Aegilops speltoides* and *Ae. tauschii*, the diploid ancestors of polyploid wheat. Theor Appl Genet 111:1617–1622

Aniol A (1983) Aluminium uptake by roots of two winter wheat varieties of different tolerance to aluminium. Biochem Physiol Pflanzen 178:11–20

Aniol A (2004) Chromosomal location of aluminium tolerance genes in rye. Plant Breed 123:132–136

Aniol A, Gustafson JP (1984) Chromosome location of genes controlling aluminum tolerance in wheat, rye, and triticale. Can J Genet Cytol 26:701–705

Ayliffe MA, Lawrence GJ, Ellis JG, Pryor AJ (1994) Heteroduplex molecules formed between allelic sequences cause nonparental RAPD bands. Nucleic Acids Res 22:1632–1636

Bennett MD, Smith JB (1976) Nuclear DNA amounts in angiosperms. Philos Trans R Soc Lond B Biol Sci 274:227–274

Cenci A, Chantret N, Kong X, Gu Y, Anderson OD, Fahima T, Distelfeld A, Dubcovsky J (2003) Construction and characterization of a half million clone BAC library of durum wheat (*Triticum turgidium* ssp. *durum*). Theor Appl Genet 107:931–939

Collins NC, Shirley NJ, Saeed M, Pallotta M, Gustafson JP (2008) An *ALMT1* gene cluster controlling aluminum tolerance at the *Alt4* locus of rye (*Secale cereale* L.). Genetics 179:669–682

Ehdaie B, Whitkus RW, Waines JG (2003) Root biomass, water-use efficiency, and performance of wheat-rye translocations of chromosomes 1 and 2 in spring bread wheat 'Pavon'. Crop Sci 43:710–717

Fontecha G, Silva-Navas J, Benito C, Mestres MA, Espino FJ, Hernández-Riquer MV, Gallego FJ (2007) Candidate gene identification of an aluminum-activated organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale* L.). Theor Appl Genet 114:249–260

Furukawa J, Yamaji N, Wang H, Mitani N, Murata Y, Sato K, Katsuhara M, Takeda K, Ma JF (2007) An aluminum-activated citrate transporter in barley. Plant Cell Physiol 48:1081–1091

Gallego FJ, Calles B, Benito C (1998) Molecular markers linked to the aluminum tolerance gene *Alt1* in rye (*Secale cereale* L.). Theor Appl Genet 97:1104–1109

Graybosch RA (2001) Uneasy unions: quality effects of rye chromatin transfers to wheat. J Cereal Sci 33:3–16

Gustafson JP, Ross K (1990) Control of alien gene expression for aluminum tolerance in wheat. Genome 33:9–12

Hackauf B, Wehling P (2005) Approaching the self-incompatibility locus *Z* in rye (*Secale cereale* L.) via comparative genetics. Theor Appl Genet 110:832–845

Hoffmann B (2008) Alteration of drought tolerance of winter wheat caused by translocation of rye chromosome segment 1RS. Cereal Res Commun 36:269–278

Isidore E, Scherrer B, Bellec A, Budin K, Faivre-Rampant P, Waugh R, Keller B, Caboche M, Feuillet C, Chalhoub B (2005) Direct targeting and rapid isolation of BAC clones spanning a defined chromosome region. Funct Integr Genomics 5:97–103

Keller B, Feuillet C (2000) Colinearity and gene density in grass genomes. Trends Plant Sci 5:246–251

Kim BY, Baier AC, Somers DJ, Gustafson JP (2001) Aluminum tolerance in triticale, wheat, and rye. Euphytica 120:329–337

Kim W, Johnson JW, Baenziger PS, Lukaszewski AJ, Gaines CS (2004) Agronomic effect of wheat-rye translocation carrying rye chromatin (1R) from different sources. Crop Sci 44:1254–1258

Lijavetzky D, Muzzi G, Wicker T, Keller B, Wing R, Dubcovsky J (1999) Construction and characterization of a bacterial artificial chromosome (BAC) library for the A genome of wheat. Genome 42:1176–1182

Ling P, Chen XM (2005) Construction of a hexaploid wheat (*Triticum aestivum* L.) bacterial artificial chromosome library for cloning genes for stripe rust resistance. Genome 48:1028–1036

Luo M-C, Thomas C, You FM, Hsiao J, Ouyang S, Buell CR, Malandro M, McGuire PE, Anderson OD, Dvorak J (2003) High-throughput fingerprinting of bacterial artificial chromosomes using the SNaPshot labeling kit and sizing of restriction fragments by capillary electrophoresis. Genomics 82:378–389

Madej LJ (1996) Worldwide trends in rye growing and breeding. Vortr Pflanzenzücht 35:1–6

Matos M, Camacho MV, Pérez-Flores V, Pernaute B, Pinto-Carnide O, Benito C (2005) A new aluminum tolerance gene located on rye chromosome arm 7RS. Theor Appl Genet 111:360–369

Miftahudin, Chikmawati T, Ross K, Scoles GJ, Gustafson JP (2005) Targeting the aluminum tolerance gene *Alt3* region in rye, using rice/rye micro-colinearity. Theor Appl Genet 110:906–913

Moreno-Sevilla B, Baenziger PS, Peterson CJ, Graybosch RA, McVey DV (1995) The 1BL/1RS translocation: agronomic performance of F_3-derived lines from a winter wheat cross. Crop Sci 35:1051–1055

Moullet O, Zhang H-B, Lagudah ES (1999) Construction and characterisation of a large DNA insert library from the D genome of wheat. Theor Appl Genet 99:305–313

Mugwira LM, Elgawhary SM, Patel KI (1976) Differential tolerances of triticale, wheat, rye, and barley to aluminum in nutrient solution. Agron J 68:782–787

Oettler G (2005) The fortune of a botanical curiosity—Triticale: past, present and future. J Agric Sci 143:329–346

Rabinovich SV (1998) Importance of wheat-rye translocations for breeding modern cultivars of *Triticum aestivum* L. Euphytica 100:323–340

Saisho D, Myoraku E, Kawasaki S, Sato K, Takeda K (2007) Construction and characterization of a bacterial artificial chromosome (BAC) library from the Japanese malting barley variety 'Haruna Nijo'. Breed Sci 57:29–38

Sasaki T, Yamamoto Y, Ezaki B, Katsuhara M, Ahn SJ, Ryan PR, Delhaize E, Matsumoto H (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J 37:645–653

Scherrer B, Isidore E, Klein P, Kim J, Bellec A, Chalhoub B, Keller B, Feuillet C (2005) Large intraspecific haplotype variability at the *Rph7* locus results from rapid and recent divergence in the barley genome. Plant Cell 17:361–374

Shen B, Wang DM, McIntyre CL, Liu CJ (2005) A 'Chinese Spring' wheat (*Triticum aestivum* L.) bacterial artificial chromosome library and its use in the isolation of SSR markers for targeted genome regions. Theor Appl Genet 111:1489–1494

Shi B-J, Collins NC, Miftahudin, Schnurbusch T, Langridge P, Gustafson JP (2007) Construction of a rye cv. Blanco BAC library, and progress towards cloning the rye *Alt3* aluminium (aluminum) tolerance gene. Vortr Pflanzenzücht 71:205–209

Šimková H, Šafář J, Suchánková P, Kovářová P, Bartoš J, Kubaláková M, Janda J, Číhalíková J, Mago R, Lelley T, Doležel J (2008) A novel resource for genomics of Triticeae: BAC library specific for the short arm of rye (*Secale cereale* L.) chromosome 1R (1RS). BMC Genomics 9:237

Song R, Messing J (2003) Gene expression of a gene family in maize based on noncollinear haplotypes. Proc Natl Acad Sci USA 100:9055–9060

Tenhola-Roininen T, Immonen S, Tanhuanpää P (2006) Rye doubled haploids as a research and breeding tool—a practical point of view. Plant Breed 125:584–590

Tomerius A-M, Miedaner T, Geiger HH (2008) A model calculation approach towards the optimization of a standard scheme of seed-parent line development in hybrid rye breeding. Plant Breed 127:433–440

Varshney RK, Beier U, Khlestkina EK, Kota R, Korzun V, Graner A, Börner A (2007) Single nucleotide polymorphisms in rye (*Secale cereale* L.): discovery, frequency, and applications for genome mapping and diversity studies. Theor Appl Genet 114:1105–1116

Villareal RL, Bañuelos O, Mujeeb-Kazi A, Rajaram S (1998) Agronomic performance of chromosomes 1B and T1BL.1RS near-isolines in the spring bread wheat Seri M82. Euphytica 103:195–202

Wang Q, Dooner HK (2006) Remarkable variation in maize genome structure inferred from haplotype diversity at the bz locus. Proc Natl Acad Sci USA 103:17644–17649

Wang J, Raman H, Zhou M, Ryan PR, Delhaize E, Hebb DM, Coombes N, Mendham N (2007) High-resolution mapping of the *Alp* locus and identification of a candidate gene *HvMATE* controlling aluminium tolerance in barley (*Hordeum vulgare* L.). Theor Appl Genet 115:265–276

Yu Y, Tomkins JP, Waugh R, Frisch DA, Kudrna D, Kleinhofs A, Brueggeman RS, Muehlbauer GJ, Wise RP, Wing RA (2000) A bacterial artificial chromosome library for barley (*Hordeum vulgare* L.) and the identification of clones containing putative resistance genes. Theor Appl Genet 101:1093–1099

Zhang H-B, Zhao X, Ding X, Paterson AH, Wing RA (1995) Preparation of megabase-size DNA from plant nuclei. Plant J 7:175–184