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ORIGINAL PAPER

Development of wheat–*Lophopyrum elongatum* recombinant lines for enhanced sodium 'exclusion' during salinity stress

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Abstract *Lophopyrum elongatum* (tall wheatgrass), a wild relative of wheat, can be used as a source of novel genes for improving salt tolerance of bread wheat. Sodium 'exclusion' is a major physiological mechanism for salt tolerance in a wheat–tall wheatgrass amphiploid, and a large proportion (~50%) for reduced Na^+ accumulation in the flag leaf, as compared to wheat, was earlier shown to be contributed by genetic effects from substitution of chromosome 3E from tall wheatgrass for wheat chromosomes 3A and 3D. Homoeologous recombination between 3E and wheat chromosomes 3A and 3D was induced using the *ph1b* mutant, and putative recombinants were identified as having SSR markers specific for tall wheatgrass loci. As many as 14 recombinants with smaller segments of tall wheatgrass chromatin were identified and low-resolution breakpoint analysis was achieved using wheat SSR loci. Seven recombinants were identified to have leaf Na^+ concentrations similar to those in 3E(3A) or 3E(3D) substitution lines, when grown in 200 mM NaCl in nutrient solution. Phenotypic analysis identified recombinants with introgressions at the distal end on the long arm of homoeologous group 3 chromosomes being responsible for Na^+ 'exclusion'. A total of 55 wheat SSR markers mapped to the long arm of homoeologous group 3 markers by genetic and deletion bin mapping were used for high resolution of wheat–tall wheatgrass chromosomal breakpoints in selected recombinants. Molecular marker analysis and genomic in situ hybridisation confirmed the 524-568 recombinant line as containing the smallest introgression of tall wheatgrass chromatin on the distal end of the long arm of wheat chromosome 3A and identified this line as suitable for developing wheat germplasm with Na^+ 'exclusion'.

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Introduction

Salinity is a major threat to the sustainability of wheat production in irrigated and rain-fed environments around the world (Ghassemi et al. 1995). Reduction in plant growth under salinity is due to osmotic stress from decreased soil water potential, or specific ion effects on plant cellular metabolism (Munns and Termaat 1986; Munns and Tester 2008; Munns et al. 1995). Consequently, enhancing plant ionic regulation by reducing the rate of Na^+ entry and preventing build-up of tissue Na^+ concentrations (i.e. Na^+ 'exclusion') is important to improve salt tolerance in wheat (Colmer et al. 2005). The high accumulation of Na^+ in modern commercial wheat varieties contributes to yield

penalties under moderate salinity stress (Colmer et al. 2006; Kingsbury and Epstein 1984; Salam et al. 1999). Therefore, a broadening of the range of genetic diversity and reducing Na^+ accumulation will extend the levels of salt tolerance beyond the existing cultivated wheat gene pool.

Wild relatives of wheat contain a large number of genes for desirable traits that may be exploited for wheat improvement (Colmer et al. 2006; Friebe et al. 1991). *Lophopyrum elongatum* (Host) A. Lőve [syn. *Thinopyrum elongatum* or *Agropyron elongatum*] (tall wheatgrass) has been identified as a species adapted to saline soils (Dewey 1960; McGuire and Dvorak 1981). By intergeneric hybridisation and combining genomes from hexaploid wheat (genome, AABBDD) and diploid tall wheatgrass (genome, EE), an amphiploid (AABBDDEE) has been developed with increased Na^+ 'exclusion' contributing to salt tolerance (Gorham 1994; Omielan et al. 1991; Shannon 1978; Storey et al. 1985). Although new alleles can be transferred into wheat, amphiploids often have negative quality and agronomic traits. Therefore, disomic substitution and addition lines of tall wheatgrass in bread wheat have been developed to reduce undesirable characteristics to retain the enhanced Na^+ 'exclusion' characteristic (Dvorak 1979, 1980; Dvorak and Knott 1974; Hart and Tuleen 1983; Tuleen and Hart 1988). Phenotypic analyses identified chromosome 3E from tall wheatgrass, when substituted for either wheat chromosome 3A, 3B or 3D, accounting for approximately 50% of reduced leaf Na^+ concentration also found in the amphiploid, as compared to the wheat parent (Gorham 1994; Mullan et al. 2007; Omielan et al. 1991). However, substitution lines are associated with impaired growth (McDonald et al. 2001), reduced yield (Dvorak and Sosulski 1974; McDonald et al. 2001) and unfavourable quality characteristics (Friebe et al. 1996) caused by excessive alien chromatin, or 'linkage drag'. Breaking linkage between genes controlling enhanced Na^+ 'exclusion' and undesirable agronomic and quality characteristics will reduce the size of the tall wheatgrass segment suitable for wheat improvement.

Homologous pairing of wheat chromosomes are strictly controlled by *Ph* genes on 5B (Riley and Chapman 1958; Sears and Okamoto 1958) and 3D (Mello-Sampayo 1971), and manipulation of wheat and alien chromosomes can be achieved by disruption of these genes. Lines nullisomic for *Ph1* on 5BL and *ph1b* mutants (Sears 1975, 1976) or lines that are nullisomic for *Ph2* on 3DS (Driscoll 1972; Mello-Sampayo and Canas 1973; Sears 1977, 1982) have been successful in inducing homoeologous recombination and introgressing novel alleles from wild relatives into wheat chromosomes to improve traits for biotic and abiotic stress tolerances (Friebe et al. 1996; Jauhar and Peterson 1996; Xin et al. 2001; Wang 2003; Wang et al. 2003). However, developing new wheat germplasm by alien introgression is often hindered by lack of efficient methods in selecting and identifying suitable recombinants.

Phenotypic screening followed by cytogenetic analysis has been a common strategy to identify and select desirable wheat–alien introgressions (Schwarzacher et al. 1992; Miller et al. 1995, 1996; Berzonsky and Francki 1999; Fedak and Han 2005; Zhang et al. 2007), but is inefficient for selection of desirable progeny with minimal linkage drag (Crasta et al. 2000). The application of molecular markers provides an alternative approach to identify translocation and recombinant lines with small alien segments prior to phenotypic evaluation. Simple sequence repeat (SSR) DNA markers have been used for the analysis of alien chromosome segments in wheat (Chen et al. 2005; Zhang et al. 2005) and are amenable to high-throughput screening of individuals from large population sizes (Powell et al. 1996). SSR markers have been developed specifically for detecting tall wheatgrass chromatin in wheat (Mullan et al. 2005) and these provide an opportunity to develop a DNA marker-assisted selection strategy prior to phenotypic analysis for detecting small tall wheatgrass segments with leaf Na^+ 'exclusion' in wheat.

The aim of this study was to induce recombination between chromosome 3E from tall wheatgrass with homoeologous group 3 chromosomes in bread wheat and implement SSR markers to identify recombinants with smallest tall wheatgrass segment with Na^+ 'exclusion'. The development of wheat–tall wheatgrass recombinants provides novel germplasm that could be deployed to enhance Na^+ 'exclusion' and improve salt tolerance in bread wheat.

Materials and methods

Plant materials

Genetic stocks of wheat (cv. Chinese Spring (CS)), a wheat–tall wheatgrass (*Lophopyrum elongatum* Host A. Love) amphiploid ($2n = 8x = 56$; genome AABBDDEE) and group 3 wheat–tall wheatgrass substitution lines (3E(3A), 3E(3B), 3E(3D)), were kindly provided by J. Dvořák (University of California, Davis). Seeds of the *ph1b* mutant and nullisomic 5B-tetrasomic 5D line (N5BT5D) were obtained from I. Dundas and M. Pallota, University of Adelaide. The wheat cultivar 'Camm' was obtained from the wheat seed collection, Department of Agriculture and Food, Western Australia.

Population development

Nullisomic 5B (N5B-T5D), monosomic 5B and *ph1b* mutants were used to induce homoeologous recombinations

between wheat and chromosome 3E from tall wheatgrass in five populations (Table 1). A further two F_2 populations (05Y190 and 05Y191) were derived from crosses between wheat 'Chinese Spring' and 3E(3A) and 3E(3D) substitution lines and were used as controls (Table 1). The 3E(3B) substitution line was omitted from population development as previous studies found the line to accumulate more leaf Na^+ than the 3E(3A) and 3E(3D) substitution lines (Mullan et al. 2007). F_1 plants in each population were self-pollinated to produce F_2 seeds (Table 1). Plants at F_2 were selected using DNA markers and subsequently grown at F_3 and F_4 generations. Control populations were screened using molecular markers at the F_2 generation stage only.

DNA marker-assisted selection

Plant DNA from parental lines was extracted from leaves using a phenol-chloroform-based method as described by Francki et al. (1997), whereas DNA from F_2, F_3 and F_4 lines was extracted using a high-throughput extraction protocol. Briefly, 325 µL of extraction buffer (0.15 M Tris–HCl; 0.5 M NaCl; 0.1 M EDTA; 2% SDS) was added to 0.1 g of fresh leaf material in a 1.5-mL centrifuge tube. A ball bearing was placed in each sample tube and the tissue was homogenised using a Genogrinder (Geno/Grinder, Spex Certiprep® Inc., NJ, USA). Samples were shaken at 500 strokes min^{-1} for 3 min before 110 µL potassium acetate (5 M) was added and the solution mixed by inversion. The samples were kept on ice for 10 min and centrifuged at 10,000*g* for 15 min. A 400-µL aliquot of 100% ethanol and 15 µL of 3 M sodium acetate (pH 5.2) was added to the supernatant and mixed by inversion. The samples were centrifuged at 10,000*g* for 10 min and the supernatant discarded. The DNA pellet was washed with ethanol (70%) before being air-dried and resuspended in 30 µL of R40 (10 mM Tris–HCl pH 8; 1 mM EDTA pH 8, 40 µg/ml RNAase A).

Individuals from F_2, F_3 and F_4 families were screened for chromosome 3E loci using four SSR markers (edm8, edm28, edm96 and gwm383) (Roder et al. 1998; Mullan et al. 2005). The PCR conditions and fragment analysis were as described by Mullan et al. (2005).

Selected F_4 recombinants were further screened for wheat loci using 55 SSR markers from genetic maps (Roder et al. 1998; Guyomarc'h et al. 2002; Pestsova et al. 2000; Gupta et al. 2002; Somers et al. 2004; http://www.scabusa.org; http://wheat.pw.usda.gov/) and deletion bin maps (Sourdille et al. 2004). PCR amplification of wheat SSR markers was performed for 35 cycles using either regular annealing temperatures of 30 s at 94°C, 30 s at 55/60°C, 30 s at 72°C or touchdown annealing temperatures consisting of 30 s at 94°C, 30 s at 55–47/60–50/65–55°C (1°C increments) and 30 s at 72°C. The remaining cycles for

Table 1 Populations developed for inducing homoeologous recombination between wheat and tall wheatgrass group 3 chromatin, and selection of recombinants at F_2, F_3 and F_4 using four EST-SSR markers identifying tall wheatgrass loci edm8, edm28, edm96 and gwm383

Population	Pedigree	F_1	F_2			F_3			F_4	
		No. of individuals	No. of plants screened	No. of recombinants	% recombination	No. of F_2 lines screened	No. of plants screened	No. of homozygous recombinant lines	No. of plants available	No. of plants characterised
03Y568	N5BT5D/Camm//3E(3A)	96	329	58	17.6	28	224	9	17	1
03Y570	N5BT5D/Camm//3E(3D)	96	344	76	22.1	48	572	15	22	6
03Y571	3E(3D)//N5BT5D/Camm	96	314	77	24.5	47	472	17	29	4
04Y180	*ph1bph1b*/3E(3D)	80	288	43	14.9	10	80	6	12	2
04Y181	N5BT5D/3E(3A)	80	348	63	18.1	25	200	4	8	1
05Y190	CS/3E(3A)	80	167	25	15.0	–	–	–	–	–
05Y191	CS/3E(3D)	80	127	21	16.5	–	–	–	–	–

both regular and touchdown annealing temperatures included 30 s at 94°C, 30 s at 47/50/55°C and 30 s at 72°C. PCR products were separated on 8% polyacrylamide gels in 0.5× Tris–borate–EDTA using either a Protean II xi Cell gel system (BioRad, Hercules, USA) at constant voltage (90 V) for 16 h or a 3× wide gel system (C.B.S. Scientific Company Inc., Delmar, USA) at 200 V for 2.5 h. Gels were stained with ethidium bromide and visualised under UV light with a Gel Doc System (BioRad, Milan, Italy).

F_4 recombinants were screened using PCR primers for *SOS1* gene on 3AS (*SOS1-11*), 3DS (*SOS1-12*) and 3ES (*SOS1-14*) as described by Mullan et al. (2007). The PCR conditions and fragment analysis were as in Mullan et al. (2007).

Genomic in situ hybridisation

Plant chromosome preparation and in situ hybridisation followed the procedure described by Francki and Langridge (1994). The hybridisation probe was from total genomic DNA from *Pseudoroegneria stipifolia*, sheared into fragments ranging from 0.5 to 2 kbp by autoclaving for 5 min and labelled with biotin-11-dUTP using a nick translation kit (Roche Diagnostics, Germany). Unlabelled sheared wheat genomic DNA was used as blocking DNA with a probe:block ratio of 1:100. Chromosome preparations were counter-stained with propidium iodide and analysed with an epifluorescence Zeiss Axio Imager microscope. Images were captured with a charge-coupled device camera operated with Image-Pro Express 5.1 software (Media Cybernetics Inc., Bethesda, MD) and processed with Photoshop v8.0 software (Adobe Systems, San Jose, CA, USA).

Hydroponic screening in saline conditions

Wheat–tall wheatgrass amphiploid seeds were germinated 3 days prior to all other cytogenetic lines, so as to obtain plants at a similar stage of development when treatments were imposed (McDonald et al. 2001). There were six replicate plants for each genotype, completely randomised in pots. Plant development, hydroponic conditions, salt treatments, harvesting, tissue Na^+ analysis, data collection and analysis were conducted as previously described by Mullan et al. (2007). Data were analysed by ANOVA using Genstat 9.0 software to classify recombinant lines as having high, low or intermediate leaf Na^+ concentration. Recombinant lines with significantly greater ($P < 0.05$) Na^+ concentration than their respective 3E substitution line were classified as high accumulators, those with Na^+ levels significantly lower ($P < 0.05$) than Chinese Spring wheat and not different from the substitution lines ($P > 0.05$) were classified as low leaf Na^+ accumulators, and an intermediate classification was assigned to lines with no significant difference from either Chinese Spring wheat or the substitution lines ($P > 0.05$).

Results

Selection of recombinants and low-resolution breakpoint analysis

Four SSR markers capable of detecting loci diagnostic for tall wheatgrass chromatin (edm8, edm28, edm96 and gwm383) were used to detect the presence of tall wheatgrass segments in segregating populations and a total of 1,917 F_2 plants were screened. F_2 lines were identified as putative wheat–tall wheatgrass recombinants on the basis of the presence of one to three tall wheatgrass-derived SSR loci. Typical examples of recombinant lines detecting 1, 2 or 3 tall wheatgrass loci for markers edm96, edm8, edm28 and gwm383 are shown in Fig. 1. F_2 lines that showed tall wheatgrass loci for all four markers were identified as retaining the entire 3E chromosome and were not selected for further analysis. Similarly, F_2 lines that did not show tall wheatgrass loci for any of the four markers were not selected. The frequency of recombination between tall wheatgrass 3E and wheat chromosomes was estimated from total number of F_2 individuals from each population and the presence or absence of SSR markers detecting 3E loci. Crosses derived from 3E(3A) or 3E(3D) and 'Chinese Spring' parents showed high (15–16.5%) recombination frequencies (Table 1). F_2 individuals derived from crosses between 3E(3A) and 3E(3D) substitution lines and *ph1b* mutant, nullisomic or monosomic 5B lines, showed 14.9–24.5% recombination frequency (Table 1).

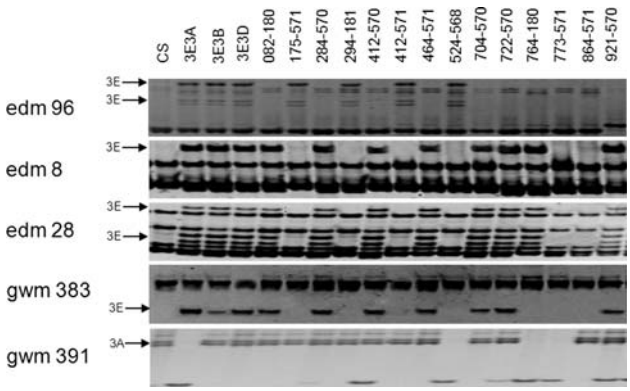


Fig. 1 Selection of putative recombinant lines using SSR markers. Putative recombinant lines were identified by the presence of one, two or three tall wheatgrass-specific (E genome) markers. The gwm391 marker (Roder et al. 1998) shows absence of bands in wheat–tall wheatgrass group 3 substitution lines (3E(3A), 3E(3B), 3E(3D)) and some recombinant lines, indicating portions of wheat chromosomes replaced with tall wheatgrass chromatin

A total of 158 putative F_2 recombinant lines from the 03Y568, 03Y570, 03Y571, 04Y180 and 04Y181 populations were selected and self-pollinated. At least eight individuals from each F_3 family were re-screened for 3E loci. Homozygous F_3 families were identified when the dominant SSR markers amplified 3E loci in all individuals. Pearson's χ^2 analysis confirmed an expected 1:2 ratio ($P < 0.05$) for homozygous:heterozygous F_2 recombinant lines in all populations. A total of 51 homozygous F_3 lines were selected from 03Y568, 03Y570, 03Y571, 04Y180 and 04Y181 populations (Table 1).

Wheat–tall wheatgrass breakpoint analysis was achieved by screening for the presence or absence of wheat loci using 12 SSR markers in the selected F_3 families. Selection of wheat SSRs for low-resolution breakpoint analysis was based on their representation in each deletion bin along chromosomes 3A, 3B and 3D. A portion of a wheat chromosome was identified as being replaced by tall wheatgrass chromatin when a wheat SSR marker locus allocated to a deletion bin was absent. A typical example is shown in Fig. 1, in which a wheat locus for marker gwm391 on chromosome 3A is absent in 3E(3A) substitution and recombinant line 524-568. This marker was mapped in the telomeric deletion bin of chromosome 3AL (Roder et al. 1998; Sourdille et al. 2004, http://wheat.pw.usda.gov) and indicated that a segment of tall wheatgrass replaced wheat chromatin in the distal region of 3AL. Low-resolution breakpoint analysis identified 14 putative recombinants that appeared to have large differences in the size of tall wheatgrass segments from the 51 homozygous F_3 lines. Amongst the 14 putative recombinant lines, four recombinants identified tall wheatgrass chromatin in the A genome (175-570, 294-181, 524-568, 773-571) and seven with the D genome (284-570, 412-571, 464-571, 704-570, 722-570, 864-571, 921-570). Two lines had tall wheatgrass chromatin recombined in both the B and D genomes (082-180, 412-570), and one line (764-180) had tall wheatgrass segments recombined in the A, B and D genomes. The 14 putative F_4 recombinant lines are shown in Fig. 2 and were selected for physiological characterisation and high-resolution breakpoint analysis.

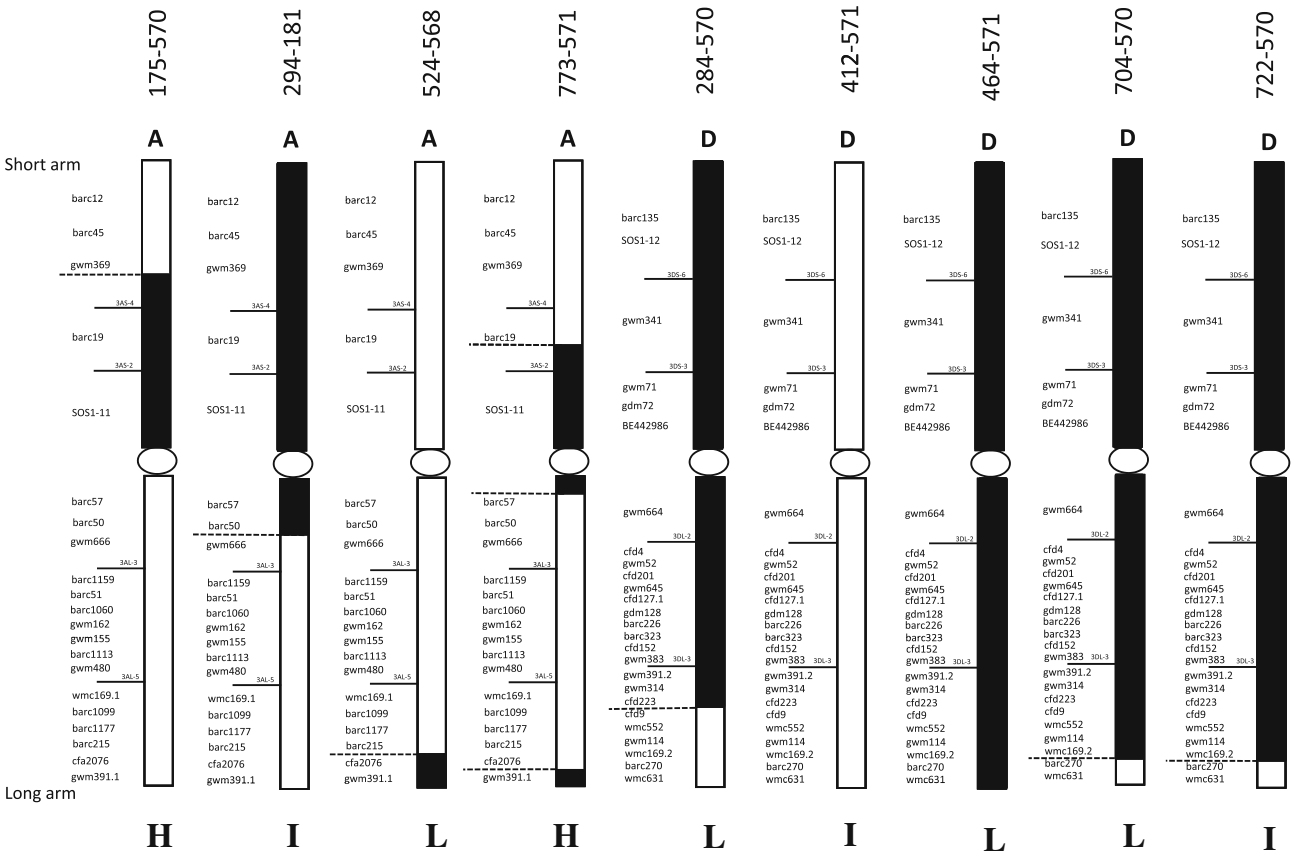
Physiological characterisation of leaf Na^+ concentrations in recombinant lines

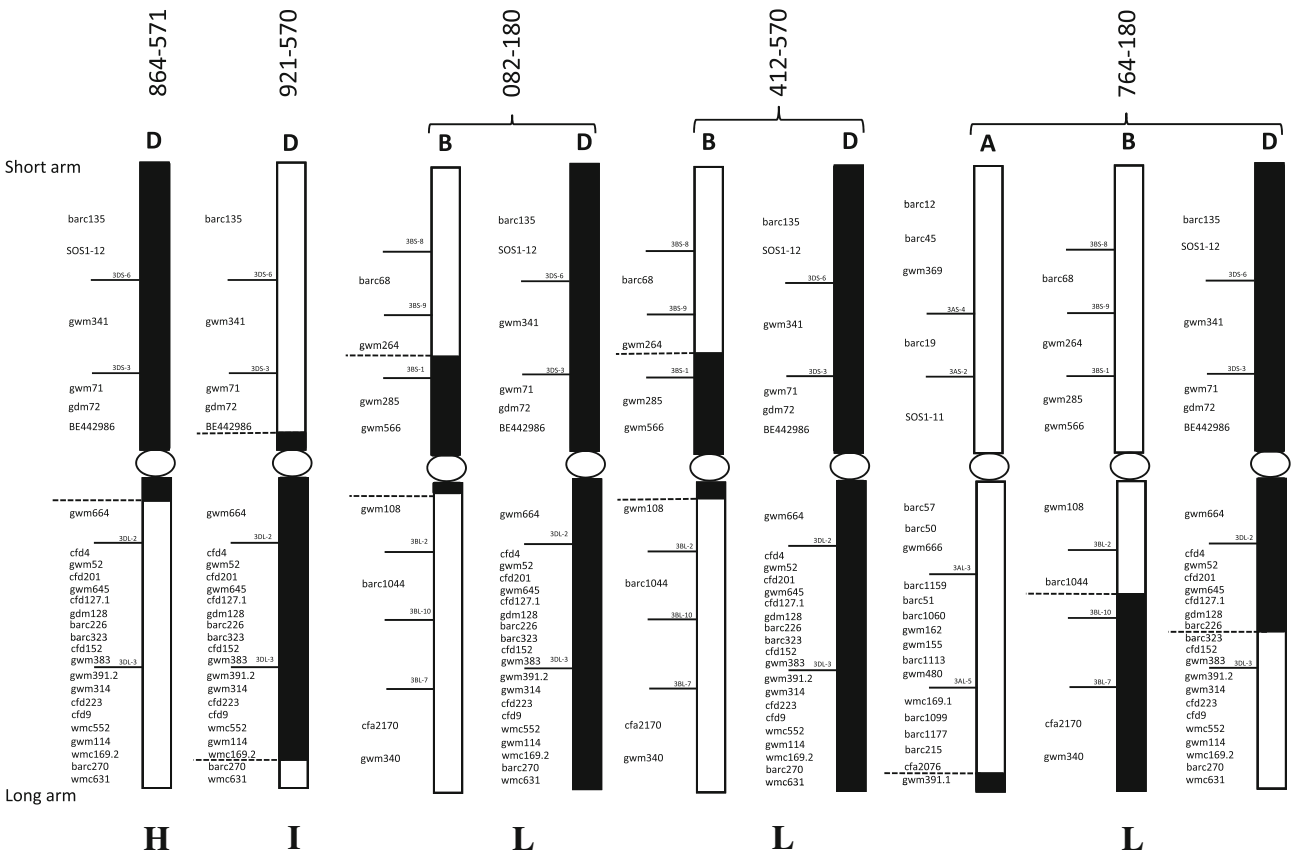
The 14 F_4 wheat–tall wheatgrass recombinant lines were evaluated for leaf Na^+ concentration in a hydroponic system containing 200 mM NaCl. Wheat 'Chinese Spring' showed a sevenfold greater leaf Na^+ concentration than the wheat–tall wheatgrass amphiploid (Table 2). The group 3 substitution lines represented approximately 50% of the difference between wheat and the amphiploid, with a significant ($P < 0.05$) difference between the 3E(3A) and 3E(3D) substitution lines and 'Chinese Spring' (Table 2). Seven recombinant lines (082-180, 284-570, 412-570, 464-571, 524-568, 704-570, 764-180) also showed significantly ($P < 0.05$) lower leaf Na^+ concentration than 'Chinese Spring' (Table 2). Additionally, the average Na^+ concentration for recombinant lines 082-180 and 524-568 was not significantly ($P > 0.05$) different from the wheat–tall wheatgrass amphiploid. The recombinant line, 524-568, was of particular interest as it had a significant reduction in leaf Na^+ concentration and the smallest segment of tall wheatgrass chromatin recombined on the long arm of chromosome 3A, determined by low-resolution breakpoint analysis (Fig. 1). Intermediate levels of leaf Na^+ concentration were identified for recombinant lines 294-181, 722-570 and 921-570, and high levels for 175-570, 773-571 and 864-571 (Table 2).

High-resolution chromosomal breakpoint analysis for selected recombinants

The phenotype and size of the alien introgressions indicated that gene(s) for reducing Na^+ accumulation in leaves, reside(s) on the long arm on chromosomes 3A and 3D. Therefore, a total of 55 wheat SSR markers, allocated to the long arms of wheat chromosomes 3A, 3B and 3D by genetic and deletion bin mapping (Sourdille et al. 2004; Somers et al. 2004), were used for high-resolution breakpoint analysis in the 14 selected F_4 lines. A wheat–tall wheatgrass chromosomal breakpoint in each recombinant was positioned between two genetically linked wheat markers when one marker showed the presence and the other showed the absence of a wheat locus. Graphical genotyping and high-resolution breakpoint analysis for the long arm of homoeologous group 3 chromosomes in the 14 F_4 recombinants are shown in Fig. 2.

High-resolution breakpoint analysis of recombinant lines together with phenotypic analysis provided a more precise location of genes controlling leaf Na^+ concentration. Four recombinant lines had tall wheatgrass chromatin introgressed in wheat chromosome 3A (Fig. 2), whereby two of these lines showed similar or significantly higher Na^+ accumulation than wheat (Fig. 2; Table 2). Interestingly, lines 175-570 and 773-571 had two and three chromosomal breakpoints, respectively. Recombinant line 294-181 contains an introgression at the proximal end of the long arm of 3A, replacing the short arm of wheat chromosome 3A and an intermediate level of leaf Na^+ accumulation. A wheat–tall wheatgrass breakpoint in the line with the smallest alien segment and significantly ($P < 0.001$) reduced Na^+ accumulation than wheat were identified in line 524-568 (Fig. 2; Table 2). High-resolution analysis positioned the wheat–tall wheatgrass chromosome breakpoint at 524-568, between wheat SSR markers, barc215 and cfa2076 (Fig. 2).


175-570
294-181
524-568
773-571
284-570
412-571
464-571
704-570
722-570
Short arm
Long arm
H
I
L
H
L
I
L
L
I


864-571
921-570
082-180
412-570
764-180
Short arm
Long arm
H
I
L
L
L

◀ **Fig. 2** Karyotypes of recombinant lines with different segments of 3E tall wheatgrass chromatin introgressed into wheat chromosomes 3A, 3B and 3D. The *black* and *white shaded* regions represent tall wheatgrass and wheat chromatin identified by the absence and presence of wheat SSR markers in each line, respectively. Deletion bins are shown by *horizontal solid lines*. Wheat SSR markers are assigned to the *left*. Wheat–tall wheatgrass chromosomal breakpoints are indicated by *horizontal dashed lines*. High (*H*), low (*L*) and intermediate (*I*) leaf Na^+ concentrations, determined in plants exposed to 200 mM NaCl in nutrient solution culture, are shown at the *bottom* of each recombinant line

Seven recombinant lines were identified as having tall wheatgrass chromatin introgressed into chromosome 3D and most were estimated to have large segments (>50%) of tall wheatgrass chromatin (Fig. 2). The recombinants indicated that the region controlling leaf Na^+ concentration resided on the tall wheatgrass segment in the interstitial region of the long arm of 3D between wheat SSR markers, gwm664 and cfd9. The phenotype with low leaf Na^+ accumulation was observed in three lines (464-571, 284-570, 704-570) that contain tall wheatgrass chromatin between marker loci gwm664 and cfd9 (Fig. 2; Table 2). Conversely, wheatgrass chromatin in the 864-571 recombinant line was not detected between these marker loci and this line had a high Na^+ concentration phenotype (Fig. 2; Table 2). Three of the D genome recombinant lines (722-570, 921-570, 412-571) recorded intermediate leaf Na^+ accumulation (Table 2). High-resolution breakpoint analysis was unable to identify the location of the 3E introgression in line 412-571 or the wheat–tall wheatgrass breakpoint in line 464-571 on wheat chromosome 3D.

Three recombinant lines, 082-180, 412-570 and 764-180, have low Na^+ accumulation phenotypes and these contain tall wheatgrass chromatin introgressed into more than one of the wheat group 3 chromosomes (Fig. 2; Table 2). These recombinants have tall wheatgrass segments in common with recombinants containing low Na^+ accumulation with individual introgressions on the long arms of either 3A or 3D.

Putative orthologs of the *Arabidopsis SOS1* gene that contributes to controlling shoot Na^+ accumulation (Shi et al. 2002) have been identified on wheat and tall wheatgrass chromosomes (Mullan et al. 2007). The *SOS1* orthologues on chromosomes 3A and 3D of wheat and 3E of tall wheatgrass were screened against DNA from recombinant lines with high and low leaf Na^+ concentration. The tall wheatgrass-specific locus (*SOS1-14*) was not detected in any recombinant line (Fig. 2). Similarly, the presence of the wheat *SOS1-11* and *SOS1-12* markers on the short arm of chromosome 3A and 3D could not be correlated with either high or low leaf Na^+ concentration in the recombinant lines (Fig. 2).

GISH analysis of recombinant 524-568

High-resolution chromosomal breakpoint and phenotypic analysis identified the recombinant line 524-568 as having the smallest segment of tall wheatgrass chromatin with

Table 2 Mean leaf Na^+ concentration and ANOVA significance tests between wheat (Chinese Spring), wheat–tall wheatgrass amphiploid (Amphiploid), recombinant lines and the 3E(3A) and 3E(3D) tall wheatgrass substitution lines to identify lines significantly lower than wheat or significantly higher than the 3E substitution lines

Genotype	Mean leaf Na^+ concentration ($\mu molg^{-1}$ DW) ± SE	Significant difference with Chinese Spring	Significant difference with 3E(3A)	Significant difference with 3E(3D)	Classification
Chinese Spring	722 ± 97	–	0.019	0.005	High
Amphiploid	106 ± 26	<0.001	<0.001[b]	0.001[b]	Low
3E(3A)	484 ± 63	–	–	0.641	Low
3E(3D)	438 ± 57	–	0.641	–	Low
175-571	1430 ± 278	<0.001[a]	<0.001	–	High
294-181	536 ± 122	0.06	0.623	–	Intermediate
524-568	264 ± 50	<0.001	0.029[b]	–	Low
773-571	911 ± 108	0.058	<0.001	–	High
284-570	501 ± 59	0.025	–	0.563	Low
412-571	531 ± 58	0.064	–	0.323	Intermediate
464-571	388 ± 92	0.001	–	0.65	Low
704-570	357 ± 46	<0.001	–	0.381	Low
722-570	552 ± 139	0.085	–	0.261	Intermediate
864-571	753 ± 74	0.754	–	0.002	High
921-570	600 ± 135	0.223	–	0.104	Intermediate
082-180	278 ± 60	<0.001	0.034[b]	0.096	Low
412-570	366 ± 109	<0.001	0.234	0.467	Low
764-180	377 ± 27	<0.001	0.279	0.535	Low

Recombinant lines were assigned leaf Na^+ accumulation classifications as: concentrations of leaf Na^+ significantly higher than the 3E substitution lines (High), significantly lower leaf Na^+ than wheat (Low) or having no significant difference with either wheat or the 3E substitution lines (Intermediate)

[a] Significantly greater leaf Na^+ accumulation than wheat

[b] Significantly lower leaf Na^+ accumulation than 3E(3A)

lower leaf Na^+ concentration than the 3E(3A) and 3E(3D) substitution lines. GISH was used to confirm the position of the introgressed segment in wheat. Figure 3 shows GISH analysis using labelled *P. stipifolia* as a hybridisation probe where the position of the introgressed tall wheatgrass segment (yellow fluorescence) was confirmed at the distal end of the long arm of wheat chromosome 3A (red fluorescence). Total genomic DNA from *P. stipifolia* was able to better discriminate alien from wheat chromatin than *L. elongatum* (data not shown).

Discussion

This study developed a series of wheat–tall wheatgrass recombinant lines involving chromosome 3E to identify germplasm with the smallest segment of tall wheatgrass chromatin and low leaf Na^+ concentration, suitable for wheat improvement for tolerance to salinity stress. DNA marker-assisted selection in early generations allowed rapid and reliable assessment of large population sizes for smaller segments of chromosome 3E in wheat. The selection of recombinant lines with differing sizes of tall wheatgrass segments was phenotyped for high, low or intermediate levels of leaf Na^+ concentration. High-resolution chromosomal breakpoints of wheat–tall wheatgrass recombinant lines with high and low leaf Na^+ concentrations using wheat SSR markers identified the region controlling Na^+ 'exclusion' on the distal end of the long arm of homoeologous 3A and 3D replaced by tall wheatgrass chromatin. In this study, the recombinant line, 524-568, was identified as containing the smallest portion of tall wheatgrass chromatin on the distal end of the long arm of wheat chromosome 3A and with low leaf Na^+ accumulation, making this line most suitable for wheat germplasm development.

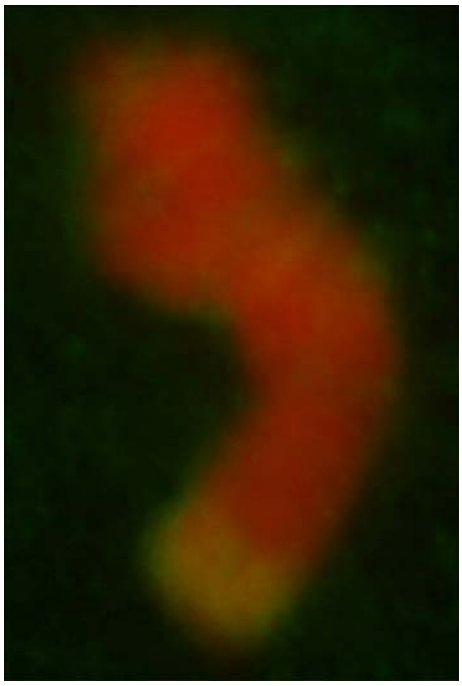


Fig. 3 Genomic in situ hybridisation of line 524-568 showing hybridisation of the *Pseudoroegneria stipifolia* probe to a tall wheatgrass chromosome introgression on the distal end of wheat chromosome 3A

Several studies have used either C-banding and/or in situ hybridisation to detect alien chromatin in wheat (reviewed in Friebe et al. 1996). Although the recent increase in genomics resources can add value to the analysis of wide crossing strategies, thus leading to the capability of performing high-density genome-wide marker-based screens, more recent studies continue to use cytogenetic and phenotyping approaches to characterise alien introgressions to select lines in early generations and implement molecular markers as a secondary screening tool to refine chromosomal breakpoint resolution (Fedak 1999; Shen et al. 2004; Jauhar 2008). This approach is often inefficient in selecting early generation material amongst large population sizes. PCR markers for alien introgression can be more efficiently utilised if implemented earlier in the selection process to reduce the number of plants required for phenotypic and cytogenetic analyses. Therefore, implementation of high-throughput SSR markers and low-resolution genotyping was the key in early generation selection in this study, followed by phenotypic characterisation and high-resolution genotyping. In this manner, early generation screening with tall wheatgrass SSRs identified putative recombinant lines that either did not have tall wheatgrass introgressions or appeared to have full 3E chromosomes, thereby rapidly eliminating undesirable genotypes prior to physiological screening. The four tall wheatgrass SSR loci distributed across chromosome 3E, followed by analysis with a minimal set of 12 wheat SSR markers, were valuable selection tools to identify 14 putative recombinant lines from 1972 individuals. The individuals had relatively small segments of tall wheatgrass chromatin in early generation selection for subsequent phenotypic and high-resolution chromosome breakpoint analysis.

Homoeologous recombination between wheat and tall wheatgrass was achieved by manipulation of the homologous chromosome pairing genes. Previous studies have reported varying frequencies of recombination between wild relatives and wheat, including very low recombination frequencies with *Agropyron cristatum* (0–0.02%) (Jubault et al. 2006), rye (0.1–0.4%) (Lukaszewski 2000; Anugrahwati et al. 2008) and barley (1.8%) (Taketa et al. 2005), as well as higher recombination frequencies between wheat and *Thinopyrum bessarabicum* (12.7%) (King et al. 1993) and *Thinopyrum junceum* (10%) (Wang 2003). In this study, levels of homoeologous recombination were higher

than expected. Moreover, the high frequency of double recombinants detected is further evidence for the high level of recombination between wheat and tall wheatgrass. It has been previously reported that the genomes of wheat and tall wheatgrass may have significant cross-compatibility and chromosome morphology (Dvorak et al. 1984) and this factor may, therefore, contribute to the high recombination between chromosomes from these two species. We cannot, however, disregard the effect of wheat *Ph* genes on chromosomes 5BL and those replaced by tall wheatgrass chromosomes in 3E(3A) and 3E(3D) substitution lines as parents in population development that contribute to high rates of recombination. It is interesting to note that there was no substantial increase in recombination frequency due to the simultaneous absence of *Ph1* on 5BL (replaced by *ph1b*) and the *Ph* genes on 3AS and 3DS chromosomes (replaced by 3E), compared to the already high levels of recombination detected in the control populations, 05Y190 and 05Y191, with the normal *Ph1* gene present Therefore, the majority of recombination may be due to the effects of tall wheatgrass 3E replacing wheat *Ph* genes in the 3E(3A) and 3E(3D) substitution lines.

It was expected that the 14 recombinant lines selected by molecular markers would have either a high or low leaf Na^+ accumulation phenotype, similar to the wheat and 3E(3A)/3E(3D) substitution lines, respectively. The analysis of seedlings in hydroponic solution not only identified phenotypes similar to the parents, but also a number of mid-range values (classified as intermediate phenotypes in this study) indicating that low Na^+ accumulation in the 3E(3A) and 3E(3D) substitution lines is likely to be under polygenic control. Moreover, the leaf Na^+ accumulation in the recombinant line, 175-571, is significantly greater than the wheat parent (high leaf Na^+ concentration), indicating transgressive segregation and providing further evidence that leaf Na^+ accumulation in 3E(3A) and 3E(3D) is a quantitative trait. The analysis of the phenotypes for selected recombinants is likely to be under control of gene combinations from wheat and tall wheatgrass that interact in a complex manner. The identification of genes responsible and their interactions would provide further scope on the regulation of leaf Na^+ accumulation.

Comparative genomic analysis identified an orthologue of the *Arabidopsis* Na^+ transporter gene *SOS1*, located in wheat and tall wheatgrass on the short arm of group 3 chromosomes (Mullan et al. 2007). In an attempt to investigate gene interactions, we used genetic information relevant to *SOS1* expecting that gene orthologues from wheat and tall wheatgrass may contribute towards controlling leaf Na^+ accumulation in recombinant lines. However, there was no clear association between the wheat or wheatgrass *SOS1* orthologue, with either low or high leaf Na^+ concentrations in the recombinant lines. Therefore, it is unlikely that this gene has a major role in controlling Na^+ accumulation in wheat–wheatgrass aneuploid lines. Moreover, comparative analysis of amino acid residues between *Arabidopsis*, rice and wheat indicated that functional domains of *SOS1* were not conserved between species and so orthologues may have different functions in wheat and tall wheatgrass (Mullan et al. 2007). Therefore, it is likely that other genes on wheat and wheatgrass interact in a complex manner to control Na^+ accumulation in wheat–tall wheatgrass aneuploid lines. The differences in transcriptional regulation of *HKT1* gene (Mullan et al. 2007), located on wheat chromosome 7B in 3E substitution lines under salt stress (Mullan et al. 2007) provides further evidence that genes on group 3 and other wheat chromosomes interact synergistically to control Na^+ accumulation. However, we are unable to resolve whether the genes are contributed by new alleles on tall wheatgrass segment that function to restrict Na^+ entry, or the absence of wheat genes that usually enhance Na^+ entry into leaves during salt stress.

Tall wheatgrass-specific SSRs were used initially in early generation screening, but were not used in high-resolution chromosomal breakpoint analysis of recombinant lines. The reason is largely due to uncertainty of the comparative order of tall wheatgrass loci compared with wheat (Mullan et al. 2005). Any unexpected rearrangements, including translocations, deletions and inversions during introgression may not have necessarily allowed the accurate estimation of the size and position of tall wheatgrass segments recombined in wheat chromosomes. Therefore, wheat SSRs were used as the main tool for low- and high-resolution breakpoint analysis for a more accurate estimate of the size of the tall wheatgrass introgressions in recombinant lines. In some instances (for example, recombinant line 412-571), wheatgrass chromatin was detected in recombinant lines, but failed to place a chromosomal breakpoint using wheat SSR analysis. This is likely due to a very small tall wheatgrass chromosome segment being in a position on the wheat chromosome that was not represented by high-resolution breakpoint analysis. Interestingly, recombinant lines contained a high occurrence of large introgressions in the D rather than the A genome, implying a high level of genomic sequence similarity between *T. tauschii* and *L. elongatum*. Variation in the size of tall wheatgrass introgressions between the wheat genomes is likely due to differences in homology between the three wheat genomes and tall wheatgrass and the subsequent effect on cross-compatibility and chromosome morphology (Dvorak 1980). Furthermore, whilst large and multiple genome introgressions increase the opportunity for undesirable linkage drag (Dvorak and Sosulski 1974; Friebe et al. 1996; McDonald et al. 2001), these may also increase the dosage of beneficial genes contributed by tall wheatgrass. This is evident in the recombinant line 082-180 that contains a tall

wheatgrass introgression on the B and D genomes and exhibits very low leaf Na^+ accumulation. The recombinant lines containing large sizes of introgressed chromatin and retaining low leaf Na^+ accumulation will need to undergo further development to reduce the size of alien fragments and any associated linkage drag.

In summary, high-resolution breakpoint analysis and phenotypic evaluations enabled a more precise chromosomal position of genes controlling the low leaf Na^+ accumulation phenotype in the recombinant lines. The chromosomal regions most likely to contribute the desired phenotype are located in the distal region of 3AL and proximal region on 3DL. The recombinant line 524-568 generated in this study provides novel germplasm to improve Na^+ 'exclusion' ability in wheat, a trait that contributes to salt tolerance in wheat (Munns 2005). Future germplasm development will use 524-568 in recurrent backcrossing to incorporate the tall wheatgrass segment into locally adapted bread wheat varieties and assess regulation of leaf Na^+ concentrations and salt tolerance in seedlings using hydroponics and adult plants in field evaluation. The DNA makers developed in this study can be used to track the alien segment in germplasm development.

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