

***TaALMT1* and *TaMATE1B* allelic variability in a collection of Brazilian wheat and its association with root growth on acidic soil**

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Abstract Repeated blocks in the *TaALMT1* promoter as well as a transposon insertion in the *TaMATE1B* upstream region have been correlated with the level of gene expression, organic acid efflux, and ultimately aluminum (Al^{3+}) resistance in wheat (*Triticum aestivum* L.). In this study, we investigated the allelic polymorphism related to the *TaALMT1* and *TaMATE1B* promoter regions in 300 Brazilian wheat genotypes and the correlation of that variation with root growth on acid soil. In addition, SSR markers

were used to determine the genetic variability of the genotypes. Seven *TaALMT1* promoter alleles (Types I–VII) were detected based on size of PCR products. The most common alleles were Type V and Type VI (71.3 and 11.9 %, respectively), and these are generally associated with higher levels of *TaALMT1* expression and Al^{3+} resistance. The promoter alleles Type I and Type II, which are usually associated with Al^{3+} sensitivity, were detected in 12.2 % of the genotypes. The insertion in the *TaMATE1B* promoter, associated with greater Al^{3+} resistance, was identified in 80 genotypes. Combination among the alleles allowed the separation in 12 haplotypes were 68 genotypes presented the *TaALMT1* promoters Type V and Type VI along with the transposon insertion in the *TaMATE1B* promoter. However, the most represented haplotype was Type V without the insertion (176 genotypes). Short-term soil experiment, performed in 33 genotypes representing the 12 haplotypes, revealed that the higher relative root length was observed in some genotypes presenting *TaALMT1* promoters Type V or Type VI and the transposon insertion in the *TaMATE1B* promoter. Moreover, when comparing genotypes inside the same haplotype, the transposon insertion was significantly advantageous for a few materials. However, the majority the genotypes presenting the insertion in the *TaMATE1B* promoter did not outperform the genotypes without the insertion but showing the same *TaALMT1* promoter. Analysis using SSR markers, with an average PIC of 0.60, showed

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high genetic diversity among the genotypes belonging to different haplotypes. The alleles and the genetically diverse genotypes reported here should be considered for wheat-breeding programs aiming increments in wheat Al^{3+} resistance.

Keywords Acid soil · Aluminum resistance · Citrate transporter · Genetic diversity · Malate transporter · Wheat (*Triticum aestivum* L.)

Introduction

Many plant species cope with the aluminum (Al^{3+}) present in acid soils ($pH \leq 5.5$) using mechanisms classified as resistance (exclusion of Al^{3+} from root and shoot tissues) or tolerance (safe accommodation of Al^{3+} once it enters the cell). An Al^{3+} resistance mechanism present in many species is the efflux of organic acids (citrate, malate or oxalate) from root apices into the apoplasm (Liu et al. 2014). This mechanism relies on the formation of a complex between the organic acid(s) released by the root tips and the Al^{3+} present in the soil; these complexes are less damaging to the root tip, consequently reducing Al^{3+} toxicity to the plant (Kochian et al. 2015).

In wheat (*Triticum aestivum* L.), two organic anions (malate and citrate) have been reported to detoxify the Al^{3+} around the root tip (Delhaize et al. 1993; Ryan et al. 2009), and the membrane transporters encoded by the genes *TaALMT1* (chromosome 4D) and *TaMATE1B* (chromosome 4B) are responsible for the efflux of these anions by the root apex (Sasaki et al. 2004; Raman et al. 2005; Tovkach et al. 2013). Although differences in the *TaALMT1* and *TaMATE1B* structural region can be detected (Sasaki et al. 2004; Raman et al. 2005, 2008; Tovkach et al. 2013), it is the level of gene expression that shows higher correlation to the Al^{3+} resistance/sensitivity. For instance, the overexpression of those genes have been reported to transgenically enhance the amount of malate or citrate released by the root tip (Sasaki et al. 2004; Delhaize et al. 2004; Zhang et al. 2008; Pereira et al. 2010; Ryan et al. 2011; Tovkach et al. 2013). Ultimately, the increase in organic acid release enhances the Al^{3+} resistance. The link between higher *TaALMT1* expression and higher Al^{3+} resistance was also observed in conventional wheat lines of non-

Japanese origin where the number of repeats of four blocks (A, B, C and D) in the *TaALMT1* promoter was correlated with the level of *TaALMT1* expression, malate efflux, and Al^{3+} resistance (Sasaki et al. 2006). It is the presence of these tandem repeated elements in the *TaALMT1* promoter region that contribute directly to the Al^{3+} resistance of hexaploid wheat (Ryan et al. 2010). Similarly, greater Al^{3+} resistance in wheat has also been correlated with citrate efflux from root tips and the expression of the *TaMATE1B* gene (Tovkach et al. 2013). Genotypes with a transposon-like insertion upstream of the *TaMATE1B* coding region have stronger expression at the root apex and greater citrate efflux (Tovkach et al. 2013; Garcia-Oliveira et al. 2014). The transposon-like insertion near *TaMATE1B* as well as the repeated elements in the *TaALMT1* promoter can be discriminated by PCR (Sasaki et al. 2006; Garcia-Oliveira et al. 2014). Up to date, nine alleles for the *TaALMT1* promoter have been described this way (Sasaki et al. 2006; Raman et al. 2008; Ryan et al. 2010; Delhaize et al. 2012b), while the transposon insertion in the *TaMATE1B* promoter can be distinguished by the presence or absence of a PCR product (Tovkach et al. 2013; Garcia-Oliveira et al. 2014). In general, genotypes with the Type V and Type VI alleles of *TaALMT1* (greater number of repeats in the promoter) show intermediate to high levels of expression and are more resistant to Al^{3+} (Sasaki et al. 2006; Raman et al. 2008). The transposon-like insertion near *TaMATE1B* is associated with constitutive citrate efflux by the root tip and higher Al^{3+} resistance (Tovkach et al. 2013; Garcia-Oliveira et al. 2014).

Although a number of wheat genotypes have been analyzed for the *TaALMT1* and *TaMATE1B* promoter polymorphism (Sasaki et al. 2006; Raman et al. 2008; Ryan et al. 2010; Garcia-Oliveira et al. 2014), only a few Brazilian wheat genotypes have been characterized, even though Brazilian wheat is an important source of Al^{3+} resistance. For instance, Brazilian genotypes BH 1146, Carazinho, Frontana, Fronteira, IAC 5 - Maringá, Toropi, Trintani, Trintecinco, and Veranópolis have been used in different studies investigating Al^{3+} resistance mechanisms, and all of them have been recognized as important Al^{3+} -resistant genotypes (de Sousa 1998; Garvin and Carver 2003; Raman et al. 2008; Ryan et al. 2009; Tovkach et al. 2013). Because 60 % of the soil in tropical and subtropical regions is acidic (Von Uexküll and Mutert

1995), improved Al^{3+} resistance remains an important objective for wheat-breeding programs. In Brazil, 63 % of the territory is composed by acidic soil (FAO 2000), and Al^{3+} toxicity is an important constraint in south and central regions (Echart and Cavalli-Molina 2001; Yamada 2005) where practically all wheat is grown.

Different methodologies can be used to assess the Al^{3+} resistance/tolerance in wheat, and a high correlation has been reported between field trials and root length in hydroponics (Baier et al. 1995) and in pots in glasshouses (Tang et al. 2003). Although studies have demonstrated that Brazilian wheat show a diverse level of Al^{3+} resistance (Camargo and Oliveira 1981; Camargo et al. 1987, 2006; Bertan et al. 2006; Raman et al. 2008), no reports have evaluated the genetic variability among Al^{3+} -resistant Brazilian wheat cultivars. Genetic diversity studies have been performed in wheat genotypes from Brazil using SSR markers (Huang et al. 2002; Warburton et al. 2006; Zhou et al. 2007; Hu et al. 2008; Schuster et al. 2009) as these markers have been reported to be highly suitable and very effective for the assessment of genetic diversity in wheat (Röder et al. 1998; Huang et al. 2002). Although most papers have used a small number of Brazilian genotypes, a highly variable wheat germplasm adapted for use in Brazil has been reported (Schuster et al. 2009). Because breeding programs depend on the genetic variability that can be exploited in their germplasm, it is important to access the genetic diversity among Brazilian genotypes with superior Al^{3+} resistance.

In this context, the objective of this work was to assess the allelic polymorphism in the *TaALMT1* and *TaMATE1B* promoters in a collection of 300 Brazilian wheat genotypes and to link these polymorphisms with root growth on acidic soil and with genetic variability detected by SSR markers. Our goal also extended to the identification of *TaALMT1* and *TaMATE1B* promoters that are associated with the highest levels of Al^{3+} resistance.

Materials and methods

Genotypes

Seeds of the 300 Brazilian wheat genotypes (Table 1), belonging to different wheat-breeding programs, were provided by the Embrapa Active Wheat Germplasm Bank.

DNA extraction

Seeds were pre-germinated in the dark at 23 °C for 48 h. Five to ten germinated seeds were transferred to pots containing a mix of soil, vermiculite, and sand (1:1:1). The pots were placed on a laboratory bench under ambient light and irrigated daily. After 7 days, leaves of the seedlings were collected, transferred to a 2-ml Eppendorf tube containing three stainless steel beads (2.3 mm diameter), and frozen in liquid nitrogen. Leaf tissue was triturated in a Mini-BeadbeaterTM (Biospec Products) for 1 min. Total DNA was extracted using a CTAB-based protocol (Doyle and Doyle 1987). After quantification on an agarose gel, 100–150 ng of DNA was used for PCR.

Detection of the allelic variability in the *TaALMT1* and *TaMATE1B* promoters

To amplify the different alleles of the *TaALMT1* promoter, primers LPF-F (CCTGGTTTTCTTGAT GGGGGCACA) and LPF-R (TGCCCACCATCTCG CCGTCGCTCTCTCT) were used according to Sasaki et al. (2006). If promoters Type I or II (1190 or 1221 bp) were identified, an additional amplification with primers SPF-F (GCTCCTACCACTATGGT TGCG) and SPF-R (CCAGGCCGACTTTGAGC-GAG) was performed to allow better allele discrimination (612 or 643 bp). To detect the presence of the transposon insertion in the *TaMATE1B* promoter, primers TaMATE1-4B-SLT-F (ATCCATCCTCCTT CCCTCAC) and TaMATE1-4B-SLT-R (ATGAATG CTGTGTCCACCAA) were used according to Garcia-Oliveira et al. (2014). The reactions were carried out in a final volume of 20 µl containing 1× PCR buffer, 0.3 mM each dNTPs, 1× Q solution, 0.3 µM each primer, and one-unit Taq DNA Polymerase (Qiagen). Amplification programs were performed as described by Sasaki et al. (2006) and Garcia-Oliveira et al. (2014). PCR fragments were run on a 1 % (LPF primers), 1.25 % (SPF primers), or 1.5 % (*TaMATE1B* primers) agarose gel, stained with ethidium bromide, and detected as shown in Fig. 1. Since a negative amplification with the *TaMATE1B* primers is related to the absence of the insertion but could also be associated with poor DNA quality or mistakes in PCR preparation, we executed controls by adding a positive sample in each set of amplification, replicating the

Table 1 Allelic variation of the *TaALMT1* and *TaMATE1B* promoters in a collection of Brazilian wheat genotypes

Genotypes	<i>TaALMT1</i> allele ^a	<i>TaMATE1B</i> allele ^b
Bagé, Branco Ligeiro, BRS 264, Caçador 2, CD 104, Embrapa 10 - Guajá, Glória, IAC 22 - Araguaia, IAC 287 - Yaco, IAS 30 - São Sepé, Negroz 11-45, Ocepar 7 - Baturia, Palotina, Pampa, Tiacena 1, Trigo Libanes, and Veadeiros	I	—
Hulha Negra, IAC 6 - Brasil, Tiacena 2, and Tiacena 3	I	+
BRS 208, Candiota, Dom Marco, Esteana 11 a 42, Herval, IAS 58, IAS-C 46 - Curitiba, Minuano, Rio Sulino, and Trintani	II	—
Fortaleza, Horto, IAC 2 - Kibeiro, IAPAR 33 - Guarapuava, IAS 15 - Campeiro, Trintecinco ^c Ocepar 17	II III	+
B 20, CD 118, BRS 207, IAPAR 28 - Igapó, IAPAR 29 - Cacatu, Ocepar 18, Ocepar 19, PAT 24, Trigo BR 20 - Guató, Trigo BR 26 - São Gotardo, and Trigo BR 31 - Miriti	IV	—
IAS 14 - Contestado	IV	+
Aceguá, Alegrete, B 5, B 8, B 15, Beckman, BH 1146, Branco Pelado, BRS 49, BRS 177, BRS 179, BRS 194, BRS 220, BRS 229, BRS Angico, BRS Camboatã, BRS Gaivota, BRS Guabiju, BRS Guamirim, BRS Louro, BRS Tangará, BRS Timbaúva, BRS Umbu, Butuí, Candeias, Carazinho ^c , CD 105, CD 114, Centidor 2118-46 B, CEP 11, CEP 13 - Guaíba, CEP 14 - Tapes, CEP 17 - Itapua, CEP 19 - Jatai, CEP 21 - Campos, CEP 24 - Industrial, CEP 27 - Missoes, Charrua, Cincana, CNT 2, CNT 3, CNT 4, CNT 5, CNT 7, CNT 8, CNT 9, CNT 10, Confiança, Cotiporã, Embrapa 21, Embrapa 22, Embrapa 24, Embrapa 27, Embrapa 41, Embrapa 42, Embrapa 52, Encruzilhada, Farrapo, Floreana, Florestana, Frontana, Fronteira, Fundacep 29, Fundacep 30, Garibaldi Vermelho, General Vargas, Giruá, IAC 1 - Cacique, IAC 7 - Bartira, IAC 17 - Maracaí, IAC 18 - Xavantes, IAC 24 - Tucuruí, IAC 160 - Juruá, IAC 161 - Taiamã, IAC 227 - Anhumas, IAPAR 3 - Aracatu, IAPAR 6 - Tapejara, IAPAR 18 - Marumbi, IAPAR 21 - Taquari, IAPAR 22 - Guaraúna, IAPAR 32 - Guaratã, IAPAR 40 - Mirim, IAPAR 41 - Tamacoré, IAPAR 42 - Ibiara, IAPAR 60, IAPAR 78, IAS 2, IAS 6, IAS 13 - Passo Fundo, IAS 20 - IASSUL, IAS 23, IAS 24, IAS 25, IAS 28 - Ijuí, IAS 36 - Jarau, IAS 49 - Pioneiro, IAS 51 - Albatroz, IAS 54, IAS 55, IAS 56, IAS 59, IAS 62, IAS 64, IAS-C 47 - Florestal, IAS-C 48 - Guarapuava, IPR 84, IPR 85, Jacuí, Lagoa Vermelha, Lanceiro, Londrina ^c , Mascarenhas, MG 1, Milagre, Milanes, Negroz 22-45, Negroz 3028-46, Nova Prata, Ocepar 8 - Macuco, Ocepar 9 - Perdiz, Ocepar 13 - Acauã, Ocepar 15, Ocepar 16, Ocepar 20, Ocepar 23, OR 1, Panda, PAT 18, PAT 7219, PAT 7392, Patriarca, Peladinho, Pinhal Grande, Pitana, Pitoco Pelado, Preludio, Quartz, S 12, Safira, Sales, Santa Barbara, Sema 220, Serrano, Trigo BR 1, Trigo BR 2, Trigo BR 3, Trigo BR 4, Trigo BR 5, Trigo BR 8, Trigo BR 10 - Formosa, Trigo BR 11 - Guarani, Trigo BR 12 - Aruanã, Trigo BR 13, Trigo BR 14, Trigo BR 15, Trigo BR 16 - Rio Verde, Trigo BR 17 - Caiuá, Trigo BR 18 - Terena, Trigo BR 19, Trigo BR 21 - Nhandeva, Trigo BR 22, Trigo BR 23, Trigo BR 25, Trigo BR 28, Trigo BR 29 - Javaé, Trigo BR 30 - Cadiuéu, Trigo BR 32, Trigo BR 33 - Guará, Trigo BR 34, Trigo BR 38, Trigo BR 39 - Paraúna, Trigo BR 41 - Ofaié, Trigo BR 43, Trigo Chapéu, Tucano, and Veranópolis	V	—
B 4, B 9, Carazinho ^c , Colconcol, Combate, Coxilha, Embrapa 15, Erexim, IAC 72 - Tapajós, IAPAR 34 - Guaraji, IAPAR 46, IAS 16 - Cruz Alta, IAS 17, IAS 22 - Tibaji, IAS 32 - Sudeste, IAS 34 - Xapécó, IAS 53, IAS 57, IAS 60, Londrina ^c , MGS 1 Aliança, MGS 3 Brilhante, Minuano 82, Nobre, Ônix, PAT 1, PAT 9, PAT 19, RS 1 - Fênix, RS 2 - Santa Maria, RS 3 - Palmeira, S 76, Santiago, Surpresa, Trapeano, Trigo BR 7, Trigo BR 24, Trigo BR 27, Vacaria, and Vila Rica	V	+
Frocor, IAC 3 - Anhanguera, IAC 23 - Tocantins, IAC 350, IAPAR 53, Ocepar 10 - Garça, Ocepar 11 - Juriti, and Trigo BR 6	VI	—
C 33, Cinquentenário, Colonista, Dom Feliciano, IAC 5 - Maringá, IAC 8 - Paraguaçu, IAC 21 - Iguacu, IAC 286 - Takaoka, IAPAR 30 - Pirata, IAS 3 - São Borja, IAS 7, IAS 8 - Piratini, IAS 9, IAS 50 - Alvorada, IAS 52, Jesuíta, Nordeste, Novo Sulino, Novo Surto, PAT 10, PG 1, Planalto, RS 4 - Ibiraiaras, Seberi, Toropi, Trigo BR 9 - Cerrados, Trigo BR 37, and Trintecinco ^c	VI	+

Table 1 continued

Genotypes	<i>TaALMT1</i> allele ^a	<i>TaMATE1B</i> allele ^b
Centelha	VII	+

^a According to Sasaki et al. (2006) and Raman et al. (2008)

^b Presence (+) or absence (−) of the Sukkula-like transposon in the promoter of *TaMATE1B* as described by Garcia-Oliveira et al. (2014)

^c Different *TaALMT1* or *TaMATE1B* promoter alleles were detected for genotypes Carazinho, Londrina and Trintecinco. Because of that, percentage of the allelic distribution was calculated based on 303 alleles

PCR (using the same DNA) and re-extracting and re-amplifying DNA from 121 samples.

Sequencing and sequence analysis

To confirm the PCR fragment amplified for cultivar Centelha (Type VII), we sequenced the fragment. PCR products were precipitated, quantified on a 0.8 % agarose gel, and cloned into the pGEM-T easy vector (Promega). The plasmids were sequenced with primers LPF-F, LPF-R, T7 (TAATACGACTCATTATAGGG), SP6 (ATTTAGGTGACACTATAG), and VII-seq (CAGGTGCAAGGTTGTTAGAAGA) (3.2 μM), sequencing buffer and BigDye Terminator version 3.1. The sample was precipitated again, resuspended in Hi-Di formamide, denatured, and run on an ABI 3130 Sequence Analyzer. Sequencing Analysis Software version 5.1.1 and the Staden sequencing analysis package (Staden 1996) were used to analyze and align the sequences. The resulting sequence was deposited at GenBank with accession number KF662919.

Soil experiment

Short-term growth experiments were conducted to measure the root growth of 33 genotypes selected from each of the 12 haplotypes. Except for genotypes Ocepar 17, IAS 14 - Contestado, and Centelha, which are the unique representatives from their haplotypes, the selection inside each haplotype was random. The acidic soil (dystrophic red latosol of clayey texture) was collected at a depth of 0–20 cm from Embrapa Trigo (latitude-28,216979, longitude-52,408428). The soil pH in the water was 4.2, and the exchangeable aluminum (extracted by 1 mol L^{−1} KCl) was 58.5 mmol_c dm^{−3} representing 78.9 % of the total cations exchange capacity (CEC). Half of the soil was

limed, increasing the pH to 6.2 and reducing the soluble aluminum to 0 %. Five germinated seeds of each genotype containing roots of similar sizes were transferred to pots (25 cm high × 5 cm diameter) containing 450 g of limed and acidic soil and incubated in a glasshouse at 18 °C/24 °C (night–day) and natural light for 8 days. The pots were structured in randomized block designs, and the five plants constituting each repetition were watered to 90 % of field capacity every day. At the end of the experiment, the roots were carefully removed from the soil and washed. The length of the longest root on each plant was measured. The RRL was estimated as (root length in acidic soil/root length in limed soil) × 100. Errors associated with deriving the RRL were calculated by $SE_{RRL} = RRL [(SE_x/x)^2 + (SE_y/y)^2]^{1/2}$ where x represents the mean root length in the acidic soil and y the mean root length in the limed soil. Statistical analyses were performed as detailed by Zhou et al. (2013).

Diversity analysis with SSR markers

Twelve SSR markers (Table 2) were used along with a M13 primer labeled with a fluorescent dye (FAM, NED, PET or VIC) as described in Schuelke (2000). For each SSR primer, part of the reaction mixture was identical (1 × buffer, 0.2 μM reverse primer, 0.02 μM forward primer, and 0.2 μM labeled M13 primer), but other reagents were optimized for each primer and added as follows: mix A (1.5 mM MgCl₂, 0.2 mM each dNTP, 0.5 U of *Taq* polymerase) for marker wms120; mix C (2.5 mM MgCl₂, 0.2 mM each dNTP, 0.75 U of *Taq* polymerase) for barc71, barc130, wms539, wms3, and wms261; and mix D (2.5 mM MgCl₂, 0.35 mM each dNTP, 0.75 U of *Taq* polymerase) for wmc215, barc96, wms18, psp3000, wmc331, and wms155. The amplification program

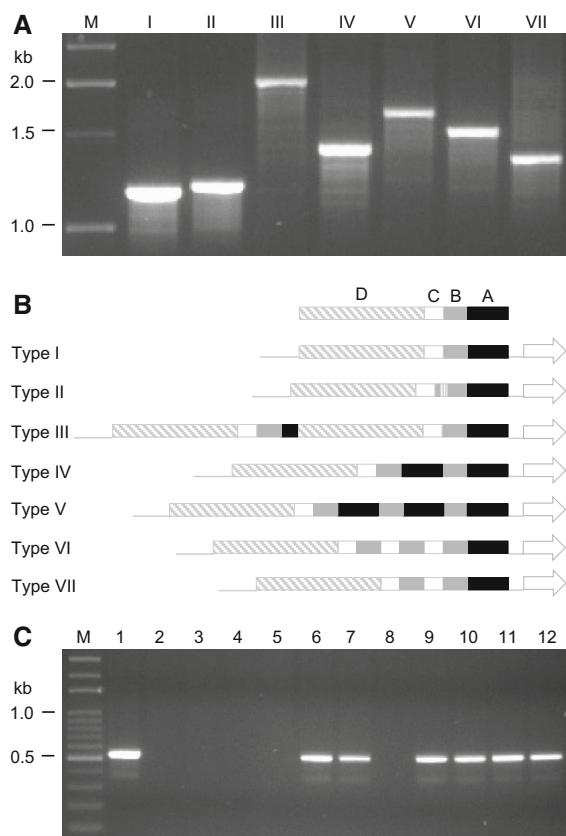


Fig. 1 *TaALMT1* and *TaMATE1B* promoter alleles identified in this study. **a** Type I–VII as described in Sasaki et al. (2006) and Raman et al. (2008). ‘M’ indicates marker (shown at left in kb), ‘I’ indicates the Type I promoter (1190 bp/cultivar Caçador 2); ‘II’, Type II (1221 bp/cultivar IAS 15 - Campeiro); ‘III’, Type III (1993 bp/Ocepar 17); ‘IV’, Type IV (1470 bp/cultivar Ocepar 19); ‘V’, Type V (1750 bp/IAS 2); ‘VI’, Type VI (1600 bp/cultivar IAS 52); and ‘VII’, Type VII (1395 bp/cultivar Centelha). **b** Schematic representation of the *TaALMT1* promoters alleles including the Type VII identified in cultivar Centelha (GenBank KF662919) and the Type I and V alleles identified in its parents (cultivars Centenário and Fronteira). Block A is represented in black (172 bp), block B in gray (108 bp), block C in white (97 bp) and block D in downward diagonal stripes (528 bp). A repeat (31 bp) within block B in the promoter Type II is represented by vertical stripes. The second repeat of block A in the promoter Type III is shorter (70 bp). Lines represent sequences not identified as repeats by Sasaki et al. (2006) (210 bp to the right and 75 bp to the left). Arrows indicate the coding region (not proportional). **c** Presence or absence of a transposon insertion in the *TaMATE1B* promoter as described in Garcia-Oliveira et al. (2014). ‘M’ indicates marker (shown at left in kb), ‘I’ indicates cultivar IAC 5 - Maringá; ‘2’, BRS 207; ‘3’, CNT 10; ‘4’, BRS 194; ‘5’, Trigo Chapéu; ‘6’, MGS 1 Aliança; ‘7’, Toropi; ‘8’, B 5; ‘9’, Cinquentenário; ‘10’, Fortaleza; ‘11’, MGS 3 Brilhante; and ‘12’, Horto

was 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by four cycles at decreasing annealing temperatures of 1 °C per cycle and then 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. After amplification, the reactions were multiplexed (up to 4 primers combinations with each one containing different fluorescent dyes), diluted in water, mixed with Hi-Di formamide and GeneScan 500 LIZ size standard (Applied Biosystems), denatured (94 °C for 5 min), and run on an ABI 3130xl Genetic Analyzer containing a 36 cm capillary array with POP7 polymer. The program GeneMapper v3.5 was used to analyze the data. The SSR loci were used to build a similarity matrix using the simple matching coefficient and dendrogram construction was carried out using the hierarchical UPGMA method (Unweighted Pair Group Method with Arithmetic Mean) using the obtained matrix. The data were analyzed using the PowerMaker program (Liu and Muse 2005), and the polymorphism information content (PIC) value of each polymorphic marker was calculated according to Anderson et al. (1993).

Results

Analysis of *TaALMT1* and *TaMATE1B* allelic variability

PCR primers specific for the *TaALMT1* and *TaMATE1B* promoter regions were used to detect their allelic variability. The different types of *TaALMT1* and *TaMATE1B* promoters detected in this study are listed in Table 1. Seven different *TaALMT1* promoter alleles were detected based on amplicon size (Fig. 1) being promoters Type V (71.3 %) and Type VI (11.9 %) the most frequently detected. Those two types of promoter are usually associated with Al³⁺ resistance (Sasaki et al. 2006; Raman et al. 2008). On the other hand, promoters Type I and Type II, generally associated with Al³⁺ sensitivity, were identified in 21 (6.9 %) and 16 (5.3 %) genotypes, respectively. *TaALMT1* promoters Type III and VII were detected in one genotype each (cultivars Ocepar 17 and Centelha, respectively), representing only 0.6 % of the detected alleles. The Type IV promoter was also uncommon (detected in 4.0 % of the analyzed genotypes).

Table 2 Primers and detailed characteristics of the SSR markers used in this study

Primer ^a	Forward sequence ^b	Reverse sequence	Dye	Chromosome ^c	Number of alleles	PIC
wms18	TGGCGCCATGATTGCATTATCTTC	GGTTGCTGAAGAACCCTTATTAGG	FAM	1B/4B	9	0.54
psp3000	GCAGACCTGTGTCATTGGTC	GATATAGTGGCAGCAGGATACG	NED	1B	10	0.79
wms120	GATCCACCTTCCTCTCTCTC	GATTATACTGGTGCCGAAAC	FAM	2B	9	0.64
wms539	CTGCTCTAAGATTTCATGCAACC	GAGGCTTGCCCTCTGTAG	VIC	2D	15	0.69
wms261	CTCCCTGTACGCCCTAAGGC	CTCGCGCTACTAGCCATTG	PET	2D	11	0.58
wms155	CAATCATTTTCCCCCTCCC	AATCATTTGGAAATCCATATGCC	VIC	1D/3A	8	0.69
wms3	GCAGCGGCACTGGTACATTT	AATATCGCATCACTATCCCA	NED	3D	7	0.46
bare71	GCGCTTGTTCCCTCACCTGCTCATA	GCGTATATTCTCTCGTCTTCTTGTGGTT	NED	3D	18	0.61
wmc331	CCTGTGTCATACTTGACCTTTT	GGAGTTCAATCTTTCATCACCAT	PET	4D	6	0.51
wmc215	CATGCATGGTTGCAAGCAAAAG	CATCCCGGTGCAACATCTGAAA	FAM	3A/5A/5D	7	0.58
bare130	CGGCTAGTAGTTGGAGTGTGG	ACCGCTCTAGTTATTGCTCTC	PET	5D	3	0.46
bare96	AGCCTTGTTGTCCGTATTAT	GCGGTTTATATTTTGTGGTTGAGCATTTT	VIC	5B/6D	10	0.69

^a wms = Xgwm^b M13 tail was added to the forward primer^c Accordingly to GrainGenes (www.wheat.pw.usda.gov). More than one chromosome as reported by different papers

The presence of the transposon insertion in the *TaMATE1B* promoter was observed in 80 genotypes where 68 of them showed *TaALMT1* promoters Type V and VI. Considering the *TaALMT1* and *TaMATE1B* allelic variation, the genotypes were separated in 12 haplotypes (Table 1). Only two haplotypes (*TaALMT1* promoter Type III and presence of the insertion in the *TaMATE1B* promoter as well as *TaALMT1* promoter Type VII and absence of the *TaMATE1B* promoter insertion) were not detected. Three haplotypes were represented by only one genotype, while the most frequent one was the *TaALMT1* promoter Type V with the absence of insertion in the *TaMATE1B* promoter (176 genotypes). Interestingly, most of the genotypes presenting the *TaALMT1* promoter Type VI also showed the insertion in the *TaMATE1B* promoter (28 in 36 genotypes), while in all others *TaALMT1* promoter alleles the absence of the insertion was more frequent.

Some discrepancies related to the type of *TaALMT1* promoter were found in the results published here compared to previous reports. The results of most genotypes were consistent although we detected the *TaALMT1* promoter Type II in Trintani and Type VI in Cinquentenário and Trintecinco, while those same genotypes amplified alleles Type V, V and II, respectively, in Raman et al. (2008). For cultivars Trintani and Trintecinco, we further analyzed seeds multiplied in different years by the Embrapa Active Wheat Germplasm Bank and found the Type II promoter in both genotypes (data not shown). In this way, we detected two different *TaALMT1* alleles (II and VI) in cultivar Trintecinco. We also identified two other genotypes where *TaMATE1B* alleles differed among the different entries. In that case, seeds from packets labeled as Carazinho and Londrina showed mixed alleles for the *TaMATE1B* promoter (Table 1). Different entries of the same genotype containing different *TaALMT1* promoter alleles or Al³⁺ resistance phenotypes have been described previously (Sasaki et al. 2006; Raman et al. 2008), and this problem, regardless of the explanation, can lead to misinterpretation of the data.

Rare *TaALMT1* promoter alleles

To understand the rare distribution of *TaALMT1* promoters Type III and Type VII, we evaluated the pedigree (assessed on <http://genbank.vurv.cz/wheat/>

pedigree/) of cultivars Ocepar 17 (Kalyansona/Bluebird//Alondra/3/B7408) and Centelha (Centenário/Fronteira). Two of the parents of Ocepar 17, Alondra and Kalyansona, have Type V and Type I promoters (Raman et al. 2008), and the Type III promoter should have been inherited from the other ancestors (Bluebird or B-7408). Unfortunately, we were not able to evaluate this because there were no seeds available in our Germplasm Bank. We assume that the ancestor containing the Type III promoter was not commonly used by the different wheat-breeding programs in Brazil. The parents of Centelha were also analyzed, and Centenário had a Type I promoter (data not shown), while Fronteira had a Type V allele (Table 1), meaning that the Type VII allele originated by merging the only B–C repeat from the Type I promoter with the only B–C repeat from the Type V promoter. This hypothesis should be viewed with caution as we were not able to evaluate the exact plants used in the particular cross that resulted in Centelha.

Relative root length (RRL) in short-term soil experiment

The length of the longest root of 33 genotypes, representatives of each haplotype, was measured after 8 days of growth in a short-term soil experiments (Fig. 2). Higher RRL was observed for some genotypes harboring the *TaALMT1* Type V or VI and the insertion in the *TaMATE1B* promoter followed by genotypes carrying the *TaALMT1* Type V without the insertion (Fig. 3). The impact of the transposon insertion in the *TaMATE1B* promoter could be evaluated by analyzing genotypes whose insertion was present or absent but showing the same *TaALMT1* allele. That analysis could not be performed for the genotypes Ocepar 17 (*TaALMT1* Type III with the *TaMATE1B* promoter insertion) and Centelha (*TaALMT1* Type VII with the *TaMATE1B* promoter insertion) since they are the single representatives of their haplotypes. For the *TaALMT1* promoter Type I and Type IV, the genotypes containing the insertion in the *TaMATE1B* promoter did not present greater RRL in comparison with the ones not presenting the insertion (Fig. 3). In fact, among the genotypes with the insertion in the *TaMATE1B* promoter, only five were statistically better than the ones without the insertion. It is important to note that the plants dealt with a high level of stress in the acidic soil used since it

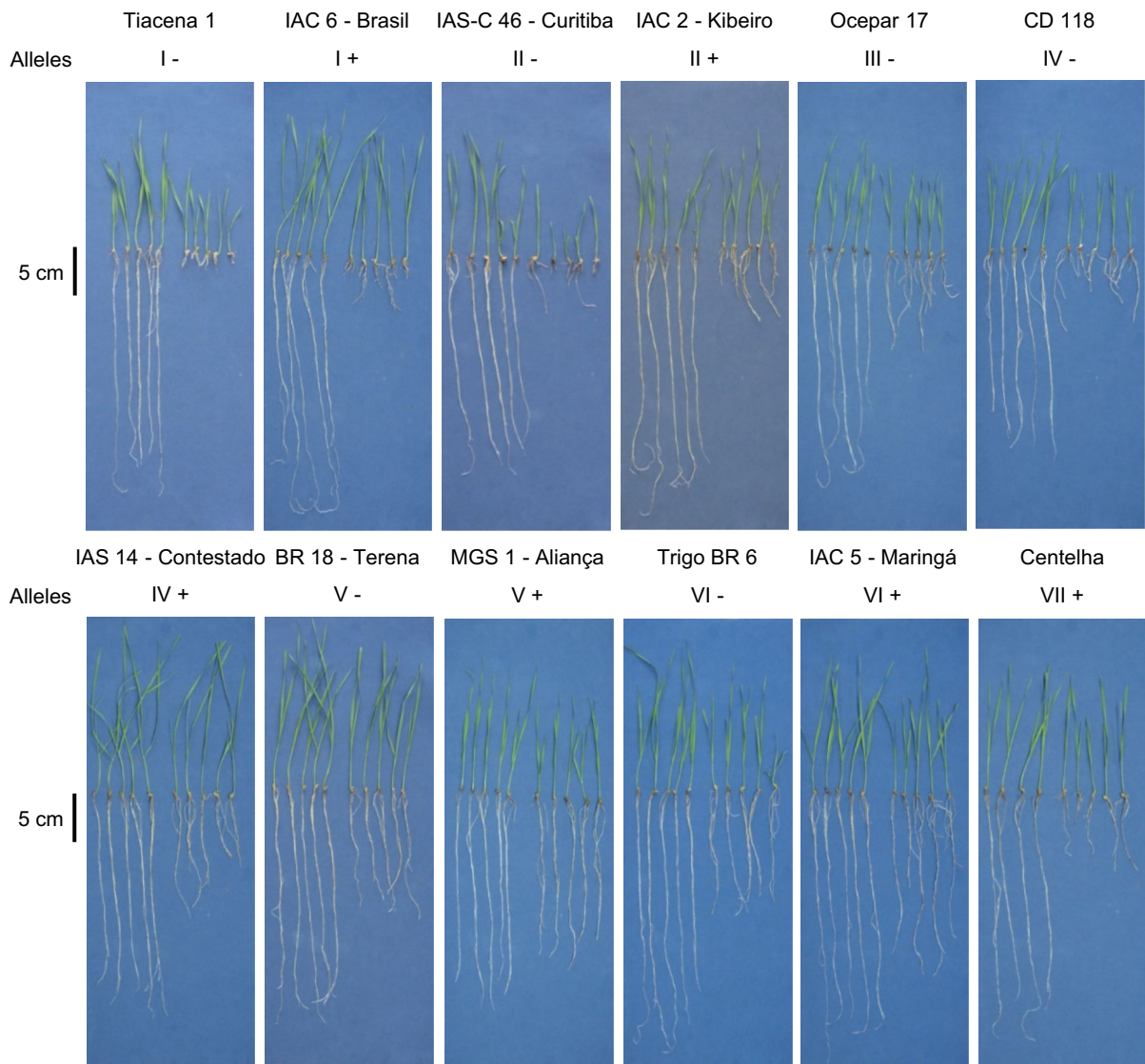


Fig. 2 Wheat plants, representative of each haplotype, after 8 days of growth in the short-term soil experiment. The plants on the *left* were grown in limed soil, and the plants on the *right*

were grown in acidic soil. ‘*Alleles*’ indicates the *TaALMT1* (Types I–VI) and *TaMATE1B* (positive ‘+’ or negative ‘–’ for the transposon insertion) alleles detected

was chemically poor (low levels of phosphorus, potassium, and organic matter) and presented a high proportion of aluminum (78.9 % of the cations).

Genetic diversity

The genetic diversity in the 300 genotypes was evaluated using 12 SSR markers spread across different chromosomes and genomes of the hexaploid wheat. The SSR markers were reasonably to

highly informative (Table 2) showing PIC values from 0.46 (wms3 and barc130) to 0.79 (psp3000) with a mean of 0.60. A total of 113 SSR alleles generated an average of 9.4 alleles per primer. Primers specific for genome B (mean PIC of 0.66) were more informative than primers specific for genome D (mean PIC of 0.55). The dendrogram constructed based on the genotypic information of the markers showed a distribution of the internationally recognized Al^{3+} resistance sources (BH

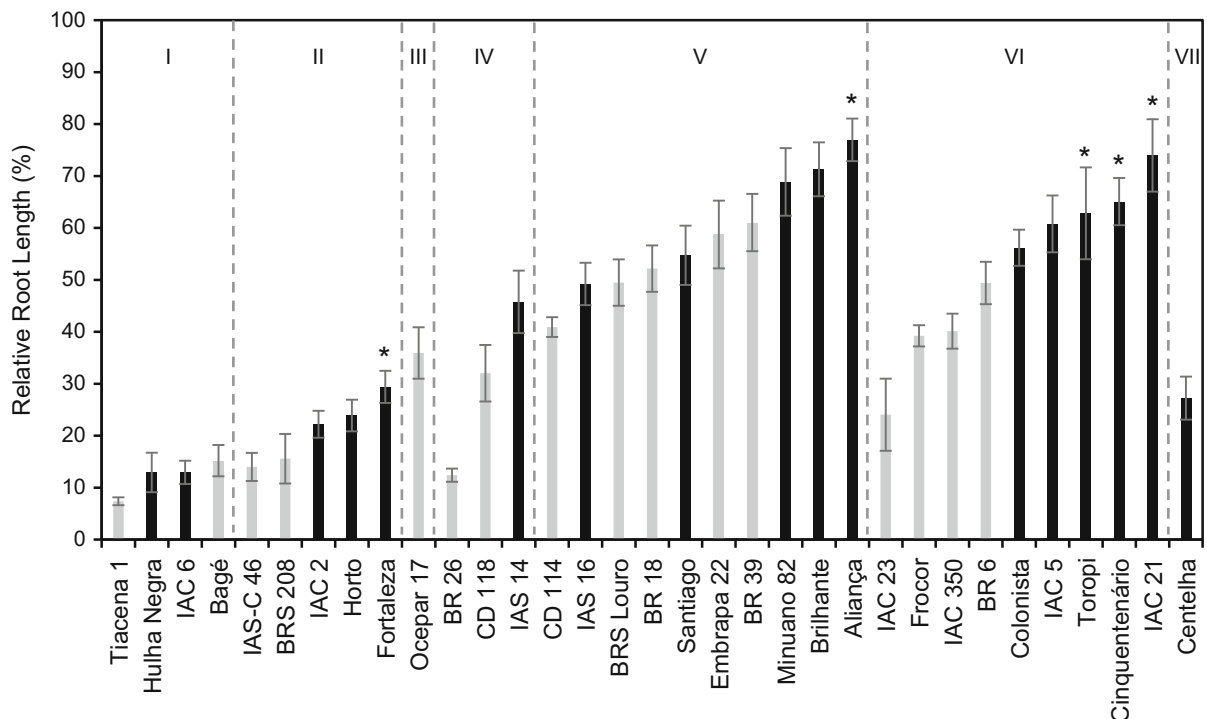


Fig. 3 Relative growth of 33 cultivars, randomly selected from each of the 12 haplotypes, in the short-term soil experiment. The bars represent the corrected mean percentage \pm SE. Numbers I to VII represent the different *TaALMT1* promoter alleles. Gray bars represent genotypes lacking the transposon insertion in the

TaMATE1B promoter while black bars represent genotypes showing the insertion. Asterisks indicate significant differences ($P > 0.05$) from genotypes with the same *TaALMT1* allele but not presenting the *TaMATE1B* promoter insertion

1146, Carazinho, Frontana, Fronteira, IAC 5 - Maringá, Toropi, Trintani, Trintecinho, and Veranópolis) among different clusters (Fig. 4), although some of them exhibited a clear relationship. Cultivars Carazinho, Frontana, and Fronteira were clustered together, as were cultivars BH1146 and IAC 5 - Maringá. The genotypes Toropi, Trintani, and Trintecinho were also closely related. That indicates those cultivars probably present similar Al^{3+} tolerance/resistance mechanisms, and crossings among genetically diverse sources should be more useful to increase Al^{3+} resistance/tolerance. Besides that, genotypes belonging to the different haplotypes reported here are distributed across the dendrogram indicating a high genetic variability. One interesting distribution is related to the 28 genotypes showing *TaALMT1* promoter Type VI and the insertion in the *TaMATE1B* promoter where 20 of them were clustered in the same group, even though they were developed by different breeding programs in Brazil.

Discussion

Soil acidity was one of the first challenges for Brazilian wheat breeders trying to introduce that crop in the South region of Brazil, and this is likely to be why Al^{3+} resistance is relatively common in Brazilian wheat genotypes (de Sousa 1998; Garvin and Carver 2003). Indeed, most Brazilian wheat varieties analyzed by others were considered resistant (de Sousa 1998; Raman et al. 2008). In this paper, we detected a high proportion (83.2 %) of *TaALMT1* promoters Type V and VI among Brazilian wheat genotypes. As reported by Sasaki et al. (2006), wheat genotypes containing those alleles generally show intermediate to high levels of *TaALMT1* expression and Al^{3+} resistance. Additionally, our study revealed that 27.0 % of the genotypes presenting *TaALMT1* promoters Type V and VI also showed the transposon insertion in the *TaMATE1B* promoter. That insertion is associated with constitutive citrate efflux by the root tip and

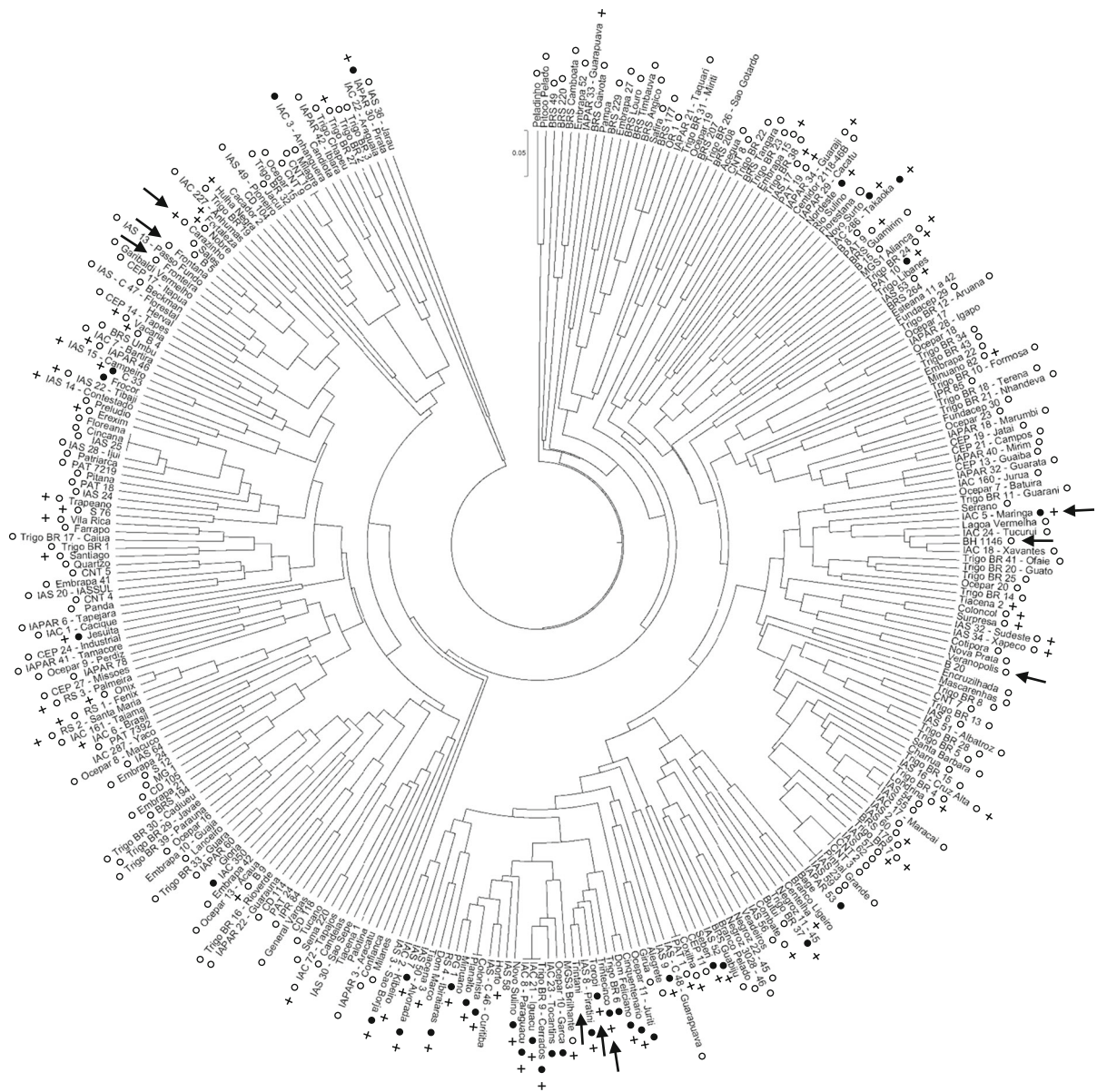


Fig. 4 Hierarchical analysis of the 300 Brazilian wheat genotypes with 12 SSR markers. The clustering dendrogram is based on a simple matching coefficient matrix. The *open circles* indicate genotypes with *TaALMT1* promoter Type V and *closed circles* genotypes with *TaALMT1* promoter Type VI. '+'

indicates the presence of the transposon insertion in the *TaMATE1B* promoter. Arrows indicate nine genotypes internationally recognized as Al^{3+} resistant (BH 1146, Carazinho, Frontana, Fronteira, IAC 5 - Maringá, Toropi, Trintani, Trintecino, and Veranópolis)

higher Al^{3+} resistance (Tovkach et al. 2013; Garcia-Oliveira et al. 2014). Aluminum resistance is not usually found in genotypes from other countries; at one time, Al^{3+} sensitivity was dominant in 250 genotypes from 21 countries from Africa, Europe, and Asia analyzed by Stodart et al. (2007).

Two genotypes analyzed here that presented *TaALMT1* promoter Type V and the insertion in the *TaMATE1B* promoter (MGS 1 Aliança and MGS 3 Brilhante) are important varieties for rainfed cropping at the Brazilian Cerrado (Scheeren et al. 2008; Coelho et al. 2010), showing that Al^{3+} resistance should have

a higher impact on wheat productivity for that condition. Heat and terminal drought are also found during the wheat-growing season in the Cerrado region (Scheeren et al. 2008), and genotypes presenting higher Al^{3+} resistance and, consequently, better root growth in acidic subsurface soil will most likely cope better when these stresses are combined. In fact, the most productive wheat genotypes in acid soil and rainfed conditions at the São Paulo state in Brazil were also Al resistant (Camargo et al. 2003). Besides that, the *TaALMT1* promoter Type V was also shown to be advantageous in lower-rainfall regions of southern Australia, and it is likely to be most beneficial under high temperature when soil moisture is expected to be limiting (Eagles et al. 2014). In this context, wheat cultivars recommended for rainfed conditions at the Brazilian Cerrado will benefit from the joint action of superior *TaALMT1* and *TaMATE1B* alleles.

Of the two Al-resistance mechanisms reported in wheat, the release of malate from root tips (encoded by *TaALMT1*) has a greater impact than the release of citrate (encoded by *TaMATE1B*); even though citrate is approximately eightfold more effective than malate in chelating Al^{3+} (Zheng et al. 1998). The reason for this is that malate release from root tips is \sim tenfold higher than citrate release (Ryan et al. 2009; Tovkach et al. 2013). Indeed, even transgenic wheat and barley lines overexpressing citrate transporters (Magalhães et al. 2007; Zhou et al. 2013, 2014) do not appear to be as resistant as lines overexpressing *TaALMT1* (Delhaize et al. 2004; Pereira et al. 2010; Zhou et al. 2014). Currently, the *TaALMT1* gene confers the most effective level of Al^{3+} resistance in transgenic barley and wheat, and the effectiveness of organic acids in chelating Al^{3+} appears to be less determinant than the amount of organic acid released from root apices (Zhou et al. 2013, 2014). Here, we were not able to detect a clear correlation between a higher relative root length (RRL) under short-term soil experiments and the insertion in the *TaMATE1B* promoter. Although, when comparing genotypes with the same *TaALMT1* alleles, the presence of the insertion in the *TaMATE1B* promoter was advantageous for a few genotypes, that advantage was not significant for most of the genotypes containing the insertion (Fig. 3). Important to note that the presence of the insertion in the *TaALMT1* promoter had no penalty for the plants since the genotypes performed at least similar to the ones not showing the insertion.

When considering the best combinations for root growth on acid soil, the genotypes containing the *TaALMT1* promoters Types V and VI and the transposon insertion in the *TaMATE1B* promoter presented the highest RRL since those genotypes are theorized to combine a higher malate and citrate efflux by the root tip (Fig. 3). That additive effect of *TaALMT1* promoter Types V or VI and the insertion in the *TaMATE1B* promoter could be used to increase the accuracy of Al^{3+} resistance prediction when analyzed together with phenotyping methods. Besides that, organic acids released by the roots have been reported to have a role in phosphorus mobilization from the soil (Kochian et al. 2004). For instance, transgenic barley expressing *TaALMT1* was more efficient at taking up phosphorus on acid soil (Delhaize et al. 2009), and theoretical modeling indicated a beneficial correlation, under certain conditions, between citrate efflux in wheat and phosphorus mobilization (Ryan et al. 2014). In this context, the incorporation of those alleles should be focused by the wheat-breeding programs targeting improvements in acid soil resistance. Additionally, breeders should also consider genes contributing to the formation of rhizosheaths (Delhaize et al. 2012a) and chromosomal regions not related to *TaALMT1* and *TaMATE1B* that have been linked to the Al^{3+} resistance/tolerance in wheat (Ma et al. 2006; Cai et al. 2008; Raman et al. 2010), even though the mechanisms underlying these regions have not yet been fully described.

It is important to point out that the primers used here to distinguish between the presence and absence of the transposon-like insertion upstream of the *TaMATE1B* coding region could not result in effective amplification if the annealing sites are mutated. In that case, other *TaMATE1B* alleles could exist. In fact, these primers do not discriminate a single nucleotide polymorphism (SNP) located \sim 2 kb upstream the *TaMATE1B* coding region that was observed by Tovkach et al. (2013). That SNP was responsible for up to threefold difference in the *TaMATE1B* expression between cultivars presenting the transposon-like insertion (Tovkach et al. 2013) and could be one explanation why wheat genotypes presenting the same *TaALMT1* allele, and the insertion in the *TaMATE1B* promoter showed different root growth in the acidic soil (Fig. 3). Besides that, other genes could also explain that difference. A number of metabolic pathways are activated in wheat under Al^{3+} stress

including the ones related to root elongation, disease resistance response, fatty acid degradation, and response to oxidative stress (Guo et al. 2007; Houde and Diallo 2008). Plant genotypes differing for those traits, but with the same *TaALMT1* and *TaMATE1B* alleles, could show different resistance to Al^{3+} . Further studies should address these differences.

Genetic analysis of wheat genotypes collected from various regions indicates that Al^{3+} resistance and the *TaALMT1* promoters could have multiple origins (Stodart et al. 2007; Zhou et al. 2007; Hu et al. 2008; Raman et al. 2008; Ryan et al. 2010), and the alleles described here for the *TaALMT1* promoter are the same as those described for wheat genotypes from different countries (Sasaki et al. 2006; Raman et al. 2008). The Type III (cultivar Ocepar 17) and Type VII (cultivar Centelha) alleles were the rarest *TaALMT1* promoters detected in Brazilian wheat genotypes and also the rarest ones detected by Raman et al. (2008). The origin of the tandem repeats in the *TaALMT1* promoter is unclear, but they most likely appeared in *T. aestivum* within the last 10,000 years, and the alleles with different repeat patterns most likely originated independently (Ryan et al. 2010). However, some of the *TaALMT1* alleles (presenting the same pattern of repeats but in a different number) are likely to have been derived from one another by unequal crossovers during recombination (Delhaize et al. 2007; Raman et al. 2008; Ryan et al. 2010). The Type VII allele (two repeats of blocks B-C) can be seen as a variant of Type VI allele that lost one B-C repeat (Raman et al. 2008); however, in this study, it appears to be a variant of Type I that acquired a new repeat because we found that the parents of Centelha, cultivars Centenário and Fronteira, had Type I and Type V alleles, respectively. Although one way to explain the origin of the Type VII promoter in Centelha is that it arose from a Type I allele, other explanations, such as heterozygosis and a mix-up of seeds, which have been detected in this study and by Raman et al. (2008), should also be considered.

A reduced amount of information is available for *TaMATE1B* alleles in comparison with the *TaALMT1* alleles. The transposon insertion in the *TaMATE1B* promoter region appears to extend the *TaMATE1B* expression to the root apex, where it confers citrate efflux and increases aluminum resistance (Tovkach et al. 2013). Constitutive citrate efflux from roots of the Brazilian wheat cultivars Carazinho, Maringa,

Toropi, and Trintecinco has been reported, while Portuguese wheat cultivars (Barbela 7/72/92 and Viloso Mole) showed different levels of that efflux (Ryan et al. 2009; Garcia-Oliveira et al. 2014). The transposon insertion in the *TaMATE1B* promoter was described for Carazinho (Tovkach et al. 2013) but not for another Brazilian cultivar, BH 1146 (Garcia-Oliveira et al. 2014). Here we confirmed that BH 1146 lack the transposon-like insertion in the *TaMATE1B* promoter. We also reported the presence of the insertion for the genotypes Carazinho, IAC 5 - Maringá, Toropi, and Trintecinco, the same ones that Ryan et al. (2009) detected constitutive citrate efflux. The insertion was not detected in Veranópolis and Fronteira, which did not present citrate efflux when analyzed by Ryan et al. (2009). In this way, the presence or absence of the *TaMATE1B* promoter insertion detailed here matched the citrate efflux results obtained by Ryan et al. (2009).

Nevertheless, discrepancies for the alleles of the other organic acid transporter gene, *TaALMT1*, were found for cultivar Trintani when compared to a previously published study (Raman et al. 2008) and for cultivar Trintecinco when comparing different seeds multiplied at the Embrapa Active Wheat Germplasm Bank. Besides that, discrepancies were also found for the genotypes Carazinho and Londrina that showed different *TaMATE1B* promoter alleles when seeds from different packets were analyzed. This is possibly due to the mix-up of seeds and misidentification and/or release of the cultivars in generations earlier than F_6 fixing different alleles in loci (especially for older cultivars—the years of release for Trintecinco and Trintani are 1936 and 1949, respectively), where some alleles could still be segregating. Regardless of the explanation, these discrepancies can lead to misinterpretations, and breeding programs should take that into consideration.

Although the Al^{3+} resistance/tolerance of Brazilian wheat has been traced to a small number of landraces introduced in South Brazil in the beginning of the twentieth century (de Sousa 1998) and the promoter alleles associated with Al^{3+} resistance in Brazilian cultivars were detected in European landraces possibly introduced in Brazil by Europeans immigrants (Raman et al. 2008), we were able to detect genetic diversity among internationally recognized Al^{3+} -resistant Brazilian wheat (Fig. 4). Some of these cultivars are more similar (Carazinho, Frontana, and

Fronteira, as well as BH1146 and IAC 5 - Maringá or Toropi, Trintani, and Trintecinco), and those genotypes are most likely to share similar Al^{3+} -resistance/tolerance mechanisms. That relationship reflects the cultivar pedigree since Fronteira is one of the genitors of Frontana (Fronteira/Mentana) while Frontana was used in the cross that generated Carazinho (Colonista/Frontana). Moreover, Trintecinco is one of the genitors of Trintani (Trintecinco/Guarany) while BH 1146 (PG 1//Fronteira/Mentana) and IAC 5 - Maringá (Frontana/Kenya 58//PG 1) share common ancestors. In fact, BH 1146, Frontana, IAC 5 - Maringá, and Trintecinco are among the most commonly used genotypes to generate Brazilian cultivars (de Sousa and Caierão 2014). Besides that diversity among recognized Al^{3+} -resistant cultivars, our analysis revealed that other genotypes (presenting *TaALMT1* promoters Type V or VI and the insertion in the *TaMATE1B* promoter) were quite different (Fig. 4), and these genetically diverse Al^{3+} -resistant cultivars are important for the improvement of Al^{3+} resistance or as a source for studying the Al^{3+} resistance mechanisms. The SSR markers used here are located in different chromosomes and genomes of the hexaploid wheat (Table 2), and one of them, wmc331, is located in the chromosome 4DL, and it was reported to be closely linked to the *TaALMT1* gene (Raman et al. 2005). In our analysis with these SSR markers, in which the highest number of Brazilian wheat genotypes has been genotypically analyzed, we were able to detect an average PIC of 0.60 (Table 2), indicating high genetic diversity among the analyzed genotypes. Although we used a small number of SSR markers, the polymorphism detected here is consistent with the results reported by Schuster et al. (2009), where a mean PIC of 0.49 was found.

In conclusion, for the first time a large number of Brazilian wheat genotypes was genotypically analyzed with markers linked to the *TaALMT1* and *TaMATE1B* promoter. A high proportion of *TaALMT1* promoter Types V and VI was found although the majority of the genotypes did not present the transposon insertion in the *TaMATE1B* promoter. Besides that, genotypes showing specific combinations of *TaALMT1* and *TaMATE1B* alleles presented higher root growth on acid soil. In this context, to increase the frequency of the insertion in the *TaMATE1B* promoter is a target to be pursued by different wheat-breeding programs in Brazil. Analysis using SSR markers

indicated an important genetic variability among different Al^{3+} -resistant sources and among genotypes harboring superior *TaALMT1* and *TaMATE1B* alleles. This information is useful for studies regarding Al^{3+} resistance mechanisms and for wheat breeders targeting an improvement in root growth under acidic soil.

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