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Marker-assisted breeding for *TaALMT1*, a major gene conferring aluminium tolerance to wheat

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Abstract

Aluminium toxicity in acid soils is the main limitation to crop production worldwide. In wheat (*Triticum aestivum* L.), the Al-activated malate transporter (*TaALMT1*) gene located on chromosome 4DL is associated with malate efflux and Al-tolerance. To introgress Al-tolerance from the breeding line CAR3911 into the high yielding Al-sensitive cultivar Kumpa-INIA, phenotypic and molecular characterizations of gene/QTL underlying Al-tolerance in CAR3911 followed by marker-assisted backcrossing (MAS-BC) were undertaken. Al-tolerant backcross (BC) lines were selected using the functional marker ALMT1-4 designed immediately upstream of the *TaALMT1* coding region. Foreground and background selections using ALMT1-4 and microsatellite markers were conducted. Linkage and sequence analyses suggest that the *TaALMT1* gene could underly the Al-tolerance in CAR3911, possessing the same promoter type (V) as the Al-tolerant genotypes Carazinho and ET8. The MAS-BC strategy allowed the selection of Al-tolerant lines with the smallest introgressed region (6 cM) on 4D and the highest recurrent parent genome (RPG) (98 %) covering 2 194 cM of the wheat genome. The homozygous BC_3F_2 line named Kumpa-INIA-*TaALMT1* expressed a 3-fold higher Al-tolerance than its isogenic line Kumpa-INIA at 40 μM Al in the hydroponic solution, and similarly to CAR3911 and Carazinho. The MAS-BC strategy was successful for the introgression of the *TaALMT1* gene into Kumpa-INIA in only three BC generations, shortening the breeding cycle to 24 months, which promises to increase wheat production and a greater yield stability in the acid soils of Southern Chile.

Additional key words: acid soils, backcross lines, gene expression, introgression, malate transporter, microsatellite markers.

Introduction

Acid soils (pH < 5.5) comprise 40 % of the arable land and approximately 70 % of the potentially arable areas in the world (Zheng *et al.* 1998, Hede *et al.* 2002). In these soils, Al^{3+} affects plant growth by inhibiting root cellular divisions, the absorption of water and nutrients, and ultimately reducing productivity (Raman *et al.* 2010, Zheng 2010, Inostroza-Blancheteau *et al.* 2012). Al toxicity reduces yield by 20 % in East Asia, Sub-Saharan Africa, and North America, 31 % in Latin America, and 38 % of the farmland in Southeast Asia (Wood *et al.* 2000).

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Abbreviations: BSA - bulk segregant analysis; MAS-BC - marker assisted backcrossing; QTL - quantitative trait loci; RPG - recurrent parent genome; RRE - relative root elongation; RRG - root regrowth; *TaALMT1* - Al-activated malate transporter gene 1.
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A common practice to reduce the Al^{3+} consists in neutralizing soil acidity with the addition of lime ($CaCO_3$) or dolomite ($MgCO_3$) (Hede *et al.* 2002). Although this is a common practice, the effectiveness of raising soil pH remains conditioned to the first centimeters of the arable layer (Zhou *et al.* 2007, Hu *et al.* 2008). Alternatively, the cultivation of Al tolerant genotypes combined with liming practices can provide a cost-effective solution.

Wheat (*Triticum aestivum* L.), is one of the most widely grown cereal crops, and winter wheat Kumpa-INIA is characterized by its high yield potential (Jobet and Hewstone 2003). The Kumpa-INIA has been bred for other traits, such as the introgression of high protein content from *Triticum turgidum* ssp. *dicoccoides* (Khan *et al.* 2000) and resistance to the imidazolinone herbicide (unpublished data). However, it is sensitive to Al, hindering its extensive cultivation in Southern Chile (Gallardo *et al.* 1999).

In wheat germplasm, a wide range of genotypic variation in Al-tolerance exists enabling the development of adapted cultivars to acidic soils (Raman *et al.* 2006). In some genotypes, this variation is controlled by a single major genetic locus (Somers and Gustafson 1995, Basu *et al.* 1997, Sasaki *et al.* 2004), whereas in other genotypes, the trait involves multiple genes (Papernik *et al.* 2001, Ma *et al.* 2005, Zhou *et al.* 2007). Studies on recombinant inbred lines (RILs) derived from a cross between Atlas 66 (Al-tolerant) and Chisholm (Al-sensitive) identified two quantitative trait loci (QTL). A major QTL is located on chromosome 4D that co-segregates with *TaALMT1* and a minor QTL is located on chromosome 3BL (Zhou *et al.* 2007). Likewise, the Al-tolerance present in a Chinese landrace FSW seems to be controlled by three QTL located on 4DL, 3BL, and 2A (Cai *et al.* 2008). A second mechanism that relies on citrate efflux has been described in the Brazilian cultivar Carazinho, the gene named *TaMATE1B* (for multidrug and toxic compound extrusion) has been mapped on 4BL (Ryan *et al*. 2009).

Since the identification and characterization of the *TaALMT1* gene in wheat (Sasaki *et al.* 2004, 2006), subsequent QTL studies have confirmed its co-location with the Al-tolerant locus on 4DL (Raman *et al.* 2005, 2006, 2010, Zhou *et al.* 2007) showing that the *TaALMT1* gene is the main factor driving the malate efflux and tolerance to Al.

Marker-assisted selection (MAS) has been shown to be effective for the introgression of qualitative traits and major QTL (Ribaut and Hoisington 1998, Bertrand *et al.* 2008). Marker assisted backcrossing (MAS-BC) is used when the recurrent parent has a large number of desirable attributes but is deficient in only a few characteristics (Tester and Langridge 2010). As *TaALMT1* represents a major Al-tolerance QTL, having comprehensive genotypic information for this locus can allow MAS for Al tolerance**.** Haplotype-specific functional markers from promoter and intronic regions of the *TaALMT1* gene (Raman *et al.* 2006, Sasaki *et al.* 2006) accompanied by molecular markers and genetic linkage maps provide the resources required for identifying and tagging *TaALMT1* alleles (foreground selection) and for recovering the recurrent parent genome (RPG) (background selection) in breeding populations (Röder *et al.* 1998, Gupta *et al.* 2002, Somers *et al.* 2004, Torada *et al.* 2006), minimizing the drawbacks of linkage drag and the need of time-consuming phenotypic assays (Ribaut and Hoisington 1998, Raman *et al.* 2003, Ma *et al.* 2005).

The aims of this study were: *1*) to characterize the Al-tolerance in the Chilean breeding line CAR3911 at the genetic and molecular levels; and *2*) to introgress the *TaALMT1* gene from CAR3911 into the high yielding Al-sensitive cv. Kumpa-INIA using a MAS-BC strategy.

Materials and methods

Plants and Al-tolerance screening: A wheat (*Triticum aestivum* L.) winter cultivar Kumpa-INIA and the spring breeding line CAR3911, both from the Wheat Breeding Program of the National Institute of Agriculture Research (INIA), Chile, were characterized in terms of their Al-tolerance in hydroponic solution. Carazinho and ET8 genotypes (Al-tolerant), Chinese Spring (Al-inter-mediate), and ES8 (Al-sensitive) were used as controls. Two hundred seeds of each genotype were surface sterilized with 1 % (m/v) NaClO for 15 min and germinated in Petri dishes. The seedlings were adapted to low pH by transferring them to a 500 µM $CaCl_2$ solution (pH 5.0) at 24 ºC in a growth chamber for 24 h. Ten plants of each genotype with similar seminal root lengths were subjected to 0, 5, 10, 15, 20, and 40 µM $AlCl_3$, which dissociated to form Al^{3+} ions when pH was adjusted to 4.5, for 72 h in a low-ionic-strength nutrient solution (Peñaloza *et al.* 2004, Martins *et al*. 2013) under greenhouse conditions with a 18-h photoperiod, an irradiance of 500 µmol m^{-2} s^{-1}, and a temperature of 24 °C. The nutrient solution was aerated and replaced daily to minimize fluctuations in pH and Al concentration. The Al tolerance status of each genotype was expressed as relative root elongation (RRE) (Raman *et al.* 2005). The significant differences among genotypes and treatments were analyzed by the analysis of variance (*ANOVA*) in the *GenStat* statistical package (*v. 14.0; VSN International*., Hemel Hempstead, UK). Significant differences between means were determined using the least significant differences (LSD = 0.05).

Mapping population and linkage analysis of Al-tolerance: To determine the genetic control of the Al-tolerance in CAR3911 a F_2 mapping population of Kumpa-INIA × CAR3911 with 170 individuals was developed. The F_2 population was screened using the

pulse-recovery method that allows discriminating between Al-tolerant, Al-intermediate, and Al-sensitive genotypes under the hypothesis of Mendelian inheritance of the Al-tolerance trait (Raman *et al.* 2002). For the pulse step, the population was subjected for 6 d to a toxic Al concentration (20 μM) determined from the RRE experiments. For the recovery step, the Al concentration was reduced to 10 μM and the root re-growth (RRG) scored after 6 d (Raman *et al.* 2002). Mendelian inheritance of the Al-tolerance was evaluated by the χ^2-analysis.

The same population was used to construct a linkage map for chromosome 4D. The simple sequence repeat markers (SSRs) WMC285, WMC720, WMC52, GWM608, CFD23, WMC473, WMC457, GWM193, GWM165, WMC331, WMC206, CFA84, WMC622, GWM194, GWM624, and GWM609 were tested for polymorphisms between parents following the PCR conditions described by Röder *et al.* (1998), Gupta *et al.* (2002), Somers *et al.* (2004) and *GrainGenes* (http://wheat.pw.usda.gov). The PCR products were separated in a denaturated 6 % (m/v) polyacrylamide gel and visualized with silver nitrate staining (Sourdille *et al.* 1998). Polymorphic SSRs and phenotypic data were analyzed in *JoinMap v. 4.0* (Van Ooijen 2006) and underwent the linkage analysis using the Kosambi function (Kosambi 1944). Linkage between markers on 4D was declared by setting the logarithm of odds (LOD) at 3.0 and the maximum recombination fraction at 0.49. Goodness of fit between the expected and observed segregation ratios was tested by the χ^2-analysis.

A bulk segregant analysis (BSA) (Michelmore *et al.* 1991) was performed to identify SSRs linked to the *TaALMT1* gene on 4D. Two bulks were constructed using an equal amount of DNA from each of the 34 Al-tolerant and 34 Al-sensitive genotypes. Polymorphic SSRs between the bulks were further analyzed in all F_2 plants to determine the genetic distance between markers and the TaALMT1 locus. The recombination frequency between the TaALMT1 locus and linked markers was obtained from maximum likelihood and converted to genetic distances using the Kosambi (1944) mapping function. The collinearity between the Kumpa-INIA × CAR3911 linkage map was compared with the consensus linkage map reported by Somers *et al.* (2004) and used to assist the recurrent parent genome (RPG) recovery on 4D.

Molecular characterization of Al-tolerant genes in CAR3911: Since the *TaALMT1* gene has been identified in other Al-tolerant wheat genotypes, primers from its promoter sequence that co-segregate with Al phenotypes were used to reveal its presence in CAR3911. PCR was performed using primer set 4 (F: 5'-GCTCCTACC ACTATGGTTGCG-3'; R: 5'-CAGATGCAATGCAAG CTCAAG-3') according to Sasaki *et al.* (2006). CAR3911 and the control genotypes with known amplification patterns of promoter types ET8 (pattern V), Chinese Spring (pattern III), Carazinho (pattern V), and ES8 (pattern I) were screened. Patterns I, III, and V are associated with the Al-sensitive, Al-intermediate, and Al-tolerant phenotypes, respectively (Sasaki *et al.* 2006). Further, PCR products from the upstream coding region of the *TaALMT1* gene were amplified in CAR3911 with primer sets 1 (F: 5'-GTCACCACGAAGAAGAGG AACACCGACC-3'; R: 5'-AGTGGAGCAGCAGCC CGCAGCTGGCGATG-3') and 2 (F: 5'-CCTGGTTTT CTTGATGGGGGCACACAC-3'; R: 5'-TGCCCACCA TCTCGCCGTCGCTCTCTCT-3') (Sasaki *et al.* 2006), and sequenced to elucidate its promoter structure.

Introgression of Al-tolerance gene from CAR3911 into Kumpa-INIA: To introgress the Al-tolerance from CAR3911 into Kumpa-INIA, a BC population of 70 BC_1 lines was developed. Since the *TaALMT1* gene with pattern V was confirmed in CAR3911 (see Results), the BC population was screened with the ALMT1-4 marker to confirm Al-tolerance/sensitive status. The Al-tolerant BC_1 lines were then analyzed for all markers on 4D using the linkage map of Kumpa-INIA × CAR3911 with the aim of accelerating the RPG recovery using the MAS-BC strategy described by Frisch *et al.* (1999) along with graphical representation of chromosomes implemented in the software *GGT 2.0* (Van Berloo 2008). The Al-tolerant BC_1 lines with the flanking markers to the *TaALMT1* gene and with the highest percentage of loci fixed for the RPG allele on 4D were selected. This subset of BC_1 lines were further analyzed across the remaining 20 chromosomes so that the individual carrying the highest percentage of alleles fixed for the RPG was advanced to the next generation. For scanning the whole genome (background selection), 354 additional SSRs were tested for polymorphism between parents according to Röder *et al.* (1998), Gupta *et al.* (2002), Somers *et al.* (2004), and *GrainGenes* databases. The markers order and genetic distances of polymorphic SSRs were those reported by Somers *et al.* (2004). The MAS-BC strategy described above was carried out until the BC_3 generation and Al-tolerant status of the BC_3F_2 lines was confirmed hydroponically using the RRE method (Raman *et al.* 2005).

Results

There were highly significant effects ($P < 0.001$) of genotype, Al treatment, and genotype × Al treatment interaction on RRE with the six genotypes displaying differential responses to the Al stress (Table 1). CAR3911 and Carazinho showed superior performances compared to the other genotypes. For example, RRE of Kumpa-INIA was inhibited ~40 % at 5 μM Al and ~63 % at 40 μM Al corroborating its high sensitivity to Al. On

the other hand, RRE of CAR3911 and Carazinho were not inhibited at 5 μM Al and only 20 % at 40 μM Al corroborating their Al-tolerance. RRE of Chinese Spring and ET8 were similar and not statistically different despite ET8 being considered Al-tolerant and Chinese Spring Al-inter-mediate (Table 1). However, the assay clearly identified ET8 as significantly more tolerant (approximately 3-fold) than ES8, as expected.

Table 1. The effect of Al on root length and relative root elongation (RRE) in six wheat genotypes after 72 h. Means ± SD (n = 10). Means with common letters are not significantly different at $P > 0.05$ according to the LSD test.

Al [μM]	Genotype	Root length [cm]	RRE [%]
0	Chinese Spring	5.91 ± 0.99	100.0a
	ET8	6.04 ± 0.64	100.0a
	ES8	5.73 ± 0.82	100.0a
	Carazinho	7.11 ± 1.31	100.0a
	CAR3911	7.21 ± 2.17	100.0a
	Kumpa-INIA	5.87 ± 0.87	100.0a
5	Chinese Spring	5.39 ± 0.76	91.2b
	ET8	5.36 ± 0.55	89.2b
	ES8	1.52 ± 0.61	26.5d
	Carazinho	7.53 ± 1.15	105.9a
	CAR3911	7.37 ± 2.27	102.2a
	Kumpa-INIA	3.58 ± 0.60	60.9c
10	Chinese Spring	4.87 ± 0.74	82.4b
	ET8	5.12 ± 1.15	84.8b
	ES8	1.49 ± 0.64	26.0d
	Carazinho	7.50 ± 1.05	105.5a
	CAR3911	7.46 ± 2.39	103.5a
	Kumpa-INIA	3.45 ± 0.70	58.8c
15	Chinese Spring	3.87 ± 0.59	65.5b
	ET8	4.17 ± 1.09	69.1b
	ES8	1.42 ± 0.56	24.8d
	Carazinho	6.25 ± 2.54	87.9a
	CAR3911	6.76 ± 1.03	93.8a
	Kumpa-INIA	3.03 ± 0.64	51.6c
20	Chinese Spring	3.54 ± 0.90	59.9b
	ET8	3.86 ± 0.42	63.9b
	ES8	1.29 ± 0.35	22.5d
	Carazinho	5.84 ± 1.03	82.1a
	CAR3911	6.33 ± 2.18	85.9a
	Kumpa-INIA	2.49 ± 0.99	42.4c
40	Chinese Spring	3.51 ± 0.81	59.4b
	ET8	3.58 ± 0.65	59.3b
	ES8	1.11 ± 0.40	19.4d
	Carazinho	5.76 ± 0.71	81.0a
	CAR3911	5.64 ± 2.03	78.2a
	Kumpa-INIA	2.18 ± 0.40	37.1c

To elucidate the genetic architecture of Al tolerance in CAR3911, the Kumpa-INIA × CAR3911 F_2 mapping population and BC lines were subjected to 20 μM Al [at this concentration, RRE of Kumpa-INIA was significantly reduced compared to CAR3911 (Table 1, Fig. 1)]. In the pulse stage, RRG of the tolerant plants continued unimpeded as expected showing a white color. The Al-intermediate plants showed a small reduction in RRG with atrophy of lateral meristems. The Al-sensitive plants were strongly affected having their RRG completely inhibited. In the recovery stage, among the 170 F_2 plants, 44 were classified as Al-tolerant, 82 as intermediate, and 44 as Al-sensitive. This distribution fitted a 1:2:1 ($\chi^2 = 0.156$, $P = 0.925$) Mendelian ratio for monogenic segregation and a bi-modal distribution (data not shown) confirming that a single major locus controls Al tolerance in CAR3911.

The linkage map of 4D was constructed with six polymorphic SSRs and used to identify the most tightly linked markers to the TaALMT1 locus. The map covered 78 cM of the 4D map reported by Somers *et al.* (2004) and shared significant marker collinearity (Fig. 2*A*). The markers WMC457, WMC720, and WMC331 showed tight linkage with the TaALMT1 locus (LOD > 3) (Fig. 2*B*). The *ANOVA* analysis for markers WMC457, WMC331, and WMC720 showed a high association with the Al tolerance variation ($P < 0.001$). BSA determined the genetic distance of markers WMC457 and WMC331 at 3.5 and 2 cM from the TaALMT1 locus, respectively (Fig. 2*B*). In addition, the functional marker ALMT1-4, when tested in the F_2 mapping population, exhibited the complete association with the Al tolerance variation suggesting that the *TaALMT1* gene might exert the main control of the Al response in CAR3911.

To confirm the presence of the *TaALMT1* gene in CAR3911 and characterize its promoter structure, markers ALMT1-4 and primer sets 1 and 2 were used (Sasaki *et al.* 2006). The control genotypes Carazinho, ET8, Chinese Spring, and ES8 showed the expected pattern types (Fig. 3). The marker ALMT1-4 amplified 986 bp products in the Al-tolerant genotypes (type V) and 426 bp products in the Al-sensitive ones (type I). Chinese Spring is considered Al-intermediate (type III); it showed both 1 229 and 426 bp products (Fig. 3). On the other hand, CAR3911 showed the type V. The analysis of the 1.7-kb promoter sequence from CAR3911 revealed 100 % homology with *TaALMT1* and the identical promoter structure as that observed in ET8 (Fig. 1 Suppl.). The sequence has been deposited in GenBank with acc. No. KF245637.

Among the 70 BC_1 lines, the marker ALMT1-4 distinguished unequivocally Al-tolerant (n = 34) and Al-sensitive (n = 36) genotypes, correlating 100 % of the root re-growth response with patterns V (Al-tolerant) and I (Al-sensitive) (data not shown). Among the 34 Al-tolerant BC_1 individuals assessed on 4D, only BC_1-14 and BC_1-28 showed recombination events in the flanking markers WMC331 and WMC457 with an introgressed segment of 6 cM. Based on the graphical genotype analysis, the rate of RPG recovered on 4D ranged from 16.3 % (BC_1-9) to 80 % (BC_1-14) (Fig. 2*C*). The BC lines BC_1-14 and BC_1-28 were selected for the background (remaining 20 chromosomes) RPG recovery analysis using 151 polymorphic SSRs from the consensus linkage map constructed by Somers *et al.* (2004). The line

BC_1-14 exhibited 70, 71, and 77 % of background RPG recovered on A, B, and D genomes, respectively, with an average of 72.6 %, whereas the line BC_1-28 recovered 68, 72, and 74 % of background RPG on A, B, and D genomes, respectively, with an average of 71.3 %. Thus, the lines BC_1-14 and BC_1-28 were backcrossed to Kumpa-INIA to produce the BC_2 generation.

Forty-eight Al-tolerant BC_2 lines derived from BC_1-14 carried the type V promoter. The lines BC_2-14-10 (A: 88.4 %, B: 81.8 %, D: 89.9 %) and BC_2-14-1 (A: 82.6 %, B: 86.4 %, D: 86.3 %) recovered average RPGs of 86.7 and 85.1 %, respectively, being the highest among the BC_2 lines. The line BC_2-14-10 showed a major percentage of RPG on the A and D genome than $BC_2$14-1 and, as a result, backrossed to generate the BC_3 population.

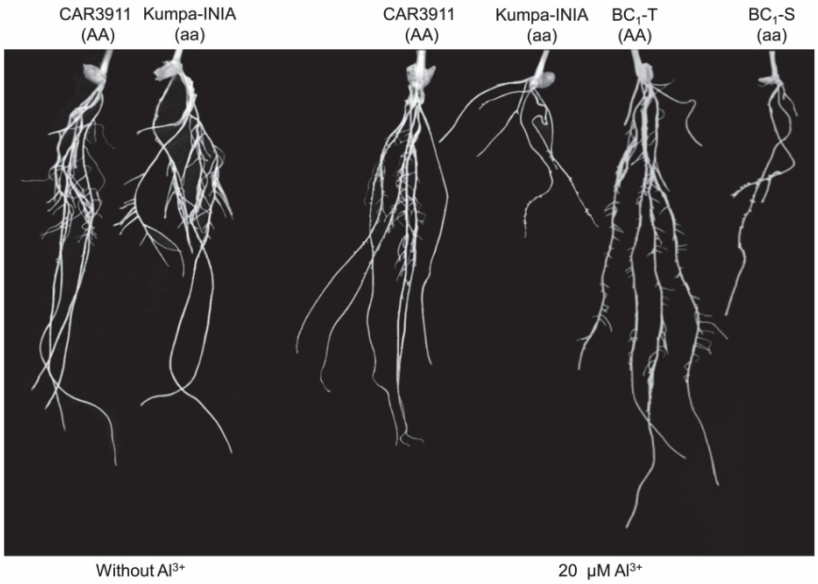

Fig. 1. Al-induced root growth inhibition when CAR3911, Kumpa-INIA, BC_1-T, and BC_1-S lines were exposed to 20 µM Al for 6 d and recovery at 10 µM Al for 6 d using the pulse-recovery method of Raman *et al.* (2002).

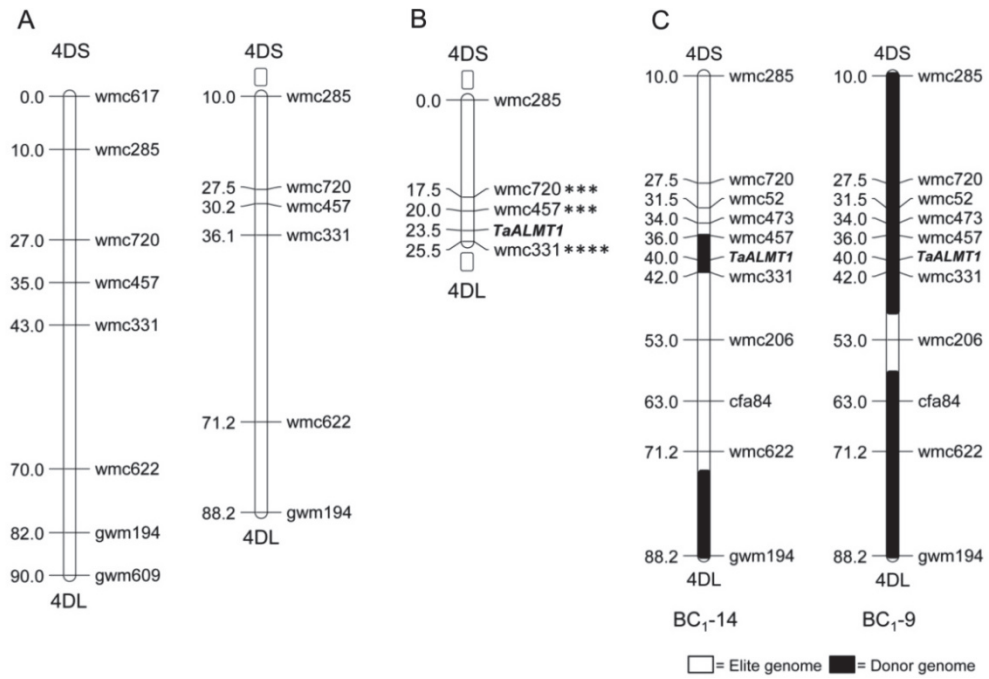

Fig. 2. A genetic linkage map in Kumpa-INIA × CAR3911 and location of the ALMT1 locus on chromosome 4D. Collinearity between the Kumpa-INIA × CAR3911 linkage map on 4D and the consensus linkage map of Somers *et al.* (2004). *A* - Bulk Segregant Analysis-based linkage map showing SSRs significantly associated with RRE and Al tolerance. *B* - Percentage of RPG (elite = Kumpa-INIA) recovered on 4D as assessed by MAS-BC in BC_1 lines (donor = CAR3911). *C* - BC_1-14 shows a higher percentage of RPG compared with BC_1-9. *** - $P < 0.001$, **** - $P < 0.0001$.

Fifty-six Al-tolerant BC_3 lines derived from BC_2-14-10 carried the type V promoter. The best candidate line was BC_3-14-10-28 (A: 97.1 %, B: 100 %, D: 96.9 %) which averaged a background RPG of 98 % (Fig. 4). The most significant progress was observed on B genome with 18.2 % of RPG recovered between the BC_2 and BC_3 generations. However, chromosome 5A maintained a heterozygous segment on the marker WMC475. Subsequently, the line BC_3-14-10-28 was allowed to self-pollinate to generate a homozygous BC_3F_2 Al-tolerant line that was named Kumpa-INIA-*TaALMT1*.

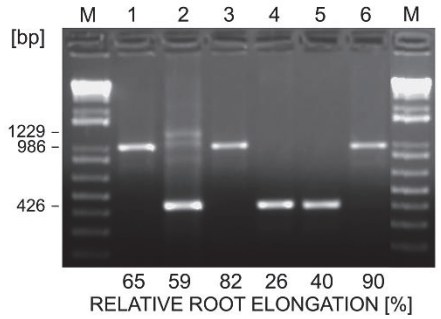

Fig. 3. PCR products generated from the *TaALMT1* upstream coding region using primer sets 4 (Sasaki *et al.* 2006). Polymorphic products were observed between Al-tolerant cultivars (pattern V, 986 bp) and Al-sensitive (pattern I, 426 bp) in an agarose gel. M - 1 kb plus DNA ladder, 1 - ET8, 2 - Chinese Spring, 3 - Carazinho, 4 - ES8, 5 - Kumpa-INIA, 6 - CAR3911.

Aluminium tolerance was determined hydroponically using Kumpa-INIA-*TaALMT1*, Kumpa-INIA, and CAR3911. The line Kumpa-INIA-*TaALMT1* expressed similar RRE to CAR3911 in all the Al treatments (Table 2). RRE of Kumpa-INIA was inhibited as in the previous evaluation (Table 1) even at 5 μM Al. RRE of CAR3911 (76.9 %) and Kumpa-INIA-*TaALMT1* (75.2 %) were not statistically different even at 40 μM Al. This result indicates that the Al-tolerance from CAR3911 was completely transferred to Kumpa-INIA indicating that *TaALMT1* is likely the major gene conferring Al tolerance in non-Asian wheat germplasm.

Table 2. Evaluation of Al tolerance of Kumpa-INIA-*TaALMT1*, its isogenic line Kumpa-INIA, and CAR3911 (control) exposed to six Al concentrations for 72 h and measured as root length and RRE. Means ± SD (n = 10). Means with common letters are not significantly different at $P > 0.05$, according to the LSD test.

Al [μM]	Genotype	Root length [cm]	RRE [%]
0	Kumpa-INIA-*TaALMT1*	7.26 ± 1.46	100.0a
	CAR3911	7.01 ± 1.22	100.0a
	Kumpa-INIA	5.68 ± 0.99	100.0a
5	Kumpa-INIA-*TaALMT1*	7.13 ± 2.02	98.2a
	CAR3911	7.22 ± 1.63	102.9a
	Kumpa-INIA	3.80 ± 1.03	66.9b
10	Kumpa-INIA-*TaALMT1*	7.29 ± 1.31	100.4a
	CAR3911	7.33 ± 0.95	104.5a
	Kumpa-INIA	3.32 ± 0.86	58.5b
15	Kumpa-INIA-*TaALMT1*	6.59 ± 1.56	90.8a
	CAR3911	6.46 ± 1.71	92.2a
	Kumpa-INIA	2.98 ± 1.24	52.4b
20	Kumpa-INIA-*TaALMT1*	6.18 ± 1.28	85.1a
	CAR3911	6.23 ± 2.04	88.9a
	Kumpa-INIA	2.39 ± 1.37	42.0b
40	Kumpa-INIA-*TaALMT1*	5.46 ± 1.55	75.2a
	CAR3911	5.39 ± 2.12	76.9a
	Kumpa-INIA	2.24 ± 0.97	39.4b

Discussion

Al toxicity is a main yield-limiting factor in crops, threatening sustainable food production in many developing countries (Ryan *et al.* 2010). In wheat, Al-tolerance is a multigenic trait with at least three QTL on 4DL, 4BL, and 3BL chromosomes (Riede and Anderson 1996, Papernik *et al.* 2001, Ma *et al.* 2005, Zhou *et al.* 2007, Cai *et al.* 2008). However, in a wide range of wheat genotypes, the *TaALMT1* gene located on 4DL explains a large proportion of the Al-tolerance variation (Sasaki *et al.* 2004, 2006, Ryan *et al.* 2010). In this study, we report the characterization of the Al-tolerance in the breeding line CAR3911 and its introgression into the Al-sensitive, high yielding winter cultivar Kumpa-INIA. The phenotypic and molecular characterizations suggest that the Al-tolerance in CAR3911 could be controlled by the *TaALMT1* gene on 4DL as reported in other Al-tolerant cultivars (Delhaize *et al.* 1991, Riede and Anderson 1996, Papernik *et al.* 2001, Raman *et al.* 2005, Zhou *et al.* 2007). Interestingly, CAR3911 and Carazinho expressed similar and higher RRE than ET8, even though CAR3911 and ET8 shared the identical promoter structure across the 1.7-kb sequence (Fig 1. Suppl.). The pedigree information of CAR3911 is unclear but we can speculate that it could be of Brazilian origin because most of the Al-tolerant Brazilian landraces and their derivatives have been used in wheat breeding programs worldwide (Raman *et al.* 2008), and common to that genetic base is the Al-activated malate release mechanism (Zhou *et al.* 2007). If that is correct, CAR3911 could harbor the citrate efflux mechanism present in Carazinho, which could explain its superior RRE compared to ET8 which does not exude citrate. Ryan *et al.* (2009) observed similar differences in RRE as we observed among Carazinho and cultivars lacking citrate efflux, such as ET8 and Chinese Spring. Interestingly, Kumpa-INIA-*TaALMT1* expressed similar RRE to CAR3911 in all the Al treatments (Table 2) even though the genomic region

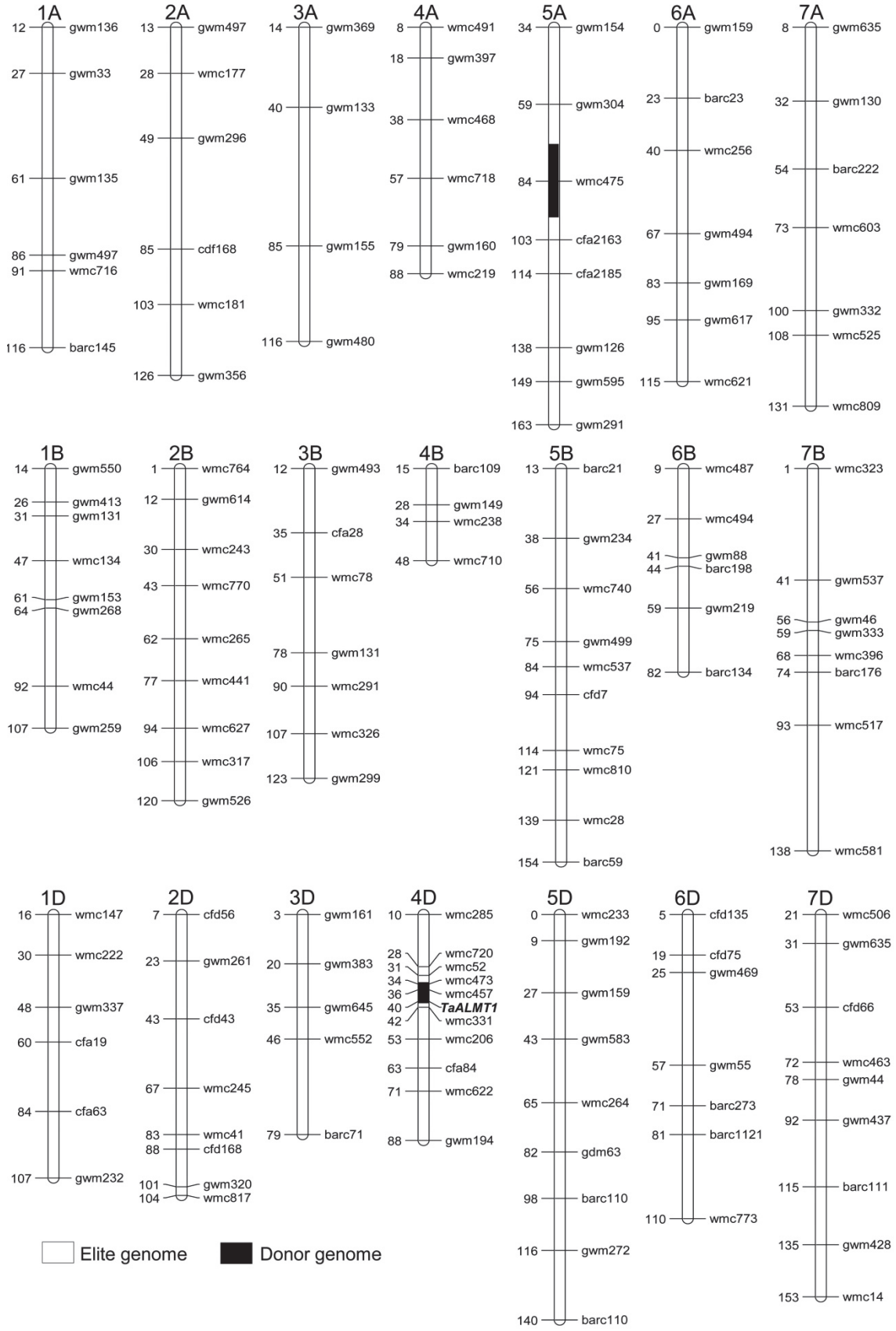

Fig. 4. Graphical representation of the 21 chromosomes of the Al-tolerant BC_3-14-10-28 line with 98 % of RPG.

where TaMATE1B resides (4B) was not introgressed from CAR3911. Indeed, Kumpa-INIA showed a superior Al tolerance compared to ES8 (Table 1) suggesting that Kumpa-INIA could also possess the TaMATE1B locus that controls citrate secretion. Therefore, full-length sequencing the *TaMATE1B* gene from CAR3911 and Kumpa-INIA will be needed in the future.

Landraces are important sources of stress-related genes for genetic improvement of modern cultivars, but generally, their poor overall agronomic performance involves the transfer of undesirable alleles using conventional backcrossing. For example, Carazinho and Atlas 66 have many undesirable agronomic traits that complicate their use as donors of Al-tolerance because of the risk of linkage drag (Zhou *et al.* 2007). On the other hand, CAR3911 compared with Carazinho possesses a superior agronomic performance and tolerance to diseases, especially yellow rust, and is therefore a more suitable donor parent of Al-tolerance for the elite cultivar Kumpa-INIA. Nevertheless, the fact that both parents have been bred by the same breeding program reduced significantly the percentage of polymorphic markers (41 %) probably because of their similar pedigrees.

The 4D linkage map constructed in this study allowed the accurate selection of Al-tolerant BC lines. The marker WMC331 exhibited a tight linkage to the *TaALMT1* gene in other mapping populations (Ma *et al.* 2005, Raman *et al.* 2005, Zhou *et al.* 2007), and our study validated its utilization as a predictive marker along with ALMT1-4 and WMC457. Their combined utilization facilitated the identification of rare recombination events flanking the *TaALMT1* gene with a minimal linkage drag and a higher RPG recovery in the BC populations (Figs. 2 and 4). MAS-BC allowed recovering 98 % of RPG within 24 months taking into account that Kumpa-INIA is a winter cultivar requiring at least 45 d of vernalization. Comparable results could be only achieved after 20 generations of conventional backcrossing (Ribaut and Hoisington 1998, Bertrand *et al.* 2008).

Unexpectedly, the rate of RPG recovered was smaller in the BC_1 population (on average 31-72 in various lines) than the predicted average of 75 %. Possibly, loci separated 40 cM away or even less caused the under-/over-estimation of RPG. For example, when two heterozygous loci are located at a 40 cM distance, the GGT analysis will consider the complete chromosomal segment as heterozygous (Servin and Hospital 2002). To overcome this limitation, it is recommended to include markers distributed every 20 cM, which minimizes the probability of double crossover (Servin and Hospital 2002). As result of the low polymorphism between CAR3911 and Kumpa-INIA in the study presented here, several genomic regions will need additional loci (such as those located on chromosomes 2A, 3A, and 7B). Nevertheless, this does not undervalue our MAS-BC strategy that was demonstrated to be a better alternative than conventional backcrossing.

In summary, we show that the promoter region of the *TaALMT1* allele from CAR3911 was identical to ET8 suggesting that this gene likely underlied the Al-tolerance mechanism in CAR3911. We validated the usefulness of ALMT1-4, WMC331, and WMC457 as predictive markers for the conversion of Kumpa-INIA into an Al-tolerant cultivar and we demonstrated that MAS-BC was more efficient and effective than conventional backcrossing. Currently, seed multiplication of Kumpa-INIA-*TaALMT1* is under way in order to evaluate its Al-tolerance in multi-location field trails.

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