Theor Appl Genet (2016) 129:1317–1331
DOI 10.1007/s00122-016-2705-3

ORIGINAL ARTICLE

Alleles of organic acid transporter genes are highly correlated with wheat resistance to acidic soil in field conditions

Jorge G. Aguilera[1] · João A. D. Minozzo[1,2] · Diliane Barichello[1,4] · Claúdia M. Fogaça[1,3] · José Pereira da Silva Jr.[1] · Luciano Consoli[1] · Jorge F. Pereira[1]

Received: 17 December 2015 / Accepted: 6 March 2016 / Published online: 23 March 2016

Abstract

Key message ***TaALMT1*** **and** ***TaMATE1B*** **promoter alleles are highly correlated with wheat growth in acidic soil with a high concentration of toxic aluminium.**

Abstract The aluminium (Al^{3+}) resistance of 338 wheat genotypes with different geographic origins was correlated with morphological traits and *TaALMT1* and *TaMATE1B* alleles. Both of these genes encode malate and citrate transporters associated with Al^{3+} resistance mechanisms in wheat. Based on comparisons with the sensitive and resistant controls, the relative root growth was evaluated in hydroponic experiments and the plant performance was visually accessed in the field. The correlation between Al^{3+} tolerance in the hydroponic and field tests was moderate ($r = 0.56$, $P < 0.001$). Higher selection pressure was observed in the field because a smaller number of genotypes was classified as resistant. The combination between the six *TaALMT1* alleles and the two *TaMATE1B* promoters allowed the identification of 11 haplotypes that showed a high ($r = 0.71$, $P < 0.001$) correlation with Al^{3+} resistance in the field, with the *TaALMT1* alleles accounting for most of the correlation. The Brazilian wheat genotypes presented the best performance in soil, including eight cultivars with promoters usually associated with Al^{3+} resistance and another six genotypes classified as moderately resistant but containing alleles usually associated with Al^{3+} sensitivity. Although an increase in favourable alleles was observed over the past few decades, the average Al^{3+} resistance in the field was not significantly different from that of older cultivars. The ease identification of the *TaALMT1* and *TaMATE1B* alleles and their higher association with Al^{3+} resistance along with the best genotypes identified here may be used for wheat-breeding programmes interested in increasing wheat Al^{3+} resistance.

Introduction

Aluminium is a common metal in the Earth's crust that is usually found in insoluble forms when the soil pH is neutral or slightly acidic. However, when the soil pH drops below 5.0, the solubility of aluminium increases and toxic aluminium ions (especially the trivalent cation Al^{3+}) can limit root growth and negatively interfere with water and nutrient uptake, consequently leading to reduced crop yields due to drought and mineral deficiencies (Kochian et al. 2015). Plant species have developed different mechanisms to cope with Al^{3+} toxicity. The most studied mechanism is the exudation of organic acids from the root apex. Once in the rhizosphere, the organic acids chelate Al^{3+}, thereby reducing its toxicity (Ryan et al. 2001).

Communicated by P. Langridge.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-016-2705-3) contains supplementary material, which is available to authorized users.

✉ José Pereira da Silva Jr.
jose.silva-junior@embrapa.br

[1] Embrapa Trigo, Rodovia BR 285 km 294, Passo Fundo, RS 99050-970, Brazil

[2] Universidade de Passo Fundo, Rodovia BR 285, Passo Fundo, RS 99052-900, Brazil

[3] Fundação Estadual de Pesquisa Agropecuária, RSC 470 km 170, Veranópolis, RS 95330-000, Brazil

[4] Instituto Agronômico do Paraná, Rodovia Celso Garcia Cid km 375, Londrina, PR 86047-902, Brazil

Although increments in organic acid production by plant cells have been associated with increased Al^{3+} resistance in plants (de la Fuente et al. 1997; Barone et al. 2008), organic acid transport out of the root cells has a greater association with Al^{3+} resistance (Sasaki et al. 2004, 2006; Raman et al. 2008; Pereira et al. 2010; Zhou et al. 2013, 2014). In wheat (*Triticum aestivum* L.), two genes (*TaALMT1* and *TaMATE1B*) that encode organic acid transporters were demonstrated to play a role in Al^{3+} resistance. *TaALMT1* is located in chromosome 4D and encodes a malate transporter belonging to the ALMT (*aluminium-activated malate transporter*) family that is expressed in the wheat root tip (Sasaki et al. 2004). *TaMATE1B* is located on chromosome 4B and encodes a citrate transporter of the MATE (*multi-drug and toxin extrusion*) family that is expressed in the root tip depending on the allele (Ryan et al. 2009; Tovkach et al. 2013). Both genes exhibit a correlation between the level of expression, the amount of organic acid exudate by the roots and the level of Al^{3+} resistance. The gene expression level is regulated by sequences in the promoter region (Sasaki et al. 2004, 2006; Ryan et al. 2009; Tovkach et al. 2013). For instance, the level of *TaALMT1* expression is correlated with tandem repeats in the promoter (named here I to VII). Promoters containing a higher number of repeats (V and VI) have higher levels of gene expression and are usually Al^{3+} resistant, whereas promoters without repeats (I and II) have low levels of gene expression and are typically Al^{3+} sensitive (Sasaki et al. 2006; Raman et al. 2008). The *TaMATE1B* gene expression level is correlated with two polymorphisms in the promoter region (Tovkach et al. 2013). The first polymorphism is a single nucleotide polymorphism (SNP) that is responsible for a two-fold increase in gene expression and the second is a transposon-like insertion that is associated with a 20-fold increase in *TaMATE1B* expression. In fact, the transposon-like insertion not only increases but also changes the location of the expression to the root apex, thereby increasing the citrate efflux and consequently Al^{3+} resistance (Tovkach et al. 2013).

The repeats located in the *TaALMT1* promoter and the insertion in the *TaMATE1B* promoter can be easily detected by PCR. The allelic variations in these promoter regions have been studied in a number of genotypes and have revealed interesting aspects of Al^{3+} resistance in wheat (Sasaki et al. 2006; Raman et al. 2008; Han et al. 2013; Garcia-Oliveira et al. 2014; Pereira et al. 2015). For instance, wheat lines of Japanese origin do not show a clear correlation between the number of repeats in the *TaALMT1* promoter region and Al^{3+} resistance; this finding is in contrast to the effects observed with lines of non-Japanese origin (Sasaki et al. 2006). An Al^{3+}-tolerant Brazilian cultivar named Trintecinco was shown to possess *TaALMT1* allele II (usually associated with sensitivity) (Raman et al. 2008), whereas the majority of the Brazilian wheat cultivars contains promoters V and VI (typically associated with Al^{3+} resistance) (Pereira et al. 2015). *TaALMT1* allele V was less frequently identified in a collection of cultivars and landraces of Chinese wheat, whereas promoter VI was not detected and allele III was identified only in Chinese and Brazilian wheat to date (Han et al. 2013; Pereira et al. 2015). *TaALMT1* promoter VII is rarely found; indeed, it has been detected in only three genotypes to date (Raman et al. 2008; Pereira et al. 2015). Moreover, the presence of the transposon-like insertion in the *TaMATE1B* promoter is less frequent in Brazilian wheat genotypes but is widespread among Portuguese wheat cultivars (Garcia-Oliveira et al. 2014; Pereira et al. 2015). This insertion appears to be important for the root growth of some genotypes analysed in short-term soil experiments. However, most genotypes presenting the insertion in the *TaMATE1B* promoter did not outperform the genotypes without the insertion that possessed the same *TaALMT1* allele (Pereira et al. 2015). Therefore, evaluating the allelic distribution of the *TaALMT1* and *TaMATE1B* promoters is crucial to increase our knowledge concerning the contribution of the different alleles to wheat Al^{3+} resistance. Moreover, the *TaALMT1* and *TaMATE1B* alleles have been associated with only Al^{3+} resistance in hydroponics or in short-term soil experiments to date. No correlation has been described between these alleles and wheat plants growing in acidic soils under field conditions.

Phenotyping using hydroponics is widely used to evaluate a number of traits including Al^{3+} resistance because this method is fast, non-destructive and allows greater control over the environmental conditions (Shavrukov et al. 2012). Indeed, phenotyping for Al^{3+} resistance in Brazilian wheat has typically been performed under hydroponic conditions (Camargo et al. 1987, 1998, 1999, 2006). Studies assessing Al^{3+} resistance under field conditions for a large number of Brazilian wheat genotypes are rare (de Sousa 1998) even though field evaluation in acidic soils is more realistic. The downside is that soil evaluation presents issues related to non-uniform nutrient distribution, uncontrollable factors (i.e., drought or excess heat) and Al^{3+} toxicity occurring together with calcium deficiency and manganese toxicity. A high correlation for Al^{3+} resistance phenotyping between field and hydroponic conditions has been reported for wheat (Baier et al. 1995); however, the genotypes evaluated under the field and hydroponic conditions differed in that study. To obtain a clear correlation, the same genotypes need to be used for the comparison of Al^{3+} resistance phenotyping between different methods.

In Brazil, acidic soils are predominant in wheat-growing areas (Echart and Cavalli-Molina 2001), and Brazilian wheat genotypes have been considered good sources of Al^{3+} resistance. Some genotypes have been used in

Table 1 Geographical origin of the 338 wheat genotypes evaluated in this work

Geographical region	Country
South America (228)	Brazil (213), Ecuador (2), Argentina (5), Uruguay (1), Peru (2), Paraguay (1), Colombia (2), Chile (1), Bolivia (1)
North and Central America (60)	Mexico (45), Canada (10), USA (4), Guatemala (1)
Africa (8)	South Africa (2), Tunisia (1), Kenya (2), Ethiopia (1), Egypt (1), Zimbabwe (1)
Europe (13)	Italy (2), Greece (2), Portugal (1), Romania (1), Netherlands (1), Iceland (1), Switzerland (1), Norway (1), Ukraine (1), Germany (1), Turkey (1)
Asia (21)	Japan (5), Pakistan (2), India (3), China (9), Afghanistan (1), Nepal (1)
Oceania (5)	New Zealand (1), Australia (4)
Middle East (3)	Israel (2), Syria (1)

different breeding programmes worldwide to develop new lineages, including BH 1146, Carazinho, Cotiporã, Frontana, IAC 5—Maringá and Trintecinco (Hettel 1989). BH 1146, Frontana, IAC 5—Maringá, and Trintecinco are among the most common genotypes used by Brazilian breeders (de Sousa and Caierão 2014). Moreover, a number of the Brazilian cultivars exhibiting Al^{3+} tolerance have PG1 as a common ancestor. PG1 is a selection of Polyssú that is one of the parents of Fronteira, which is also used as a good source of Al^{3+} resistance (de Sousa 1998). The constant use of the same or similar sources in breeding programmes can result in a lower genetic basis for Al^{3+} resistance. Phylogenetic analysis using microsatellite markers revealed similarities among some of the Brazilian cultivars used as Al^{3+} resistance sources, and these genotypes were most likely to share similar resistance/tolerance mechanisms (Pereira et al. 2015). Thus, the phenotyping of a large collection of wheat materials can result in the identification of sources containing potential new alleles/mechanisms that could be useful for breeders.

In this context, the goal of this work was to correlate the *TaALMT1* and *TaMATE1B* allelic variability with wheat resistance to toxic aluminium under hydroponic and field conditions. We used a diverse wheat collection representing different countries and more than 90 years of breeding. Our goal also extended to the identification of resistant sources not commonly used by breeders, the comparison of hydroponic and field data and the evaluation of the *TaALMT1* and *TaMATE1B* alleles over time to associate their frequency with Al^{3+} resistance over the past nine decades.

Materials and methods

Plant materials

A total of 338 wheat accessions were used in this study, including commercial cultivars, landraces, synthetic hexaploid wheats and varieties from the Embrapa Trigo (Brazilian Agricultural Research Corporation in Passo Fundo, Rio Grande do Sul, Brazil) breeding program; these accessions represented 40 countries belonging to five continents and are presented in Table 1 and Table S1. All analyses, including the molecular and phenotypic evaluations, were performed after accession purification and multiplication of one selected seed. The Al^{3+} resistance evaluation was performed in nutrient solution and in the field using cultivar IAC 5-Maringá as the resistant control and Anahuac 75 as the sensitive control.

Evaluation of aluminium resistance in nutrient solution

Al^{3+} resistance was evaluated by measuring the relative root growth (RRG) after 4 days. Wheat seeds were surface-sterilized with a 0.2 % NaClO solution for 5 min and incubated in the dark at 23 °C for 48 h. Twelve germinated seeds with roots that were approximately the same size (5–10 mm) and had similar viability were transferred to an aerated nutrient solution (pH 4.0) consisting of 0.40 mM $CaCl_2{\cdot}2H_2O$, 0.65 mM KNO_3, 0.25 mM $MgCl_2{\cdot}6H_2O$, 0.04 mM NH_4NO_3, and 0.01 mM $(NH_4)_2SO_4$ and grown for 4 days at 23 °C under a 12/12 h light/dark cycle in a growth cabinet. Two treatments were used: control (without $AlCl_3{\cdot}6H_2O$) and stressed (74 µM Al added in the form of $AlCl_3{\cdot}6H_2O$) as described by Voss et al. (2006). The nutrient solution was changed every 24 h to minimize changes in the pH and Al^{3+} concentration. The length of the main root was measured at the end of the experiment. The RRG for each genotype was estimated to be 100× (RLT/RLC), where RLT represent the root length under the Al^{3+} treatment and RLC was the root length in the control solution. Errors associated with deriving the RRG were calculated as $SE_{RRG} = RRG\ [(SEx/x)^2 + (SEy/y)^2]^{1/2}$, where x represented the mean RLC and y was the mean RLT. For all experiments, the RRG of each genotype was compared to the RRG of the resistant and sensitive controls to determine

the classification index of aluminium toxicity in hydroponics (CIATH); this step allowed the genotypes to be separated into four classes: sensitive (S); moderately sensitive (MS); moderately resistant (MR); and resistant (R). The limits for each class were calculated as follows: limit inferior for R and superior for MR [RRG resistant control—(0.5 × ((RRG resistant control—RRG sensitive control)/3))]; limit inferior for MR and superior for MS [RRG resistant control—(1.5 × ((RRG resistant control—RRG sensitive control)/3))]; and limit inferior for MS and superior for S [RRG sensitive control + (0.5 × ((RRG resistant control—RRG sensitive control)/3))].

Evaluation of aluminium resistance in the field

The reaction of the genotypes to Al^{3+} toxicity under field conditions was evaluated over a 2 year period (2012 and 2013) at Embrapa Trigo's experimental field (latitude 28°15′S, longitude 52°24′W). The soil pH of the test areas, measured in water, ranged from 4.3 to 4.7 and the soil contained 29 to 54 mmol/dm^3 Al^{3+} during the 2 years of the test. The soil was classified as Haplorthox and contained ~45 % clay. The chemical soil analysis showed the following nutrient contents: P, 17.4 mg/dm^3; K, 118.5 mg/dm^3; Ca, 5.98 mmol/dm^3; Mg, 2.7 mmol/dm^3; and organic matter, 25.25 g/dm^3. The percentage of saturation of CTC-Al was 75 to 87.9 %. The genotypes were planted in a randomized complete block design with plots consisting of six rows that were 3 m in length with three replications containing five different material lines and one line formed by one of the controls placed alternately in every plot. The plant response was evaluated visually at different growth stages (tillering, silking and maturation). The evaluation method was based on de Sousa (1998) with some modifications. The field scores were as follows: 0.5, outstanding resistance; 1, resistant: good vegetative development, normal and abundant tillers, and normal spikes; 2, moderately resistant: normal plant, slightly less vigorous and fewer tillers, and slightly impaired development compared with the previous group; 3, moderately susceptible: intermediate or deficient plant growth and normal spikes; 4, susceptible: deficient plant growth, no tillering and small spikes; and 5, highly susceptible: dead plant or very deficient development and no spikes. We estimated the overall average (AVE) based on the overall average of the three observations over the 2 year period; the AVE was used to determine the classification index of aluminium toxicity in the field (CIATF) as follows: 0.05–1.25, resistant (R); 1.26–2.5, moderately resistant (MR); 2.51–3.75, moderately susceptible (MS) and 3.76–5.00 susceptible (S) as proposed by de Sousa (1998).

DNA extraction and *TaALMT1* and *TaMATE1B* allelic variability

Leaves were collected from all genotypes (~150 mg), transferred to a 2 ml Eppendorf tube containing three stainless steel beads (2.3 mm diameter), immediately frozen in liquid nitrogen and triturated in a Mini-Beadbeater™ (Biospec Products) for 2 min. Total DNA was extracted using a CTAB-based protocol (Doyle and Doyle 1987). After quantification in an agarose gel, 100–150 ng of DNA was used for the PCR. The *TaALMT1* promoter alleles were detected using the primers LPF-F (CCTGGTTTTCTTGATGGGGGCACA) and LPF-R (TGCCCACCATCTCGCCGTCGCTCTCTCT) as described by Sasaki et al. (2006). Another amplification with the primers SPF-F (GCTCCTACCACTAT GGTTGCG) and SPF-R (CCAGGCCGACTTTGAGCG AG) was performed in cases where allele I or II was identified. To detect the *TaMATE1B* alleles, the primers TaMATE1-4B-SLT-F (ATCCATCCTCCTTCCCTCAC) and TaMATE1-4B-SLT-R (ATGAATGCTGTGTCCA CCAA) were used according to Garcia-Oliveira et al. (2014); the presence of the PCR fragment was associated with the presence of the transposon insertion in the *TaMATE1B* promoter region. All amplification reactions were performed in a final volume of 20 µl containing 1 × PCR buffer, 0.30 mM of each dNTP, 1 × Q solution, 0.3 µM of each primer, 1 U Taq DNA polymerase (Qiagen) and 100 ng of DNA. The amplification programs were performed as described by Sasaki et al. (2006) and Garcia-Oliveira et al. (2014). The amplicons were separated by electrophoresis and visualized in a 1 % agarose gel (LPF primers), 1.25 % agarose gel (SPF primers) or 1.5 % agarose gel (*TaMATE1B*). The gels were stained with ethidium bromide.

Statistical analyses

The data obtained from the different growth stages (tillering, silking and maturation) and the overall average (AVE) were subjected to analysis of variance (ANOVA). The means were compared by the Scott and Knott ($P < 0.01$) test. Data averages from tillering, silking, maturation, AVE, CIATF, RRG, CIATH, the different *TaALMT1* and *TaMATE1B* alleles and the combinations of alleles (haplotypes) were employed in the correlation analysis and principal components analysis (PCA) to estimate the relative contribution of characteristics to the diversity according to the method of Singh (1981) using the GENES program (Cruz 2006) and SAS package (SAS Institute 1997).

Results

Al^{3+} resistance in nutrient solution

We used hydroponics to evaluate the RRG of the genotypes. The duration (4 days) and stress level (74 μM $AlCl_3$) of the treatment reduced the root length in some genotypes but did not substantially reduce the root length in others. On average, the RRG was 52.18 % with a range from 23.3 % in Ocepar 7—Batuira to 95.3 % in Fundacep 30 (see the frequency distribution in Fig. 1). The genotypes used as the tolerant and sensitive controls were evaluated in each experiment to allow the comparison of the RRG between them and the tested plants. In this way, the RRG of each genotype was compared to the RRG of the resistant and sensitive controls to determine the classification index of aluminium toxicity in hydroponics (CIATH). This index allowed the separation of the genotypes into four categories: 106 resistant (R), 64 moderately resistant (MR), 84 moderately susceptible (MS) and 84 susceptible (S) (Table 2). The genotypes classified as R included 91 materials from Brazil representing 85.8 % of the resistant materials and 15 materials (14.2 %) from different origins. Among the MR genotypes, 47 (73.4 %) were from Brazil and 17 (26.6 %) were from different continents. For the MS genotypes, 49 (58.3 %) were Brazilian and 35 (41.7 %) had different origins. Among the S genotypes, 26 (31.0 %) were from Brazil and 58 (69.0 %) had different origins (Table 2).

Al^{3+} resistance in acidic soil

Wheat plants were evaluated for two successive years in acidic soil. The evaluation was based on visual characteristics in three different growth stages (tillering, silking and maturation). A highly significant difference ($P < 0.001$, ANOVA) was obtained for the genotypes, which demonstrated the high rate of diversity in the responses among the genotypes as observed in the groups for the Scott-Knott test ($P < 0.01$) (Supplementary file 1). The two wheat cultivars used as the controls showed consistency in their reactions (see the frequency distribution in Fig. 1). The averages of the grades were 2.97 [ranging from 0.82 (IAC-21—Iguaçú) to 5.41 (Wadhanak)] for tillering, 3.02 [ranging from 0.75 (Toropi) to 5.25 (CIGM 93.395)] for silking, 2.88 [ranging from 0.57 (Minuano 82) to 5.02 (Emb_07105)] for maturation, and 2.96 [ranging from 0.78 (Toropi) to 4.89 (CIGM 92.1712)] for the AVE (Supplementary file 1). The high Pearson correlation coefficient ($r > 0.86$; $P < 0.001$) obtained between these traits indicated that the Al^{3+} resistance evaluation could be practiced at any of these stages (Table 3).

Using the overall average of the field evaluation (AVE), a classification index for aluminium toxicity in the field (CIATF) was obtained to allow the separation of the genotypes into four classes. In this index, scores closer to zero represented genotypes with higher Al^{3+} resistance. The CIATF showed a high correlation ($r > -0.88$; $P < 0.001$) with tillering, silking, maturation and the AVE (Table 3). Only eight genotypes were classified as resistant (Trintecinco, Toropi, PG1, BH 1146, Aceguá, C33, IAC-21—Iguaçú and PAT 7219), all of which were from Brazil; with the exception of Trintecinco and Aceguá, all cultivars shared Polyssú as their common ancestor. Among these genotypes, the AVE ranged from 0.78 to 1.25 with an average of 1.06. The numbers of moderately resistant, moderately susceptible and susceptible genotypes were much higher than the numbers observed in the hydroponic experiments, with 122 genotypes classified as MR, 117 as MS and 91 as S (Table 2). The materials classified as MR included 114 genotypes from Brazil (93.4 % of the MR materials) and only eight (6.6 %) with different origins, including one for each of the following countries: Japan (Aburakomugi, grade 2.0), Israel (Bet Dagan 131, grade 1.7), USA (Escondido 41, grade 1.8), Egypt (Giza, grade 1.3), Argentina (Klein Lucero, grade 1.3), Ukraine (Mironovskaya Yubileinaya 50, grade 1.9), Guatemala (Maya 74, grade 1.7) and Turkey (Menceki, grade 1.5). The MS genotypes contained 82 (70.0 %) Brazilian materials and 35 (30.0 %) materials with different origins; Mexico and Japan were the most represented countries with 10 and four genotypes, respectively. The materials categorized as S included nine (9.9 %) Brazilian genotypes and 82 (90.1 %) materials from different origins; Mexico was the most represented origin with 35 genotypes (31 were synthetic wheat). The Brazilian genotypes represented 63.0 % of the genotypes evaluated in this study and corresponded to 93.8 % of the total number of R/MR genotypes from the field evaluation, indicating the importance and superiority of these resistance sources for breeding programmes (Table 2).

The distribution obtained in the different CIATF classes showed that the soil classification exerted a greater selection pressure for Al^{3+} resistance because the number of R genotypes in the field (eight) was lower than the 106 R genotypes obtained from the hydroponic experiments. Indeed, all R genotypes from the field test were also classified as R in the hydroponic experiments; the other genotypes classified as R in the hydroponic experiments were distributed in the four classes of the field response (Supplementary file 2A and 2B). Similarly, 31 among the 122 materials classified as MR in the field maintained their classification in the hydroponic experiments, whereas 65 genotypes were classified as R and 26 as MS or S. This variation in the classification between the field (CIATF) and hydroponic (CIATH) tests resulted in a moderate Pearson correlation of 0.56 ($P < 0.001$) (Table 3). Among the 26 materials classified as MS or S in the hydroponic experiments but as MR in the field, the scores in the first growth stage (tillering) were

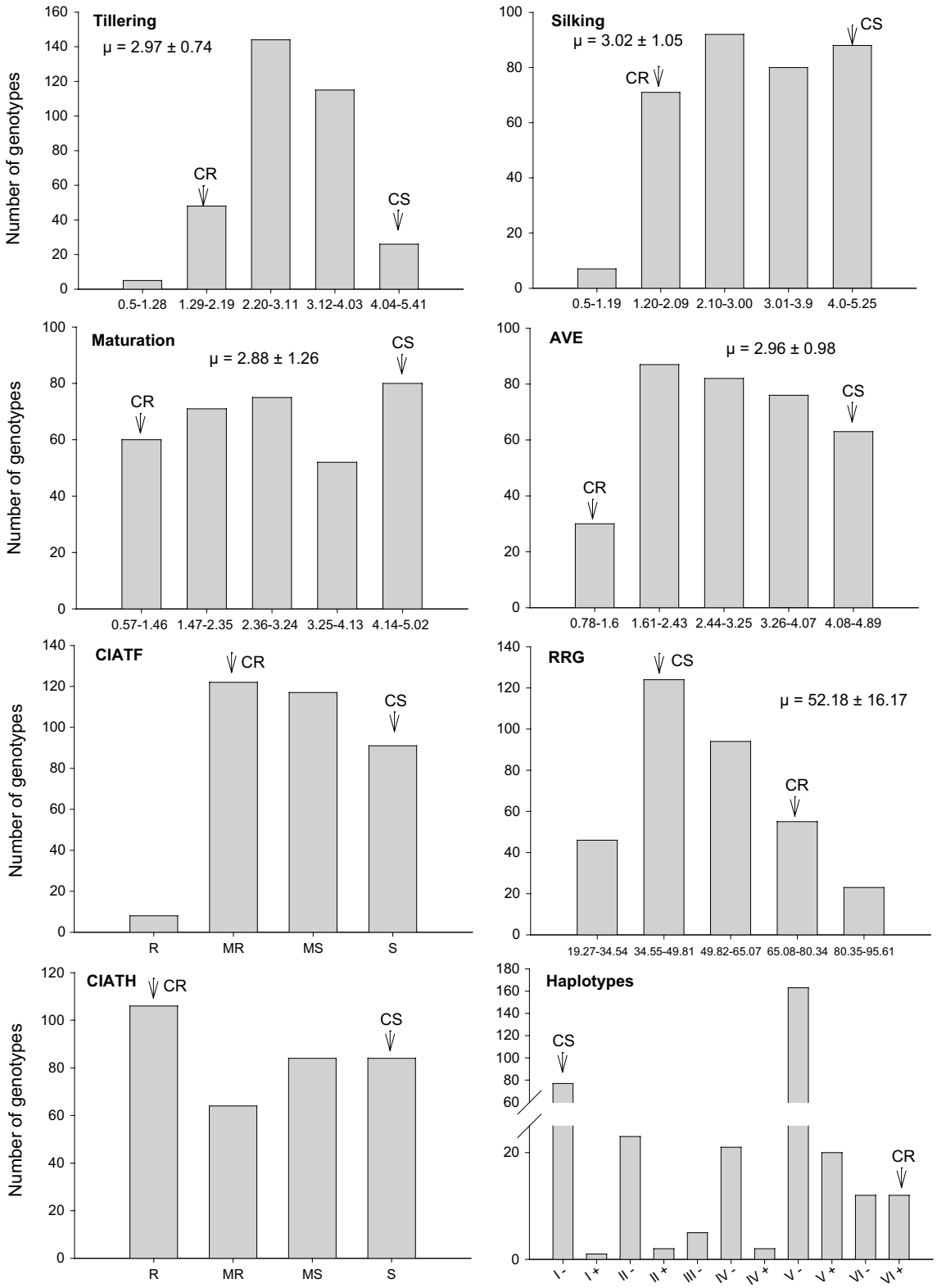

Fig. 1 Frequency distribution of grades assigned to the tillering, silking, maturation, general average (AVE), classification index of aluminium toxicity in field (CIATF), relative root growth (RRG), classification index of aluminium toxicity in hydroponics (CIATH), and haplotypes (based on the combination of the *TaALMT1* and *TaMATE1B* alleles). *Arrows* indicate the two materials used as controls: *CS* indicates the susceptible control (Anahuac 75) and *CR* indicates the resistant control (IAC 5—Maringá)

Table 2 Distribution of the 338 wheat genotypes accordingly to the geographical origin the Al^{3+} resistance in field (CIATF) and hydroponics (CIATH)

Classification	Geographical origin					
	America (without Brazil)	Brazil	Africa and Middle East	Asia and Oceania	Europe	Total
CIATH						
S	34 (45.3)[a]	26 (12.2)	5 (45.5)	13 (50.0)	6 (46.2)	84 (24.9)
MS	19 (25.3)	49 (23.0)	3 (27.3)	9 (34.6)	4 (30.8)	84 (24.9)
MR	11 (14.7)	47 (22.1)	2 (18.2)	2 (7.7)	2 (15.4)	64 (18.9)
R	11 (14.7)	91 (42.7)[b]	1 (9.1)	2 (7.7)	1 (7.7)	106 (31.4)
CIATF						
S	55 (73.3)[a]	9 (4.2)	7 (63.6)	15 (57.7)	5 (38.5)	91 (26.9)
MS	17 (22.6)	82 (38.5)	2 (18.2)	10 (38.5)	6 (46.2)	117 (34.6)
MR	3 (4.0)	114 (53.5)[b]	2 (18.2)	1 (3.8)	2 (15.4)	122 (36.1)
R	0 (0.0)	8 (3.8)	0 (0.0)	0 (0.0)	0 (0.0)	8 (2.4)

Numbers in parenthesis indicate the percentage in relation to the total number in each origin

Genotypes were separated in four classes: *S* sensitive, *MS* moderately sensitive, *MR* moderately resistant and *R* resistant

[a] position of the susceptible control (Anahuac 75)

[b] position of the resistant control (IAC 5—Maringá)

Table 3 Pearson correlation obtained by wheat traits evaluated in 338 wheat genotypes cultivated in field and hydroponics

Trait	CIATH	Tillering	Silking	Maturation	AVE	CIATF	*TaALMT1*	*TaMATE1B*	Haplotypes
RRG	0.71**	−0.50**	−0.51**	−0.52**	−0.53**	0.49**	0.47**	0.28**	0.49**
CIATH		−0.56**	−0.58**	−0.56**	−0.59**	0.56**	0.49**	0.25**	0.50**
Tillering			0.87**	0.86**	0.93**	−0.88**	−0.68**	−0.42**	−0.70**
Silking				0.94**	0.98**	−0.92**	−0.71**	−0.41**	−0.74**
Maturation					0.98**	−0.93**	−0.72**	−0.41**	−0.74**
AVE						−0.94**	−0.73**	−0.43**	−0.75**
CIATF							0.69**	0.37**	0.71**
TaALMT1								0.23**	0.99**
TaMATE1B									0.33**

** Significant at 1 % levels for teste *t*

usually higher (indicating more susceptibility) than those in the following stages (silking and maturation), indicating that some genotypes possessed the ability to recover from the initial stress in acidic soil with a high Al^{+3} concentration. This finding indicates that these genotypes recovered from Al^{3+} stress and may be one reason why the correlation between the performances in hydroponics and the field was not higher.

TaALMT1 and *TaMATE1B* allelic variability

PCR markers that can be used to discriminate between different alleles at the promoter region of the *TaALMT1* and *TaMATE1B* genes have been previously reported by others (Sasaki et al. 2006; Raman et al. 2008; Han et al. 2013; Garcia-Oliveira et al. 2014; Pereira et al. 2015). These markers were used here to evaluate the allelic distribution among the genotypes (Table 4). Six *TaALMT1* alleles (here named I to VI) were detected as follows: V in 183 (54.1 %) genotypes, followed by allele I in 78 (23.1 %), II in 25 (7.4 %), VI in 24 (7.1 %), IV in 23 (6.8 %), and III in 5 (1.5 %). Only allele VII was not found. The presence of the insertion in the *TaMATE1B* promoter [here named allele *TaMATE1*(+)] was observed in 37 genotypes (Table 4). Thus, the allele *TaMATE1B*(−) did not present the insertion in 89.1 % of the materials.

The distribution of the *TaALMT1* alleles in the genotypes from different geographic origins is shown in Table 4. Six

Table 4 Distribution of haplotypes formed by the combination of six alleles in the *TaAMLT1* promoter and two alleles in the *TaMATE1B* promoter in 338 wheat genotypes with different origins

Haplotypes		Geographical origin					Total
TaALMT1	*TaMATE1B*	America (without Brazil)	Brazil	Africa and Middle East	Europe	Asia and Oceania	
I	−	47 (62.6)[a]	13 (6.1)	4 (36.4)	4 (30.8)	9 (34.6)	77 (22.8)
I	+	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	0 (0.0)	1 (0.3)
II	−	3 (4.0)	11 (5.2)	1 (9.1)	0 (0.0)	8 (30.8)	23 (6.8)
II	+	0 (0.0)	2 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.6)
III	−	2 (2.7)	0 (0.0)	0 (0.0)	0 (0.0)	3 (11.5)	5 (1.5)
III	+	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
IV	−	5 (6.7)	12 (5.6)	1 (9.1)	2 (15.4)	1 (3.8)	21 (6.2)
IV	+	0 (0.0)	2 (0.9)	0 (0.0)	0 (0.0)	0 (0.0)	2 (0.6)
V	−	16 (21.3)	133 (62.4)	4 (36.4)	6 (46.2)	4 (15.4)	163 (48.2)
V	+	1 (1.3)	18 (8.5)	1 (9.1)	0 (0.0)	0 (0.0)	20 (5.9)
VI	−	0 (0.0)	11 (5.2)	0 (0.0)	0 (0.0)	1 (3.8)	12 (3.6)
VI	+	1 (1.3)	11 (5.2)[b]	0 (0.0)	0 (0.0)	0 (0.0)	12 (3.6)
Total		75 (22.2)	213 (63.0)	11 (3.2)	13 (3.8)	26 (7.7)	338

Numbers in parenthesis indicate the percentage in relation to the total number in each origin

[a] Position of the susceptible control (Anahuac 75)

[b] Position of the resistant control (IAC 5—Maringá)

TaALMT1 promoters were found out of the 288 genotypes from the Americas, with allele Type V being found most frequently (present in 58.3 % of the genotypes), followed by Type I (present in 20.8 % of the genotypes) and less frequently Type III (present in two genotype). In the eleven genotypes that originated from Africa and the Middle East, promoters Type III and Type VI were not found and the most frequent promoters were Type I and Type V. In the thirteen European genotypes, only three promoters were found (Type I, IV and V), with Type V the most common. In the 26 Asian and Oceania genotypes, all six *TaALMT1* promoters were found, with Type I the most common and Type IV and Type VI present in only one genotype each. *TaMATE1B*(+) was not found in any of the genotypes that originated from Asia and Oceania. This allele was found in only one genotype from the Middle East and Europe and in two genotypes from the Americas (not including Brazil). Most of the *TaMATE1B*(+) alleles were detected in Brazilian genotypes, which represented 89.2 % of the genotypes that contained that allele.

When we investigated the different combinations of the *TaALMT1* and *TaMATE1B* alleles, 11 haplotypes were detected among the 338 genotypes from different origins (Table 4). When Brazil was excluded, genotypes showing the haplotype I(−) were the most common with detection in 64 of 125 genotypes (51.2 %), followed by V(−), which was present in 30 genotypes (24.0 %). When only the Brazilian genotypes were considered, three haplotypes [I(+), III(− and +)] were not found. The most frequent combination in Brazil was V(−), which was detected in 133 of 213 genotypes. The *TaALMT1* alleles Type V and Type VI and the insertion in the *TaMATE1B* promoter usually represent genotypes with higher gene expression and organic acid efflux (Sasaki et al. 2006; Tovkach et al. 2013); this superior combination was found in 32 genotypes, most of which (90.6 %) were from Brazil. When Brazil was excluded, the superior alleles were found in one genotype from Guatemala (Maya 74), one from Argentina (Klein Lucero), and one from Israel (Bet Dagan 131). The other five genotypes containing the haplotype with *TaMATE1B*(+) but presenting *TaALMT1* alleles different from V or VI were Galego Rapado [haplotype I(+)], Fortaleza and Horto [haplotype II(+)], and IAS 14—Contestado and Emb_04101 [haplotype IV(+)]. Among all geographical regions, none of the materials showed haplotype III(+) and the majority (86.5 %) presented the *TaMATE1B*(+) allele in association with the *TaALMT1* alleles Type V and Type VI.

Relationships between Al^{3+} resistance and the *TaALMT1* and *TaMATE1B* alleles

The controls used here showed alleles corresponding to the previously published association (Sasaki et al. 2006; Tovkach et al. 2013) where the susceptible control Anahuac75 presented haplotype I(−) and the resistant control IAC 5—Maringá presented haplotype VI(+) (Supplementary file 1). The proportion of the *TaALMT1* alleles I and

Table 5 Distribution of the haplotypes formed by the combination of the six *TaALMT1* alleles and two *TaMATE1B* alleles in different Al^{3+}-resistance classes in the hydroponics and field experiments

Haplotypes			Classification index of aluminium toxicity							
			Hydroponics (CIATH)				Field (CIATF)			
TaALMT1	*TaMATE1B*	Total	S	MS	MR	R	S	MS	MR	R
I	−	77	45 (53.5)[a]	20 (23.8)	7 (10.9)	5 (4.7)	67 (72.6)[a]	9 (7.7)	1 (0.8)	0 (0.0)
I	+	1	1 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.9)	0 (0.0)	0 (0.0)
II	−	23	7 (8.3)	9 (10.7)	3 (4.6)	4 (3.8)	6 (6.6)	14 (12.0)	3 (2.5)	0 (0.0)
II	+	2	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.9)	0 (0.0)	0 (0.0)	2 (1.6)	0 (0.0)
III	−	5	3 (3.6)	1 (1.2)	1 (1.6)	0 (0.0)	3 (3.3)	2 (1.7)	0 (0.0)	0 (0.0)
III	+	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
IV	−	21	6 (7.1)	8 (9.5)	2 (3.1)	5 (4.7)	6 (6.6)	13 (11.1)	2 (1.6)	0 (0.0)
IV	+	2	0 (0.0)	0 (0.0)	0 (0.0)	2 (1.9)	0 (0.0)	0 (0.0)	2 (1.6)	0 (0.0)
V	−	163	19 (22.6)	42 (50.0)	38 (59.4)	64 (60.4)	10 (11.0)	70 (59.8)	80 (65.6)	3 (37.5)
V	+	20	2 (2.4)	2 (2.4)	8 (12.5)	8 (7.5)	0 (0.0)	2 (1.7)	18 (14.8)	0 (0.0)
VI	−	12	1 (1.2)	2 (2.4)	4 (6.2)	5 (4.7)	0 (0.0)	4 (3.4)	8 (6.6)	0 (0.0)
VI	+	12	0 (0.0)	0 (0.0)	1 (1.6)	11 (10.4)[b]	0 (0.0)	1 (0.9)	6 (4.9)[b]	5 (62.5)
Total		338	84 (24.9)	84 (24.9)	64 (18.9)	106 (31.4)	91 (26.9)	117 (34.6)	122 (36.1)	8 (2.4)

See "Materials and methods" section for the classification index of aluminum toxicity in the field (CIATF) and in hydroponics (CIATH)

Numbers in parenthesis indicate the percentage in relation to the total number in each class

Genotypes were separated in four classes: *S* sensitive, *MS* moderately sensitive, *MR* moderately resistant and *R* resistant

[a] Position of the susceptible control (Anahuac 75)

[b] Position of the resistant control (IAC 5—Maringá)

II in categories S and MS identified by the hydroponic and field tests were 79.6 and 94.2 %, respectively, which confirmed the association of these promoters with the Al^{3+}-susceptible genotypes (Table 5). The proportions of alleles V and VI in categories R and MR were 67.1 and 58.0 % in the hydroponic and soil tests, respectively, which also confirmed their association with the Al^{3+}-resistant genotypes (Table 5). When only haplotypes V(+) and VI(+) evaluated in the hydroponic tests were considered, 87.5 % of the genotypes were classified as R or MR; among the 20 genotypes carrying haplotype V(+), 16 were R or MR; all genotypes with haplotype VI(+) were R or MR. Additionally, the proportion under field conditions increased to 90.6 %, with 18 genotypes with haplotypes V(+) and 11 VI(+) classified as R or MR (Table 5).

When only the two *TaMATE1B* promoter alleles (presence or absence of the insertion) were considered, the Pearson correlation with Al^{3+} resistance was higher for the field ($-0.43 > r > -0.41$, $P < 0.001$) than the hydroponic experiments (0.28 with RRG and 0.25 with CIATH, $P < 0.001$) (Table 3). Similarly, the correlation of all *TaALMT1* alleles with the Al^{3+} resistance phenotype in the field ($-0.73 > r > -0.68$, $P < 0.001$) was higher than the hydroponic experiments (0.47 with RRG and 0.49 with CIATH, $P < 0.001$). When both genes were compared, the correlation with Al^{3+} resistance in with CIATH and CIATF was higher for the *TaALMT1* (0.49 and 0.69, respectively) than for the *TaMATE1B* allele (0.25 and 0.37, respectively). When considering the haplotypes, the correlation with CIATH and CIATF was similar (0.50 and 0.71, respectively) when only *TaALMT1* was considered (0.49 and 0.69, respectively) (Table 5). In this context, the phenotyping in the field presented a high association between the *TaALMT1* and *TaMATE1B* alleles and Al^{3+} resistance, although the additive effect of the citrate efflux was not significant.

Relative contribution of traits to variability in Al^{3+} resistance

The analysis of the relative contribution of each trait allowed us to assess their contribution according to their impact on genetic diversity. The contribution was estimated based on the methodology described by Singh (1981). The highest contributions were obtained for CIATH (18.5 %), *TaALMT1* (15.5 %) and haplotypes (12.6 %), followed by the characteristics *TaMATE1B*, maturation, CIATF, AVE, silking, RRG and tillering (Fig. 2). The combined effect of the traits CIATH, *TaALMT1*, haplotypes, *TaMATE1B*, maturation and CIATF represented 77.8 % of the assessment of diversity among the genotypes. Thus, the high correlation among the characteristics evaluated in the field (silking,

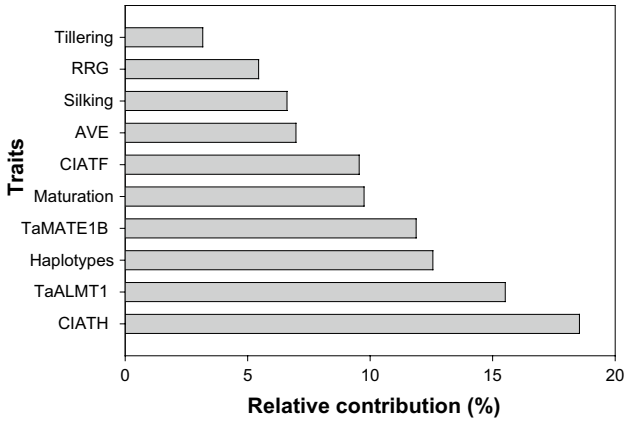

Fig. 2 Relative contribution of eight characteristics to the diversity in the Al^{3+} resistance in 338 genotypes according to the method of Singh (1981). Tillering, relative root growth (RRG), silking, overall average (AVE), classification index of aluminium toxicity in the field (CIATF), maturation, *TaMATE1B* promoter alleles, haplotypes (based on the combination of the *TaALMT1* and *TaMATE1B* alleles), *TaALMT1* promoter alleles, and classification index of aluminium toxicity in hydroponic (CIATH)

tillering and maturation) made these variables redundant. In the future, the analysis of the later stages (tillering and maturation) should not be performed.

Principal component analysis

The results of the principal component analysis showed that the first two components explained 78.8 % of the diversity among the genotypes (Supplementary file 2 and 3). The genotype distribution when both components were considered did not allow differentiation into separate clear groups. However, the genotypes could be identified based on the knowledge of the characteristics CIATH, CIATF, haplotypes and origin, which allowed better separation of the groups. The distribution based on these variables confirmed that the field classification (CIATF) better discriminated the genotypes without overlap among the groups (R, MR, MS and S) (Supplementary file 2).

Year of release for the wheat cultivars and association with the *TaALMT1* and *TaMATE1B* alleles

The distribution of the superior *TaALMT1* and *TaMATE1B* alleles over time can be evaluated based on the year of release/obtainment for each cultivar/variety and the presence of the alleles. The materials can be considered to be under positive selection if the increase in Al^{3+} tolerance is associated with the presence of specific alleles. Here, we were able to obtain the year of release or obtainment for 327 genotypes, which represented more than 90 years of wheat breeding. In general, the frequency of the *TaALMT1* alleles Type V and Type VI increased over time, whereas the *TaMATE1B*(+) allele showed a constant lower frequency (Fig. 3a). The oldest cultivars (1921–1930) presented haplotypes I/V/VI with or without *TaMATE1B*(+) [from Tunisia: Cailloux–haplotype V(−); from Brazil: Floreana–haplotype V(−) and PG1–haplotype VI(+); and from Portugal: Galego Rapado–haplotype I(+)]. Over the following three decades (1931–1960, Fig. 3b), 15 of the 24 cultivars released (62.5 %) showed haplotype V/VI(−) or haplotype V/VI(+), whereas the nine (37.5 %) remaining cultivars presented other haplotypes with *TaMATE1B*(−) or *TaMATE1B*(+). Over the next 40 years (1961–2000), 247 genotypes among the varieties and cultivars were released or obtained of which 147 (59.5 %) presented haplotype V/VI with *TaMATE1B*(−) (83.7 %) or *TaMATE1B*(+) (16.3 %), followed by 67 (27.1 %) possessing haplotype I/II(−) (100 %). The remaining genotypes showed different haplotypes that always contained *TaMATE1B*(−). A total of 52 genotypes were released or obtained between 2001 and 2013, with 37 (71.2 %) presenting haplotype V/VI(−) (96 %) or V/VI(+) (4 %) and the remaining 15 presenting haplotype I/II/IV(−) (93.3 %) or I/II/IV(+) (6.7 %). Curiously, although the presence of *TaMATE1B*(+) associated with the *TaALMT1* allele Type V or Type VI usually increased the RRG and decreased the AVE, there was a tendency for an average reduction in the RRG and average increase in the AVE over time (Fig. 3c, d).

Discussion

Aluminium (Al^{3+}) toxicity is one of the main constraints that limits crop production worldwide. Therefore, the identification of resistant sources and molecular markers linked to Al^{3+} resistance are needed to increase plant performance under acidic soil conditions. In this work, we used hydroponic and field tests to evaluate the Al^{3+} resistance of 338 wheat genotypes and correlated the resistance with markers linked to two genes encoding organic acid transporters. A wide diversity in plant performance was detected, which allowed the identification of genotypes from different geographic origins containing high variations in Al^{3+} resistance (Tables 2, 4). Although the allelic variability in *TaALMT1* and *TaMATE1B* in wheat was previously published (Sasaki et al. 2006; Raman et al. 2008; Han et al. 2013; Garcia-Oliveira et al. 2014; Pereira et al. 2015), we extended the understanding of that variation by demonstrating for the first time a correlation of these alleles with field data. A high correlation ($r = 0.71$, $P < 0.001$) between the *TaALMT1* and *TaMATE1B* haplotypes with Al^{3+} resistance in the field was obtained (Table 3).

The selection pressures under the field conditions (soil pH from 4.3 to 4.7 with Al representing 75 to 87.9 % of the

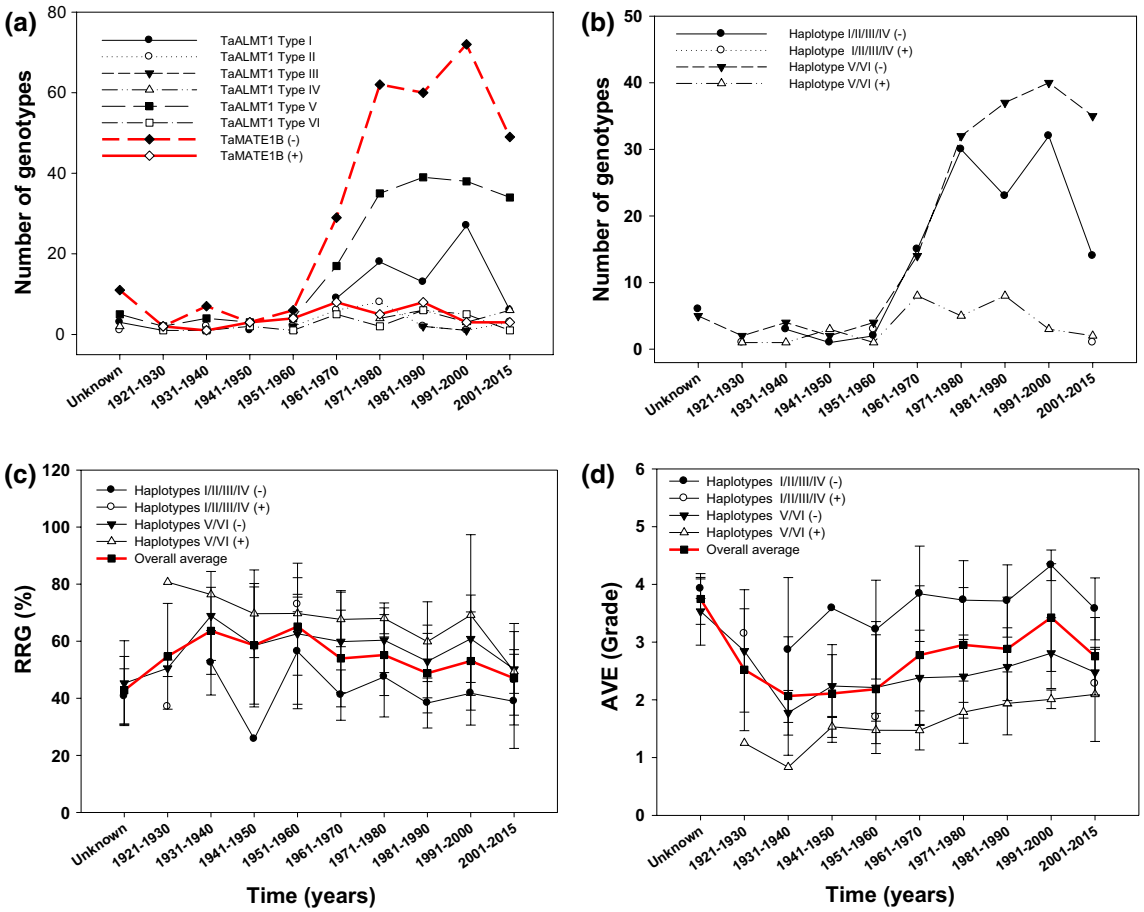

Fig. 3 Distribution of *TaALMT1* and *TaMATE1B* alleles and plant performance against Al^{3+}, in hydroponics and field, based on more than 90 years of wheat breeding. **a** *TaALMT1* and *TaMATE1B* alleles, **b** haplotypes, **c** relative root growth—RRG, and **d** overall average of the field grades—AVE. In **c** and **d** the *red line* is the average value of the characteristic in each sampled period

CTC) were higher than the stress applied in the hydroponic experiments because only eight materials were considered to be resistant in the field conditions (Tables 2, 5). The field evaluation described here was relatively fast based on the time required to reach the tillering stage. There was a high correlation (from 0.86 to 0.94, $P < 0.001$) between the phenotyping in the three growth stages (tillering, silking and tillering), indicating that only one of these stages needed be used for the evaluation of wheat Al^{3+} resistance. Additionally, the method of Singh (1981) indicated redundancy and thus a low contribution of the AVE, silking and tillering to the diversity in Al^{3+} resistance (Fig. 2), suggesting that only the evaluation at tillering was needed to facilitate the field test; this finding will reduce the laborious process of evaluating plants in the field. Moreover, the use of the controls alternately placed in every plot also facilitated the field evaluations by reducing the interference of the heterogeneous Al^{3+} distribution.

Al^{3+} resistance has been evaluated in wheat using different methods, with hydroponics being the preferred choice. The correlation between the hydroponic method and the plant performance in acidic soil has been reported to be as high as the 0.77 ($P < 0.001$) index obtained by Baier et al. (1995). However, is important to note that these authors used different genotypes to compare plant growth between the hydroponic and soil tests. We observed an intermediate correlation ($r = 0.56$, $P < 0.001$) between the hydroponic and field data (Table 3). Discrepancies between different phenotyping methods have also been reported for wheat (Hayes et al. 2004), barley (Szira et al. 2008) and oats (Nava et al. 2015). Moreover, the hydroponic and field tests showed different levels of stress; for instance, Al^{3+} could be tested in isolation in the hydroponic experiments whereas there was a mix of different factors in the field. The inconsistencies between these two methods could be partially explained by the possible non-uniformity of the nutrient distribution and other nutritional deficiencies or toxicities (e.g., phosphorus, calcium and manganese) in addition to the Al^{3+} toxicity. Additionally, the different physical/chemical characteristics in each environment (hydroponics and

soil) could lead to the development of different morphological traits in the roots. The differences in root traits could impact water and nutrient uptake by the plant and thus its performance in the soil (Shavrukov et al. 2012). Moreover, the amount of Al^{3+} used in our hydroponics experiments (74 μM) suggested by Voss et al. (2006) might not be sufficient to discriminate the wheat genotypes; thus, a wider genetic basis and adjustments in the Al^{3+} concentrations are required to improve the correlation with the field tests.

The different *TaALMT1* and *TaMATE1B* alleles were previously shown to be associated with Al^{3+} resistance (Sasaki et al. 2006; Raman et al. 2008; Tovkach et al. 2013; Garcia-Oliveira et al. 2014; Pereira et al. 2015). The *TaALMT1* alleles V and VI were correlated with higher gene expression and malate efflux by the root tip, whereas an insertion upstream of the *TaMATE1B* promoter region extended and increased the gene expression to the root apex, thereby enhancing the citrate efflux (Sasaki et al. 2006, Tovkach et al. 2013). Here, six *TaALMT1* promoter alleles were found in addition to the two *TaMATE1B* alleles. The majority of the *TaALMT1* and *TaMATE1B* alleles detected here were consistent with the alleles detailed in previous reports (Raman et al. 2008; Han et al. 2013; Garcia-Oliveira et al. 2014; Pereira et al. 2015). However, as related by Raman et al. (2008) and Pereira et al. (2015), we found inconsistent results for the *TaALMT1* alleles in the Siete Cerros, INIA F66 and Morocco cultivars compared with the results published by Raman et al. (2008). These inconsistencies could be explained by the incorrect labelling of the accessions or multilines. Most of the genotypes with alleles I/II or V/VI evaluated in this study were characterized as susceptible (S/MS) or resistant (MR/R), respectively, although the proportions were not the same (Table 5). For instance, 79.6 and 94.2 % of the genotypes classified as S/MS in the hydroponics and soil, respectively, possessed the *TaALMT1* I/II alleles, whereas 67.1 and 58.0 % of the genotypes with alleles V/VI were discriminated as MR/R. Eighty-seven genotypes in the field (42.0 %) and 68 in the hydroponic (32.9 %) experiments presented alleles V/VI and were classified as S/MS. This study is the first report of non-Japanese wheat lines carrying *TaALMT1* promoters V and VI presenting the Al^{3+}-sensitive or moderately sensitive phenotype, suggesting that some factor(s) in addition to the level of *TaALMT1* expression is (are) involved in the control of the malate efflux in these genotypes. Conversely, 21 genotypes presenting alleles I/II were classified as MR/R in the hydroponic experiments and six of them (Fortaleza, Dom Marco, Horto, IAS c46—Curitiba, Minuano and Emb_05104) were also identified as MR in the field (Supplementary file 1). Among these six genotypes, Fortaleza and Horto presented the allele *TaMATE1B*(+), which could partially explain the good performance. However, the other four genotypes lacked the insertion in the *TaMATE1B* promoter; thus, other Al^{3+} resistance mechanisms may be involved in the Al^{3+} resistance presented by these genotypes. These other mechanism could be related to a number of genes and metabolic pathways involved in Al^{3+} resistance (Raman et al. 2010; Ryan et al. 2011; Delhaize et al. 2012). More analyses should be performed to elucidate why the lower number of repeats in the *TaALMT1* promoter of these genotypes was correlated with Al^{3+} resistance or moderate resistance. *TaALMT1* alleles I/II were previously detected in Al^{3+}-resistant genotypes (Raman et al. 2008), but the mechanisms that contributed to the resistance that were not related to the organic acid efflux were not described.

In terms of the *TaMATE1B* gene, we identified the lack of the insertion in the promoter as the most frequent allele. Only 37 of 338 genotypes showed the insertion (Table 4): 33 of them originated from Brazil, two from another American country (Klein Lucero from Argentina and Maya 74 from Guatemala), one from Europe (Galego Rapado from Portugal) and one from the Middle East (Bet Dagan 131 from Israel). The insertion in the *TaMATE1B* promoter was widespread among Portuguese wheat cultivars (Garcia-Oliveira et al. 2014), and it is possible that the Portuguese immigrants who established in Brazil transported the varieties carrying that allele. However, although the majority of the 37 genotypes containing the *TaMATE1B*(+) allele were from Brazil, the frequency was low (15.5 % representing 33 genotypes among the 213 Brazilian accessions). This finding was in agreement with the report by Pereira et al. (2015) that detected the *TaMATE1B*(+) allele in 80 of the 302 Brazilian genotypes evaluated. Here, more than 86 % of the *TaMATE1B*(+) genotypes were classified as R/MR in the field or hydroponic conditions, and only one genotype (Onix) containing the *TaMATE1B*(+) allele was susceptible in the hydroponic and field experiments. We found a high association of the *TaMATE1B*(+) allele with the *TaALMT1* promoters V/VI, with 32 of 37 genotypes containing the *TaMATE1B*(+) allele showing the alleles V/VI. Among these 32 genotypes, 29 were classified as R/MR in the field. The combination of these superior alleles is interesting because they were proposed to possess higher malate and citrate efflux by the root tip. In fact, the Brazilian genotypes IAC 5—Maringá and Toropi, which are recognized as important Al^{3+}-resistant sources, showed the combination of the superior *TaALMT1* and *TaMATE1B* alleles. Moreover, the best combinations for root growth of the 33 Brazilian wheat cultivars in a short-term soil experiment were reported to be the *TaALMT1* promoters V/VI and the insertion in the *TaMATE1B* promoter (Pereira et al. 2015).

The combination of the *TaALMT1* and *TaMATE1B* alleles detected here allowed the identification of 11 haplotypes associated with higher relative contributions to the diversity of Al^{3+} resistance (Fig. 2). The correlation of

the *TaALMT1* and *TaMATE1B* alleles with the hydroponic and field data was similar (0.71) to the correlation of the *TaALMT1* alleles alone (0.69). In a recent survey, the only consistent QTL across the tested environments in an RIL population used to map genes for acidic soil tolerance in the field was centred on the *TaALMT1* gene on chromosome 4DL, which accounted for up to 38.5 % of the total phenotypic variation (Liu et al. 2015). That report and the data described here show the importance of *TaALMT1* for acid soil tolerance under field conditions. In contrast, small and not significant difference was detected between *TaALMT1* alleles I and V under mildly acid soils although no substantial disadvantage was observed for cultivars with *TaALMT1* allele V (Eagles et al. 2014). To further evaluate the impact of the *TaMATE1B*(+) allele on Al^{3+} resistance, we analysed its frequency over 90 years of breeding (the oldest cultivar Galego Rapado was obtained in 1922 and the newest cultivar BRS Marcante was obtained in 2013). An increase in the *TaMATE1B*(+) allele frequency was not detected; conversely, the number of *TaALMT1* alleles V/VI was significantly increased (Fig. 3a). This finding demonstrated that the *TaALMT1* alleles were selected by the breeding programmes but that there was little selection pressure for the *TaMATE1B*(+) allele, indicating the low adaptive power of that allele. Results recently published by our group revealed that the transposon insertion in the *TaMATE1B* promoter was significantly advantageous for a few materials; however, no clear correlation was observed with higher root growth under short-term soil experiments in 33 Brazilian wheat genotypes (Pereira et al. 2015). Despite the lower frequency of the allele (+), we confirmed that a clear correlation could not be established between the *TaMATE1B*(+) allele and higher root growth in hydroponics or better plant performance in field conditions even using a wider genetic basis. Recently, it has also been showed that *TaMATE1B*(+) conferred higher Al^{3+} tolerance when associated with an inferior *TaALMT1* allele but, when combined with a superior *TaALMT1* allele, the increase in tolerance was small (Han et al. 2016). The *TaMATE1B*(+) allele appeared to not have a good association with other traits because no benefits of citrate efflux for phosphorus nutrition were observed in studies with near-isogenic wheat lines (Ryan et al. 2014). Based on these results, the effective role of the insertion in the *TaMATE1B* promoter for traits related to the citrate efflux (e.g., Al^{3+} resistance or phosphorus nutrition) appeared to be limited. Reasons for that limited additive effect of the citrate efflux possibly include one or more of the different assumptions detailed by (Ryan et al. 2014) as, for example, low concentration and/or diffusion of citrate in the soil due to lower citrate efflux by mature roots, microbial degradation and/or soil chemistry. Clearly, these assumptions should be experimentally evaluated especially because the limited role of the citrate efflux contradicts the theoretical potential of citrate that is approximately eightfold more effective than malate in chelating Al^{3+} (Zheng et al. 1998).

Most of the Brazilian wheat varieties analysed by others were considered resistant (de Sousa 1998; Raman et al. 2008). In this study, 64.8 and 57.3 % of the Brazilian genotypes were Al^{3+}-resistant or moderately resistant in the hydroponic and soil tests, respectively (Table 2), thereby confirming the importance of Brazilian wheat as an Al^{3+}-resistant source. However, although acidic soils are common in the wheat breeding regions in Brazil, some of the Brazilian cultivars detected here as Al^{3+}-sensitive or moderately sensitivity were recommended for growth in our country and presented good yields in the past (Castro et al. 2011). Six moderately resistant foreign genotypes identified here were interesting and should be considered for further characterization. Among them, only Maya 74 (from Guatemala) and Bet Dagan 131 (from Israel) possessed Frontana as an ancestor. The other non-Brazilian genotypes classified as MR were Aburakomugi (from Japan), Escondido 41 (from USA), Giza (from Egypt) and Klein Lucero (from Argentina). These materials are good candidates for incorporation into Brazilian wheat breeding programmes. Stodart et al. (2007) found landraces that originated from different countries and represented different Al^{3+}-tolerant haplotypes compared with the materials actually used in breeding programmes. In this way, new sources can contribute with additive alleles and potentially improve the current level of Al^{3+} tolerance in modern wheat cultivars.

In conclusion, for the first time markers linked to the *TaALMT1* and *TaMATE1B* promoter regions were associated with Al^{3+} resistance in field conditions. The high correlation detected here is interesting for wheat breeding programmes targeting better wheat growth in fields containing very acidic soil. Only eight of the 338 genotypes were classified as resistant in field conditions, revealing the high selection pressures in our condition. Among these eight genotypes, Aceguá, C33, IAC-21—Iguaçú and PAT 7219 were not widely recognized as Al^{3+}-resistant sources. Six materials (Dom Marco, Fortaleza, Horto, IAS c46—Curitiba, Minuano and Emb_05104) showed *TaALMT1* alleles that were not usually associated with Al^{3+} resistance but presented good performances in the hydroponic and field tests. These results indicate the potential for new Al^{3+} resistance mechanisms, and thus these genotypes should be further characterized.

Author contribution statement L.C. and J.P.S. Jr designed the research. J.G.A., J.A.D.M., C.M.F. and D.B. performed the hydroponic experiments. J.G.A., J.P.S. Jr and J.A.D.M. performed the field tests. J.G.A. and J.F.P. evaluated the PCR markers. J.G.A., J.F.P., L.C. and J.P.S. Jr analysed the data and wrote the paper.

Acknowledgments The authors are grateful for the financial support of Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (PNPD 560516/2010-0 Grant to JPJS and postdoctoral fellowship to JGA) and Embrapa (Project "Melhoramento genético de trigo para o Brasil 2012-2016"—code number 02.11.8.001.00.03). We are thankful to Jordalan Buffet Muniz for assistance with DNA extraction and amplification and to Darci Luiz Veronese and Teles Dall Agnol for helping with the phenotyping under field conditions.

Conflict of interest We confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere. Informed consent was obtained from all individual participants included in the study. The authors declare that they have no conflict of interests.

References

Baier AC, Somers DJ, Gusiafson JP (1995) Aluminium tolerance in wheat: correlating hydroponic evaluations with field soil performances. Plant Breed 114:291–296

Barone P, Rosellini D, LaFayette P, Bouton J, Veronesi F, Parrott W (2008) Bacterial citrate synthase expression and soil aluminum tolerance in transgenic alfalfa. Plant Cell Rep 27:893–901

Camargo CEO, Ferreira Filho AWP (1999) Tolerância ao alumínio e características agronômicas em populações híbridas de trigo: estimativas de variância, herdabilidade e correlações. Sci Agric 56:449–457

Camargo CEO, Felicio JC, Rocha Junior LS (1987) Trigo: tolerância ao alumínio em solução nutritiva. Bragantia 46:183–190

Camargo CEO, Ferreira Filho AWP, de Freitas JG (1998) Avaliação de genótipos de centeio, triticale, trigo comum e trigo duro quanto à tolerância ao alumínio em solução nutritiva. Sci Agric 55:227–232

Camargo CEO, Felicio JC, Ferreira Filho AWP, Lobato MTV (2006) Tolerância de genótipos de trigo comum, trigo duro e triticale à toxicidade de alumínio em soluções nutritivas. Bragantia 65:43–53

Castro RL, Caierão E, Pires JLF, Pasinato A (2011) Ensaio estadual de cultivares de trigo do Rio Grande do Sul, 2010. Embrapa Trigo: Documentos online, Passo Fundo, p 106

Cruz CD (2006) Programa GENES: biometria. UFV, Viçosa

de la Fuente JM, Ramírez-Rodríguez V, Cabrera-Ponce JL, Herrera-Estrella L (1997) Aluminum tolerance in transgenic plants by alteration of citrate synthesis. Science 276:1566–1568

de Sousa CNA (1998) Classification of Brazilian wheat cultivars for aluminium toxicity in acid soils. Plant Breed 117:217–221

de Sousa CNA, Caierão E (2014) Cultivares de trigo indicadas para cultivo no Brasil e instituições criadoras—1922 a 2014, 2nd edn. Embrapa, Brasília

Delhaize E, Ma JF, Ryan PR (2012) Transcriptional regulation of aluminium tolerance genes. Trends Plant Sci 17:341–348

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf material. Phytochem Bull 19:11–15

Eagles HA, Cane K, Trevaskis B, Vallance N, Eastwood RF, Gororo NN, Kuchel H, Martin PJ (2014) *Ppd1*, *Vrn1*, *ALMT1* and *Rht* genes and their effects on grain yield in lower rainfall environments in southern Australia. Crop Pasture Sci 65:159–170

Echart CL, Cavalli-Molina S (2001) Fitotoxicidade do alumínio: efeitos, mecanismo de tolerância e seu controle genético. Ciência Rural 31:531–541

Garcia-Oliveira AL, Martins-Lopes P, Tolrá R, Poschenrieder C, Tarquis M, Guedes-Pinto H, Benito C (2014) Molecular characterization of the citrate transporter gene *TaMATE1* and expression analysis of upstream genes involved in organic acid transport under Al stress in bread wheat (*Triticum aestivum*). Physiol Plant 152:441–452

Han C, Dai SF, Liu DC, Pu ZJ, Wei YM, Zheng YL, Wen DJ, Zhao L, Yan ZH (2013) *TaALMT1* promoter sequence compositions, acid tolerance, and Al tolerance in wheat cultivars and landraces from Sichuan in China. Genet Mol Res 12:5602–5616

Han C, Zhang P, Ryan PR, Rathjen TM, Yan Z, Delhaize E (2016) Introgression of genes from bread wheat enhances the aluminium tolerance of durum wheat. Theor Appl Genet. doi:10.1007/s00122-015-2661-3

Hayes JE, Zhu Y-G, Mimura T, Reid RJ (2004) An assessment of the usefulness of solution culture in screening for phosphorus efficiency in wheat. Plant Soil 261:91–97

Hettel GP (1989) Wheat production advances in South America's Colossus: the gains from 20 years of Brazilian/CIMMYT collaboration. CIMMYT, Mexico

Kochian LV, Piñeros MA, Liu J, Magalhaes JV (2015) Plant adaptation to acid soils: the molecular basis for crop aluminum resistance. Annu Rev Plant Biol 66:23.1–23.28

Liu M, Yu M, Li G, Carver BF, Yan L (2015) Genetic characterization of aluminum tolerance in winter wheat. Mol Breeding 35:205

Nava IC, Delatorre CA, Pacheco MT, Scheeren PL, Federizzi LC (2015) Aluminium tolerance of oat cultivars under hydroponic and acid soil conditions. Expl Agric 1:1–13

Pereira JF, Zhou G, Delhaize E, Richardson T, Zhou M, Ryan PR (2010) Engineering greater aluminium resistance in wheat by over-expressing *TaALMT1*. Ann Bot 106:205–214

Pereira JF, Barichelo D, Ferreira JR, Aguilera JG, Consoli L, Silva Júnior JP, Cargnin A, Bonow S (2015) *TaALMT1* and *TaMATE1B* allelic variability in a collection of Brazilian wheat and its association with root growth on acidic soil. Mol Breed 35:169

Raman H, Ryan PR, Raman R, Stodart BJ, Zhang K, Martin P, Wood R, Sasaki T, Yamamoto Y, Mackay M, Hebb DM, Delhaize E (2008) Analysis of *TaALMT1* traces the transmission of aluminum resistance in cultivated common wheat (*Triticum aestivum* L.). Theor Appl Genet 116:343–354

Raman H, Stodart B, Ryan PR, Delhaize E, Emebiri L, Raman R, Coombes N, Milgate A (2010) Genome-wide association analyses of common wheat (*Triticum aestivum* L.) germplasm identifies multiple loci for aluminium resistance. Genome 53:957–966

Ryan PR, Delhaize E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Physiol Plant Mol Biol 52:527–560

Ryan PR, Raman H, Gupta S, Horst WJ, Delhaize E (2009) A second mechanism for aluminum resistance in wheat relies on the constitutive efflux of citrate from roots. Plant Physiol 149:340–351

Ryan PR, Tyerman SD, Sasaki T, Furuichi T, Yamamoto Y, Zhang WH, Delhaize E (2011) The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. J Exp Bot 62:9–20

Ryan PR, James RA, Weligama C, Delhaize E, Rattey A, Lewis DC, Bovill WD, McDonald G, Rathjen TM, Wang E, Fettell NA, Richardson AE (2014) Can citrate efflux from roots improve phosphorus uptake by plants? Testing the hypothesis with near-isogenic lines of wheat. Physiol Plant 151:230–242

SAS Institute (1997) SAS/STAT software: changes and enhancements through release 6.12. SAS Institute, Cary

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

Sasaki T, Ryan PR, Delhaize E, Hebb DM, Ogihara Y, Kawaura K, Noda K, Kojima T, Toyoda A, Matsumoto H, Yamamoto Y (2006) Sequence upstream of the wheat (*Triticum aestivum* L.) *ALMT1* gene and its relationship to aluminum resistance. Plant Cell Physiol 47:1343–1354

Shavrukov Y, Genc Y, Hayes J (2012) The use of hydroponics in abiotic stress tolerance research. In: Asao T (ed) Hydroponics—a standard methodology for plant biological researches. Intech, Osaka, pp 39–65

Singh D (1981) The relative importance of characters affecting genetic divergence. Indian J Genet 41:237–245

Stodart BJ, Raman H, Coombes N, Mackay M (2007) Evaluating landraces of bread wheat *Triticum aestivum* L. for tolerance to aluminium under low pH conditions. Genet Resour Crop Evol 54:759–766

Szira F, Bálint AF, Börner A, Galiba G (2008) Evaluation of drought-related traits and screening methods at different developmental stages in spring barley. J Agron Crop Sci 194:334–342

Tovkach A, Ryan PR, Richardson AE, Lewis DC, Rathjen TM, Ramesh S, Tyerman SD, Delhaize E (2013) Transposon-mediated alteration of *TaMATE1B* expression in wheat confers constitutive citrate efflux from root apices. Plant Physiol 161:880–892

Voss M, Cantídio NAS, Baier AC, Nascimento Júnior A, Bofff T (2006) Método de avaliação de tolerância à toxidez de alumínio em trigo, em condições de hidroponia, na Embrapa Trigo. Embrapa Trigo: Documentos online, Passo Fundo, p 16

Zheng SJ, Ma JF, Matsumoto H (1998) Continuous secretion of organic acids is related to aluminum resistance during relatively long-term exposure to aluminium stress. Physiol Plant 103:209–214

Zhou G, Delhaize E, Zhou M, Ryan PR (2013) The barley MATE gene, *HvAACT1*, increases citrate efflux and Al^{3+} tolerance when expressed in wheat and barley. Ann Bot 112:603–612

Zhou G, Pereira JF, Delhaize E, Zhou M, Magalhaes JV, Ryan PR (2014) Enhancing the aluminium tolerance of barley by expressing the citrate transporter genes *SbMATE* and *FRD3*. J Exp Bot 65:2381–2390