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Characterization, genetic diversity, phylogenetic relationships, and expression of the aluminum tolerance *MATE1* gene in *Secale* species

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Abstract

Aluminum (Al) is the main limiting factor for crop production in acidic soils. Efflux of organic acids is one of the mechanisms that determine Al-tolerance, and an Al-activated citrate transporter (multidrug and toxic compound extrusion) *MATE1* gene is involved in different species. The contribution of the rye *MATE1* gene (*ScMATE1*) depends on the rye (*Secale cereale* L.) cultivars and the crosses analyzed; there is no information about different rye species. The cDNA sequences, phylogenetic relationships, Al-tolerance, citrate exudation, and expression of the *ScMATE1* gene were analyzed in several cultivars and wild species/subspecies of the *Secale* genus. Genotypes highly tolerant to Al were found within this genus. For the first time, sequences of the cDNA of the *ScMATE1* gene were isolated and characterized in wild ryes. At least two copies of this gene were found likely to be related to Al-tolerance. The sequence comparison of 13 exons of *ScMATE1* revealed variability between species, but also inter- and intra-cultivars. Variations were found in the Al-induced expression of *ScMATE1* gene, as well as its contribution to Al-tolerance. The pattern of citrate exudation was inducible in most of the species/subspecies studied and constitutive in few. The phylogenetic analysis indicated that *ScMATE1* is orthologue of two genes (*HvMATE1* and *TaMATE1*) involved in the Al stress response in barley and wheat, respectively, but not orthologue of *SbMATE*, implicated in Al-tolerance in sorghum. *ScMATE1* is involved in the response to Al stress in ryes, but its contribution to Al-tolerance is complex, and like in other species, there are tolerant and sensitive alleles in the different cultivars and species studied.

Additional key words: Al-activated citrate transporter, citrate exudation, cultivated and wild rye.

Introduction

Acidic soils are a worldwide problem for agriculture. One of reasons is that aluminum (Al) is solubilized at acid pH producing the toxic cation Al^{3+}, which can restrict plant growth. Some plant species have developed different mechanisms to tolerate Al^{3+} toxicity. Several physiological mechanisms of Al-tolerance have been proposed, but the agronomical efficacy of promoting yield stability on acidic soils remains uncertain (Hoekenga and Magalhães 2011). One of the mechanisms that determines the resistance of some species is the efflux of one or more organic anions (*e.g.*, citrate and malate) from the root tips to the soil. The genes controlling this trait are members of the Al-activated malate transporters (*ALMT*) and citrate transporters

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Abbreviations: ALMT - Al-activated malate transporter; fd - fold differences; MATE - multidrug and toxic compound extrusion (Al-activated citrate transporter); qPCR - quantitative polymerase chain reaction; RT-PCR - reverse transcription polymerase chain reaction; sqPCR - semiquantitative PCR.
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(*MATE*) families, which encode membrane proteins that facilitate organic anion efflux across the plasma membrane. Identification of these and other resistance genes provides an opportunity to enhance the Al-tolerance of plants by marker-assisted breeding or other biotechnological methods (Ryan *et al.* 2011).

Some members of MATE protein family, including AtMATE (from *Arabidopsis thaliana*), HvMATE1 (from *Hordeum vulgare*), OsFRDL4 (from *Oryza sativa*), SbMATE (from *Sorghum bicolor*), TaMATE (from *Triticum aestivum*) and ZmMATE (from *Zea mays*), are involved in Al-activated citrate secretion (Furukawa *et al.* 2007, Magalhães *et al.* 2007, Liu *et al.* 2009, Ryan *et al.* 2009, Maron *et al.* 2010, Tovkach *et al.* 2013).

Rye (*Secale cereale* L.), one of the most Al-tolerant cereal crops, secretes both citrate and malate from roots in response to Al, and the exudation pattern described is inducible (Li *et al.* 2000). Previous studies reported that the Al-tolerance of rye is high when compared with barley and wheat, even the relatively Al-sensitive rye inbred line Riodeva (Gallego and Benito 1997) is more Al-tolerant than the tolerant cultivars of barley and wheat.

Rye *ScALMT1* gene (Al-malate activated transporter) has been reported to be a candidate gene for the previously reported *Alt4* tolerance locus on chromosome *7RS* (Matos *et al.* 2005, Fontecha *et al.* 2007, Collins *et al.* 2008, Benito *et al.* 2010). Another candidate gene for Al-tolerance in rye is *ScMATE1* (homologue of *HvMATE1*), and although in some analyses its implication was not evident (Collins *et al.* 2008), experiments with different crosses reported cosegregation of this gene with a new QTL for Al- tolerance (Silva-Navas *et al.* 2012). Yokosho *et al.* (2010) isolated two genes, named *ScFRDL1* and *ScFRDL2*, also involved in Al-tolerance in rye. Both genes were mainly expressed in roots besides their different expression patterns. These authors found that the *ScFRDL2* gene might be involved in Al-induced secretion of citrate (ScFRDL2 has 80.6 % identity with OsFRDL2, a putative Al-responsive protein in rice), whereas the *ScFRDL1* gene should be implicated in the efflux of citrate into the xylem (*ScFRDL1* has 94.2 % identity with *HvMATE1* an Al-activated citrate transporter in barley). The comparison between both DNA and protein sequences, corresponding to *ScAACT1* and *ScFRDL1,* revealed a 100 % identity. The chromosomal location and identity of the sequences support that *ScMATE1*, *ScAACT1*, and *ScFRDL1* are the same genes. Thus, in order to avoid confusion with the nomenclature, we have decided hereafter to use the first name utilized for this gene in rye: *ScMATE1* (Collins *et al.* 2008).

Previous studies about the implication of the *MATE1* gene on Al-tolerance in rye indicate that some alleles confer tolerance and that others do not. Moreover, the variability and the expression of this gene has not been examined in the wild species/subspecies of the genus *Secale,* whose germplasm could be of interest for breeding programs. Therefore, in this work, we conducted different analyses to characterize the Al-tolerance and the *ScMATE1* gene expression in several species/subspecies of the genus *Secale*.

Material and methods

Plants: In this work, three wild species of the genus *Secale*, *S. strictum* (Persl) Persl ssp. *strictum* (R1211), *S. sylvestre* Host (R892), and *S. vavilovii* Grossh. (PI618682) were analyzed, as well as three subspecies of *Secale cereale* L.: *S. cereale* ssp. *ancestrale* Zhuk. (PI445975), the cultivated *S. cereale* ssp. *Cereal*, and *S. cereale* ssp. *segetale* Zhuk. (PI326284). Ryes of accessions with codes beginning with "PI" were kindly provided by The National Small Grains Collection (NSGC) of the United States Department of Agriculture - Agricultural Research Service (USDA-ARS) and those with "R" by Genebank Gatersleben, the Institute of Plant Genetic and Crop Plant Research (IPK). Within the cultivated ryes, two landraces (Lamego and Montalegre), the cultivar Imperial, and the inbred lines Riodeva (the germplasm bank of the Department of Genetics and Biotechnology at the University of Trás-os-Montes and Alto Douro collection, Vila Real, Portugal) and P105 (kindly supplied by Dr. A. Börner from IPK) were studied. In order to make it easier, the subspecies designation will be abbreviated in the text by *S. ancestrale*, *S. cereale, S. segetale*, and *S. strictum*.

Al-tolerance screening tests: The screening method described by Aniol and Gustafson (1984) and adapted by Gallego and Benito (1997) was used for the Al-tolerance characterization. Twenty seeds were disinfected in 0.1 % (m/v) $HgCl_2$ solution for 10 min and rinsed with de-ionized water. The seeds were germinated in Petri dishes in the dark at 4 °C overnight and then incubated at 25 °C for two days. Germinated seeds were transferred to a nylon mesh floating on a continuously aerated nutrient solution containing 0.4 mM $CaCl_2$, 0.65 mM KNO_3, 0.25 mM $MgCl_2 \cdot 6\,H_2O$, 0.01 mM $(NH_4)_2SO_4$, and 0.04 mM NH_4NO_3 (pH 4.0) and grown in a chamber at a temperature of 20 °C, an air humidity of 65 %, a 16-h photoperiod, and an irradiance of 150 µmol m^{-2} s^{-1}. Four days after sowing, the seedlings were incubated for 24 h in nutrient solution with 150 µM aluminum in the form of $AlK(SO_4)_2 \cdot 12\,H_2O$ (pH 4.0). After Al exposure, the seedlings were thoroughly washed with de-ionized water. Then, roots were stained with 0.1 % (m/v) aqueous solution of Eriochrome cyanine R for 10 min, and the excess of dye washed with de-ionized water. Subsequently, the seedlings were transferred to a fresh Al-free nutrient solution for 48 h (renewed daily). After recovery, the plants were classified as tolerant or sensitive according to their root re-growth.

RNA extraction, cDNA synthesis and sequencing: Seedlings of Lamego and Montalegre landraces, Riodeva inbred line, and of the five wild species/subspecies (7-d-old) were exposed to nutrient solution with 300 µM Al for 8 or 24 h. Root apices (1 cm) and leaves either exposed or not to Al, were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until use. Homogenization was carried out using *TissueLyser II* (*Qiagen*, Hilden, Germany) and 5 mm stainless steel beads (*Qiagen*). Total RNA was extracted from both roots and leaves of about 20 different plants per genotype and per Al exposure time (0, 8, and 24 h) using *TRIzol*® kit (*Invitrogen*, Carlsbad, CA, USA). RNA quality was checked by gel electrophoresis and then quantified with a *NanoDrop® ND-1000* spectrophotometer (*NanoDrop Technologies*, Wilmington, USA). Total RNA (2 µg) was reverse transcribed with a high capacity cDNA reverse transcription kit (*Applied Biosystems*, Wilmington, CA, USA). Total RNA (2 µg) was reverse transcribed with a *high capacity cDNA reverse transcription* kit (*Applied Biosystems*, Foster City, CA, USA) according to the conditions specified by the supplier.

For *ScMATE1* gene sequencing, only cDNAs obtained from roots of plants exposed to Al for 24 h were used. The full-length *ScMATE1* open reading frame (cDNA) was isolated with two primer pairs designed from the genomic *ScMATE1* sequence of rye lines Ailés and Riodeva (Silva-Navas *et al.* 2012, Table 1 Suppl.). The resultant PCR products were cloned into *pGEM-T-Easy* cloning kit (*Promega*, Madison, USA) following the manufacturer's protocol.

Sequence analyses: Sequences were analyzed with *Chromas Lite 1.0* (*Technelysium*, Brisbane, Australia). A *BLASTN* search (http://www.ncbi.nlm. nih.gov/) was performed to confirm the DNA and amino acid sequences predicted from the analysis. Alignments between different *ScMATE1* sequences were made using the *ClustalW* algorithm (http://www.ebi.ac.uk/Tools/clustalw). The sequences obtained in this work and other sequence data from different rye cultivars previously obtained (Silva-Navas *et al.* 2012) were compared. *DnaSP v. 5.1* (Librado and Rozas 2009) was used to calculate sequence diversity parameters. Several software programs were used to predict the secondary structures and membrane topologies of the different ScMATE1 proteins (Table 2 Suppl.).

Phylogenetic analyses: Phylogenetic relationships among different *MATE1* proteins and cDNA sequences were analyzed using *MEGA 4.0* (Tamura *et al.* 2007) probing different evolutionary distances (number of differences, *p*-distance, Jukes-Cantor, Kimura 2-parameter, Tamura 3-parameter, and maximum composite likelihood), amino acid substitution models (number of differences, *p*-distance, and Poisson correction) and clustering methods (neighbor-joining, minimum evolution, maximum parsimony, and UPGMA). Bootstraps with 10 000 replicates were performed to test the robustness of the dendrograms. *LOFT 2.2* software (Van der Heijden *et al.* 2007) was used to identify levels of orthology from trees (LOFTs).

Gene expression analysis by semi-quantitative (sq) and quantitative (q) real-time PCR: For expression studies, we used cDNAs obtained from roots and leaves of plants not exposed to Al (0 h) and exposed to Al for 8 or 24 h as described above. *ScMATE1* gene expression was determined by real-time qPCR (Silva-Navas *et al.* 2012, Table 1 Suppl.) using a primer pair, designed with *Primer Express® 2.0* software (*Applied Biosystems*). Primers for the housekeeping *18S* rye gene, described by Fontecha *et al.* (2007), were used as control. PCR reactions were performed in a total volume of 0.02 cm^3 containing 0.01 cm^3 of *Fast SYBR® Green Master Mix* (*Applied Biosystems*), 6.0 pmol of each primer, and cDNA dilutions made from ~ 200 ng of RNA. Reactions were carried out using a *7900 HT* fast real-time PCR system (*Applied Biosystems*) with the following program: one step at 95 °C for 20 s and 40 cycles at 95 °C for 1 s and 60 °C for 20 s. All PCR samples and controls were prepared in duplicate in 0.1 cm^3 *MicroAmp™* optical plates (*Applied Biosystems*). Normalizations and standard deviations calculations of the samples were made according to a relative standard curve.

The *ScMATE1* expression was also determined by sqPCRs using *18S* gene as reference. The analyses were conducted using mRNAs from the five wild species/subspecies of the genus *Secale*. To detect *ScMATE1* and *18S* mRNA, the primers utilized were the same as used in the qPCR. The sq PCR was performed in a 0.02 cm^3 reaction volume containing 0.002 cm^3 of cDNA, 0.002 cm^3 of each gene-specific primer, and 0.01 cm^3 of *Taq PCR MasterMix* (*Qiagen*), using the following program: an initial step at 95 °C for 3 min, 30 cycles at 94 °C for 20 s, at 60 °C for 30 s, and at 72 °C for 35 s, followed by a final extension at 72°C for 7 min. PCR products were visualized on 1 - 2 % Tris-acetate-EDTA (TAE) agarose gels.

Citrate exudation: Citric acid was determined using enzymatic methods described by Dagley (1974) and Delhaize *et al.* (1993). Citrate efflux from intact roots of seedlings of the five wild species/subspecies (*S. ancestrale*, *S. segetale*, *S. strictum*, *S. sylvestre*, and *S. vavilovii*) and three cultivated ryes (Imperial, Riodeva, and P105) was assayed with and without Al stress. Briefly, 20 seeds from each rye were sterilized with NaClO + distilled H_2O (1:1) for 40 min. Then, seeds were washed three times with sterile water, added to flasks containing 0.2 mM $CaCl_2$ (pH 4.3) and, finally, incubated on a rotary shaker (95 rpm) at 23 °C, a 16-h photoperiod, and an irradiance of 150 µmol m^{-2} s^{-1} for 6 d. Thereafter, the solution from flasks was decanted and the seedlings were rinsed three times with the same solution as mentioned above. Later, 0.05 mM $AlCl_3$ (pH 4.3) was added to half of the seedlings. Root exudates were collected after 3, 6, and 24 h.

Number of repetitions and statistics: Screening for Al tolerance was repeated two times with the same conditions. Expression studies and citrate quantification were performed with three biological replicates for each rye sample and treatment. Analysis of variance (ANOVA) was performed using the *SPSS* statistical package for *Windows* (*v. 23.0*; *IBM Corp*., Armonk, NY, USA). Significant differences between means were determined using the Tukey test.

Results

A great variability for aluminum tolerance was found both between and within the rye species/subspecies and cultivars studied. The Al-tolerance screening method (Table 1) allowed the classification of the different rye plants as tolerant or sensitive to Al stress. Within *S. cereale*, the genotypes Montalegre, Lamego, Imperial, and P105 were classified as Al-tolerant similarly as the wild ryes *S. ancestrale*, *S. segetale*, and *S. vavilovii*. Compared with these ryes, *S. strictum* was classified as moderately Al-tolerant. On the other hand, the inbred line Riodeva and *S. sylvestre* were classified as Al-sensitive (Fig. 1 Suppl.).

Table 1. Mean lengths of root re-growth (MLRR) as a measure of Al-tolerance of the plant material used in this work. Means ± SEs, $n = 20$. Different letters indicate significant differences at $P < 0.05$, according to Tukey's test.

Rye species	Classification	MLRR [mm]
S. ancestrale	wild subspecies	5.49 ± 3.87b
S. segetale	wild subspecies	6.62 ± 4.15bc
S. strictum	wild subspecies	3.14 ± 3.08ab
S. sylvestre	wild species	0.13 ± 0.25a
S. vavilovii	wild species	5.13 ± 4.17b
S. cereale		
Imperial	cultivar	6.04 ± 5.5b1
Lamego	landrace	14.03 ± 8.06d
Montalegre	landrace	10.43 ± 7.79cd
P105	inbred line	12.30 ± 4.10d
Riodeva	inbred line	0.03 ± 0.07a

We obtained six different cDNA sequences in *S. cereale* (four from Lamego and two from Montalegre) and 13 sequences corresponding to wild *Secale* species/subspecies (two from each of *S. segetale*, *S. strictum*, and *S. sylvestre*, three from *S. ancestrale* and four from *S. vavilovii*). The sequences of the *ScMATE1* gene obtained in this study were deposited in Genbank (accessions Nos. KX632094 - KX632112). The sequence comparisons revealed intra-cultivar, inter-cultivar, intraspecific and interspecific variability. In all the ryes analyzed, a 1 665 bp coding region (including the stop codon) was found for the *ScMATE1* gene. In *S. sylvestre*, in addition to the 1 665 bp sequence, another sequence of 1 668 bp was found.

We have compared 11 different cDNA sequences of *ScMATE1* in *S. cereale*, the six mentioned above, and five (one from each of Ailés, Imperial, Linea V, Petkus, and Riodeva) from the previous work of Silva-Navas *et al.* (2012) (Table 3*A* Suppl.). Comparisons among these different *ScMATE1* sequences revealed two INDELs (insertion/deletion) of three bp each that were only observed in the exon 1 of Riodeva. Taking into account the values of nucleotide diversity (ND - 0.00755), haplotype diversity (Hd - 1) and the average number of nucleotide differences (k - 12.564), the most variable exons were 1, 4, and 8, with exon 1 displaying clearly the highest values for these diversity indexes, whereas the less variable exons were 7 and 13. In order to study the variability of the *ScMATE1* gene and its exons among different species/subspecies of *Secale*, six cDNA sequences were compared: five from wild species/ subspecies and one from cultivated rye (*S. cereale*), represented by the cultivar Imperial (Table 3*B* Suppl.). The results obtained indicated that, considering the values of ND (0.01842), Hd (1), and k (30.667), the *ScMATE1* most diverse exons in the *Secale* genus were 1, 8, and 11 and the least variable exons were 7, 1, 2 and 13. A unique INDEL, with 3 bp, was observed in the exon 1 of *S. sylvestre,* while variation for the same INDEL was found among *S. cereale* cultivars. Taking only into account the changes in the coding region, exon 1 was the most variable, both in *S. cereale* and in the whole *Secale* genus.

The variability analysis of the proteins encoded by *MATE1* gene was made using amino acid sequences from the five wild species/subspecies and two *S. cereale* cultivars (Imperial and Riodeva). The deduced proteins comprised 554 (*S. ancestrale*, *S. segetale*, *S. strictum*, *S. vavilovii*, and cv. Imperial), 555 (*S. sylvestre*), and 556 (Riodeva) amino acid residues, with a molecular masses between 58.3 and 58.4 kDa. The proteins were hydrophobic, containing the characteristic MatE domain of MATE family. Depending on the protein structure prediction software used, seven to eleven putative transmembrane helix regions (TMH1 to TMH11) were found (Fig. 2 Suppl., Table 2 Suppl.). Seven of these transmembrane helices (TMH3, TMH4, TMH5, TMH6, TMH7, TMH8, and TMH10) were predicted by all programs used.

The phylogenetic relationships among 46 MATE proteins from different plant species were established. ScMATE1 predicted proteins shared 93 % identity with HvAACT1 barley protein, 90 % with OsFRDL1, and 68 % with OsFRDL4 rice proteins, 66 % with SbMATE sorghum protein, 60 % with AtMATE and AtFRD3 *Arabidopsis* proteins. The predicted ScFRDL1 rye protein of Yokosho *et al.* (2010) was identical (100 % identity) to the predicted ScMATE1 protein in this study;

therefore, both are included in the dendrogram with the same name (ScMATE1). The different methods of distance calculations for protein sequences and the different clustering methods used to obtain the

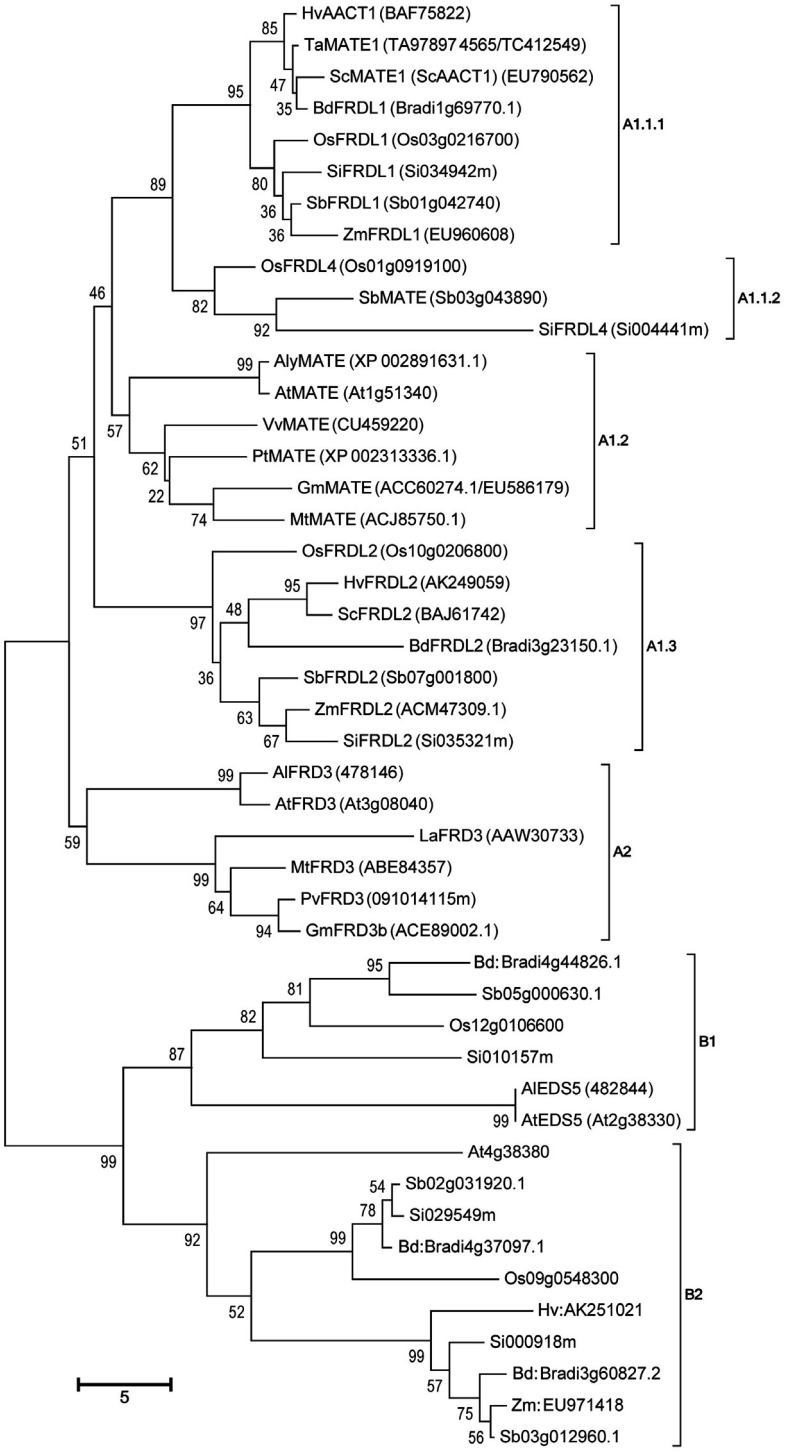

Fig. 1. Phylogenetic relationships obtained with hypothetical proteins from MATE family. Al - *Arabidopsis lyrata*, At - *Arabidopsis thaliana*, Bd - *Brachypodium distachyon*, Gm - *Glycine max*, Hv - *Hordeum vulgare*, La - *Lupinus albus*, Mt - *Medicago truncatula*, Os - *Oryza sativa*, Pv - *Phaseolus vulgaris*, Pt - *Populus trichocarpa*, Sc - *Secale cereale*, Sb - *Sorghum bicolor*, S - *Setaria italica*, Ta - *Triticum aestivum*, Vv - *Vitis vinifera*, and Zm - *Zea mays*. The evolutionary distance and the cluster method used were *p*-distance and neighbor-joining, respectively. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches. The brackets indicate groups of orthologous genes obtained using *LOFT 2.2* software.

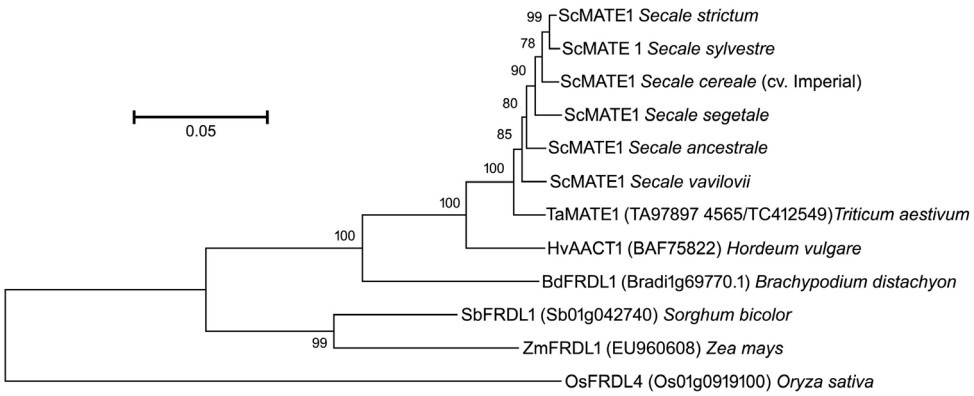

Fig. 2. Phylogenetic relationships obtained for coding region of the *ScMATE1* gene from different species/subspecies of the genus *Secale* compared with the orthologous cDNA from *H. vulgare*, *O. sativa*, *S. bicolor*, *T. aestivum*, and *Z. mays*. The evolutionary distance and the cluster method used were Kimura two parameters and neighbor-joining, respectively. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (10 000 replicates) are shown next to the branches.

phylogenetic trees of the MATE protein family, gave dendrograms with identical structure and very high bootstrap values. In all cases, two main clusters (A and B) were defined, both including proteins from monocot and dicot species (Fig. 1). Cluster A appears divided into two subclusters: A1 and A2, and the former subdivided again into several groups and subgroups. The MATE proteins of the families MATE1 (FRDL1) and MATE2 (FRDL2) belong to different groups of subclusters A1, whereas MATE3 (FRDL3) proteins are grouped in the subcluster A2. Subcluster B is also divided in two groups: B1, including MATE proteins from six different species, and B2, including MATE proteins from ten different species. This analysis revealed the existence of seven different groups of orthologous genes. With the construction of a more complete dendrogram, including 74 additional MATE protein sequences from the plant molecular database *Phytozome.net* (Fig. 3 Suppl.), the structure obtained was similar to the previous one (Fig. 1).

In order to facilitate the *ScMATE1* phylogenetic analysis among the species/subspecies of the genus *Secale*, only one cDNA or protein sequence from each species/subspecies was used (Fig. 2). As all *S. cereale* plants gave identical results, the cultivar Imperial was used as representative of the cultivated species. The cDNAs and amino acid sequences from *B. distachyon*, *H. vulgare*, *O. sativa*, *S. bicolor*, *T. aestivum*, and *Z. mays* were used as an outgroup. The dendrograms obtained from cDNAs and the ones obtained from proteins showed the same structure and, repeatedly, bootstrap values were very high. All the species/subspecies of the genus *Secale* have grouped in the same cluster. In addition, wheat, barley, and *Brachypodium* grouped together in the same cluster as the genus *Secale*.

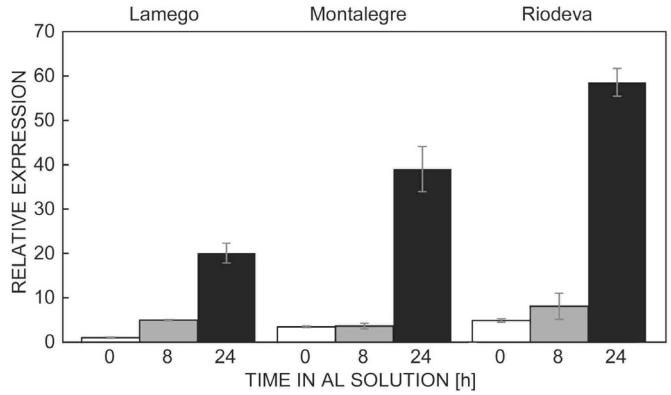

Fig. 3. Real-time qPCR showing expression patterns of root tip cDNA transcripts of *ScMATE1* gene in *S. cereale* two landraces (Lamego and Montalegre) and the inbred line Riodeva after 300 µM Al treatment. The change (fold difference) at each time point (0, 8, and 24 h) is expressed as the relative expression compared to the least fluorescence signal (Lamego without Al stress, 0 h). Significant differences among cultivars were found ($P < 0.05$).

On the first approach, the expression of the *ScMATE1* gene in roots of *S. cereale* plants (Lamego, Montalegre, and Riodeva), not treated (0 h) and exposed to Al for 8 h and 24 h, was studied. Previous studies (data not shown) showed little amount of *ScMATE1* mRNA in the leaves of *S. cereale* cultivars so that we have not included leaves

in this early study. Furthermore, the same expression values were obtained at 0, 8, and 24 h without Al which made us to use only the 0 h time point. The results indicate that there was no significant induction of the *ScMATE1* expression at 8 h in the roots of the cultivars. However, after 24 h of Al exposure, this gene was clearly expressed in Lamego, Montalegre and inbred line Riodeva with a 20, 13.5, and 12-fold difference (fd) compared to control without Al treatment (0 h) (Fig. 3). Riodeva showed the highest amount of mRNA (59-fold) and Lamego showed the smallest one (20-fold).

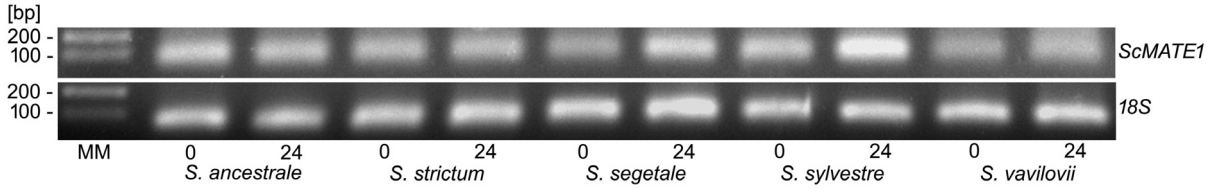

Fig. 4. Semi-quantitative PCR results showing temporal expression patterns of root tip cDNA transcripts of the *ScMATE1* gene from *S. ancestrale*, *S. strictum*, *S. segetale*, *S. sylvestre*, and *S. vavilovii* without Al (0 h) and with 300 μM Al for 24 h. Below is the expression of the housekeeping gene *18S* used as a reference.

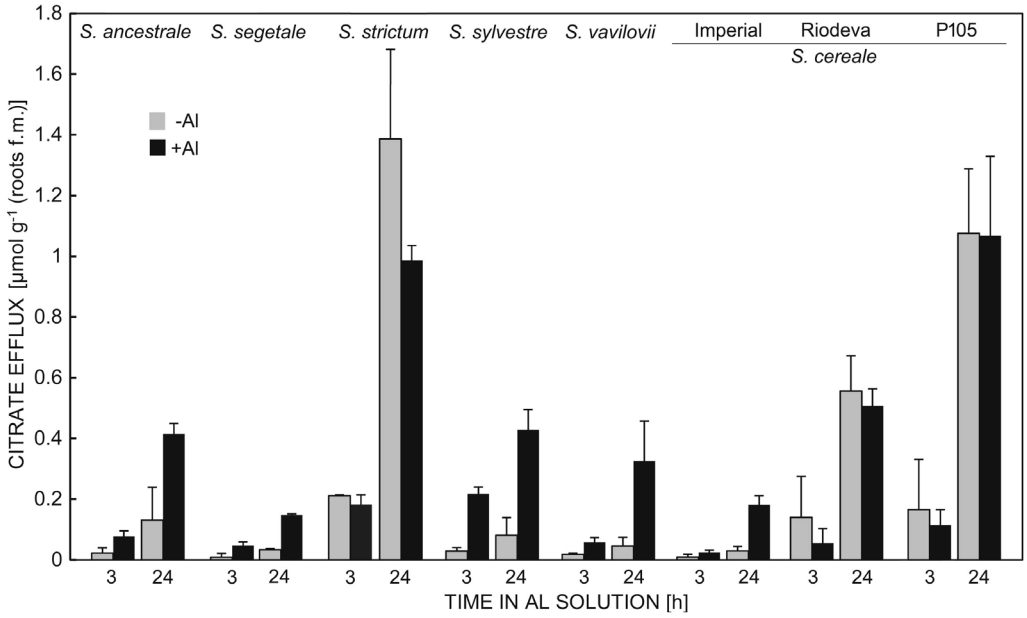

Fig. 5. Citrate exudation of *S. ancestrale*, *S. segetale*, *S. strictum*, *S. sylvestre*, *S. vavilovii*, *S. cereale* cv. Imperial and two inbred lines (Riodeva and P105). The seedlings were incubated in hydroponic culture with 0 or 0.05 mM $AlCl_3$. Data of root exudates collected at 3 and 24 h. Data are the means ±SDs, $n = 10$. Significant statistical differences were found among rye species ($P < 0.001$). Comparing with and without Al in each species, significant differences were found in *S. ancestrale* ($P < 0.05$), *S. segetale*, *S. sylvestre,* and Imperial ($P < 0.01$).

Taking into account the great differences observed between plants not treated (0 h) and plants exposed to Al for 24 h, the study of the *ScMATE1* expression in the other five species/subspecies of *Secale* was done. Since there is no data of this gene expression in the wild ryes, both leaves and roots were analyzed. The amount of *ScMATE1* mRNA was significantly higher in roots than in leaves, both in absence (0 h) and in presence of Al (24 h) in the five wild species/subspecies of *Secale*. Similar results were observed by the semi-quantitative analyses (data not shown). The ratio root/leaf detected without Al was 3.33 (*S. ancestrale*), 8.92 (*S. segetale*), 2.70 (*S. strictum*), 5.43 (*S. sylvestre*), and 8.30 (*S. vavilovii*), whereas after the treatment with Al, it was 5.30 (*S. ancestrale*), 11.99 (*S. segetale*), 1.99 (*S. strictum*), 8.75 (*S. sylvestre*), and 6.57 (*S. vavilovii*) (data not shown). The results obtained comparing plants without Al, as controls, and plants exposed to Al for 24 h indicated that the mRNA of *ScMATE1* was not induced in the roots of *S. ancestrale* (1.18 fd), *S. strictum* (1.15 fd), and *S. vavilovii* (1.19 fd) and moderately induced in *S. sylvestre* (2.10 fd) and *S. segetale* (1.73 fd) (data not shown). The data of real-time qPCR agrees with the results of sqPCR analyses (Fig. 4). The amount of mRNA of *ScMATE1* in the roots of the different rye species/subspecies, with and without Al, was very similar, except in *S. sylvestre* and *S. segetale* where a clear increase was observed in the mRNA amount, when exposed to Al (Fig. 4).

The exudation of citrate (Fig. 5) started after a lag

phase of several hours of Al exposure (usually started after 6 h – data not shown) in *S. ancestrale*, *S. vavilovii*, and *S. cereale* cv. Imperial. On the other hand, *S. sylvestre* and *S. segetale* began to exudate citrate immediately after contact with Al. After 24 h of Al exposure, induced root citrate exudation was observed in all species/subspecies. In the case of the inbred lines Riodeva and P105, the amount of citrate exudates, with and without Al, were the same over time being the citrate exudation clearly constitutive in both lines. *S. strictum* had a constitutive citric acid exudation too since the amount of his exudates was higher without Al.

Discussion

Soil acidity is a worldwide problem and when associated with Al toxicity, it is one of the main factors limiting crop production. The first injury of Al toxicity is the inhibition of the development of the root system. The root regrowth observed for Riodeva and Imperial agree with the previous data obtained by Gallego and Benito (1997) and Silva-Navas *et al.* (2012), respectively. As in previous studies (Pinto-Carnide and Guedes-Pinto 1999, Silva *et al.* 2012), the landraces Montalegre and Lamego exhibited a great Al-tolerance. The wild ryes and the inbred line P105, not previously studied, mostly showed a high degree of Al-tolerance with the exception of the *S. sylvestre*.

A great variability was observed for Al-tolerance in cultivated rye and wild rye, being intra- and inter-cultivar and intra- and inter-specific. With the exception of *S. sylvestre* and *S. vavilovii*, all the rye species/subspecies are allogamous, with heterozygous genotypes, which leads to the existence of a wide diversity of tolerant and sensitive genotypes. In the autogamous species *S. sylvestre* and the inbred lines of *S. cereale* this diversity was not found. All the plants of *S. sylvestre* were sensitive and all the plants of inbred line P105 were tolerant. Pinto-Carnide and Guedes-Pinto (1999) also observed variability in Al-tolerance among their rye as well as wheat cultivars.

Rye has a huge importance in Al-tolerance approaches, due to its high capacity to tolerate Al, as shown in the present study. The selection of crops well adapted to acidic soils is important, therefore, tolerant cultivated ryes and wild ryes prove to be a potential resource for genetic improvement programs, especially the autogamous species and the inbred lines.

Nineteen sequences isolated (13 from wild ryes and six from cultivated ryes) were similar to those previously obtained by Silva-Navas *et al.* (2012), who also found a 1 665 bp coding region of the *ScMATE1* gene in various cultivars. However, one of the sequences of *S. sylvestre* obtained had a 1 668 bp coding region. Silva-Navas *et al.* (2012) also found in Riodeva an additional cDNA with a coding region of 1 671 bp. Both results suggest that *ScMATE1* has, at least, two copies in the wild species *S. sylvestre* and in the inbred line Riodeva. Several copies of the *ZmMATE1* gene were also detected in tolerant lines of maize (Maron *et al.* 2013).

The difference in the cDNA length was found in the exon 1, where Riodeva and *S. sylvestre* showed two and one 3 bp insertions, respectively. The INDEL found in *S. sylvestre* is the same as one of the INDELs found in Riodeva. As none of these insertions changes the reading frame, the only difference in the predicted proteins is the presence of two additional amino acids in the inbred line and only one in the wild species. The detection of these two different cDNAs cannot be attributed to heterozygosity, since both types of plants are homozygous: Riodeva is an inbred line with more than forty generations of self-pollination, and *S. sylvestre* is an autogamous species. Curiously, both ryes are classified as Al-sensitive, and these insertions may have a potential relation with Al sensitivity/tolerance. Santos *et al.* (2016) also found a sequence with two 3 bp insertion (the same as in the exon 1 of the *ScMATE1* gene in Riodeva) in a sensitive genotype of *S. vavilovii*, which reinforces this point. Maron *et al.* (2013) found a correlation between the three copies of the *ZmMATE1* gene and the Al-tolerance in maize.

A great genetic variability was observed in the *ScMATE1* cDNA sequences within the subspecies *S. cereale* and even higher in the whole *Secale* genus (Table 3 Suppl.). This variability is important to reduce the vulnerability to biotic and abiotic stresses, which allows rye adaptation to adverse environments. Moreover, it has a high selection value that can be exploited through breeding programs to improve the yield/performance of related crops, and even within *Secale*, since this genus is one of the most tolerant to Al stress.

The results of the variability analysis of the deduced proteins from *ScMATE1* gene agrees with those previously obtained by Silva-Navas *et al.* (2012) with cultivated ryes. The deduced protein sequences of the *ScMATE1* alleles differed at 76 residues (28 TNSCs – total numbers of synonymous changes, 46 TNRC – total numbers of replacement changes and two insertions). All the changes detected in the different ScMATE1 proteins do not alter the transmembrane structure of this protein. Therefore, the main function of putative proteins (the citrate transport) is probably not affected.

The phylogenetic relationships among the deduced MATE1 proteins grouped the genes involved in the Al-tolerance of sorghum (*SbMATE*), rice (*OsFRDL4*), and barley (*HvMATE1* - *HvAACT1*) in the cluster A1.1 (Fig. 1) (Furukawa *et al.* 2007, Magalhães *et al.* 2007, Wang *et al.* 2007, Yokosho *et al.* 2011). However, the intron-exon structure of these three genes is quite different. The *OsFRDL1* gene of rice and the *ScMATE1* gene of rye share the same intron-exon structure as their orthologous barley gene (13 exons and 12 introns).

Moreover, according to this, the phylogenetic relationships obtained indicate that sorghum and barley genes are not orthologous, since they are clustered in different subgroups (A1.1). The orthologous of *HvMATE1* would be the sorghum *SbFRDL1* as both genes are in the same subgroup (A1.1.1) and share the same exon-intron structure. The *TaMATE1*, *ZmMATE1* (*ZmFRDL1*), *ScMATE1* and *BdMATE1* (*BdFRDL1*) genes have also been implicated in the Al-tolerance in wheat, maize, cultivated rye, and *Brachypodium distachyon*, respectively (Ryan *et al.* 2009, Maron *et al*. 2010, Silva-Navas *et al.* 2012, Contreras *et al.* 2014). However, there are no publications about the implication of the *SbFRDL1*, *SiFRDL1*, and *OsFRDL1* genes in the tolerance of their respective species. All the *MATE* genes from the A1.1.1 subgroup are probably orthologous of *HvMATE1*, and therefore have a similar function. This hypothesis is also supported by the synteny relationships. The region of the *B. distachyon* chromosome 1*,* where the *BdFRDL1* gene is located, is syntenic with the region of the rice chromosome *3,* that harbors the *OsFRDL1* gene. In addition, there are synteny relationships with the chromosome arms 4HL of barley (*HvMATE1*), 4BL of wheat (*TaMATE1*, putative location), and 7RS of rye (*ScMATE1*) (Naranjo *et al.* 1987, Gale and Devos 1998, Collins *et al.* 2008, Silva-Navas *et al.* 2012). The genes *OsFRDL4*, *SiFRDL4*, and *SbMATE* appear in a different subgroup (A1.1.2). In this case, the synteny also agrees with the orthology relationships; chromosome 1 of rice (*OsFRDL4*) is syntenic to the chromosome 3 of sorghum (*SbMATE*).

On the other hand, in the subcluster A1.3, the genes *OsFRDL2* (rice), *ScFRDL2*/*ScMATE2* (rye), *ZmFRDL2*/ *ZmMATE2*) (maize), and *BdFRDL2*/ *BdMATE2* (*B. distachyon*) were related to Al-tolerance, but only the rice and rye genes were involved in Al-induced citrate secretion (Maron *et al*. 2010, Yokosho *et al.* 2010, 2016, Contreras *et al.* 2014). Therefore, all the genes of this group are probably orthologous and could have a similar function. The *Arabidopsis AtMATE* gene (A1.2) has been related with the release of citric acid in response to Al-stress and is probably orthologous of the genes of the same group. Also, this group is more similar to the MATE1 group (A1.1) rather than the one of FRDL2 proteins (A1.3). The genes of subcluster A2 are probably orthologous to the *AtFRD3* gene of *A. thaliana*.

The taxonomy of the genus *Secale* is questioned, as different phylogenetic relationships have been obtained depending on the markers used. For this reason, the number of species proposed for this genus ranged from three to 14 (Stutz 1972, Chikmawati *et al.* 2005). In our case, all the *Secale* species/subspecies studied appear as monophyletic (Fig. 2). The wild species *S. sylvestre* and *S. strictum* are the most closely related and are both close related to the cultivated *S. cereale*. The wild rye *S. vavilovii* is the phylogenetically more distant species. Our data agrees with previous reports on various aspects: *1*) a close relationship between *S. sylvestre* and *S. strictum* has been described in different works (De Bustos and Jouve 2002, Chikmawati *et al.* 2005, Shang *et al.* 2006, Ren *et al.* 2011). *2*) *S. strictum* is considered the direct antecessor of *S. cereale* (Vences *et al.* 1987, De Bustos and Jouve 2002, Zhou *et al.* 2010, Santos *et al.* 2016). *3*) The wild subspecies *S. segetale* and *S. ancestrale* showed proximity among them and with *S. cereale* in the studies of De Bustos and Jouve (2002), Chikmawati *et al.* (2005), and Shang *et al.* (2006). These wild ryes are considered subspecies of *S. cereale* by Khush (1962) and Cuadrado and Jouve (2002). *4*) Although *S. vavilovii* is an autogamous species, different studies revealed the existence of intraspecific variability and their closely relationship with *S. cereale* (Shang *et al.* 2006, Fu *et al.* 2010, Santos *et al.* 2016). The phylogenetic relationships obtained, among the different members of the family *Poaceae*, (Fig. 2) agree with the previous data (Fig. 1) from these grasses with rye, wheat, barley, and *Brachypodium* grouped in the same cluster.

The exudation of organic acids, the most recognized mechanism responsible for Al-tolerance, is mediated by transporters that belongs to the two different gene families *ALMT* (malate) and *MATE* (citrate), both located in the plasma membrane. As referenced in the introduction of this work, the *MATE1* gene has been related to Al-tolerance in several crops. Since there is no data in wild ryes concerning the *MATE1* gene, and different alleles of cultivated ryes showed a different contribution to Al-tolerance, we decided to study the expression of this gene and the citrate exudation.

Citric acid is more efficient than malic acid in preventing Al-induced inhibition of root elongation (Delhaize *et al.* 1993, Basu *et al.* 1994, Ma *et al.* 1997). However, the most effective gene involved in Al-tolerance up to date codifies an Al-activate malate transporter protein (ALMT) in wheat, rye, and *Arabidopsis*. Organic acids can be exudate immediately after the onset of Al treatment – type I (Li *et al.* 2000, Furukawa *et al.* 2007, Ryan *et al.* 2009) or after a lag phase of several hours – type II (Magalhães *et al.* 2007, Liu *et al.* 2009, Yokosho *et al.* 2011), as described in rye (Li *et al.* 2000).

One important finding of our research is the pattern of citrate exudation being variable in *S. cereale* and in the genus *Secale* (Fig. 5). The Riodeva and P105 inbred lines of *S. cereale* and the wild subspecies *S. strictum* showed a constitutive pattern (their roots exudate citrate with as well as without Al), whereas the citrate exudations of the rye cv. Imperial and the other *Secale* wild species/subspecies were Al-inducible. However, Riodeva and *S. sylvestre* are Al-sensitive and the remaining *Secale* species/subspecies and cultivars are tolerant. These results mean that in *Secale* Al does not always induce the exudation of citrate, as it has been previously described. Furthermore, there is not a clear correlation between the Al-tolerance and the amount of citrate exuded in these ryes (Fig. 5). The amount of organic acids released varies per crop species, which makes complex the comparison of exudates amount between the tolerant and sensitive

ryes at study, since most of them belong to different *Secale* species/subspecies.

The two different citrate exudation timing patterns were observed: in *S. sylvestre* and *S. segetale* it started immediately after Al exposure (pattern I), whereas in *S. ancestrale*, *S. vavilovii* and cv. Imperial it was delayed for at least 6 h (pattern II). It was described that plants belonging to pattern II may require genes for the enhanced Al-tolerance. Probably, this possible lack of correlation between the Al-tolerance and the citrate exudation could be explained considering that the *ALMT1* gene is the most important for Al-tolerance in rye, and its presence can mask the action of the *MATE1* gene, or else, another *MATE* gene can be involved in the citrate transporter induced by Al.

The expression of *ScMATE1* in the genus *Secale* was significantly higher in roots than in leaves. In the same way, the *ScMATE1* (*ScFRDL1*), *HvMATE1* (*HvAACT1*), *BdMATE1*, and *TaMATE1* genes are also mainly expressed in the roots of cultivated rye, barley, *B. distachyon*, and wheat, respectively (Yokosho *et al.* 2010, Fujii *et al.* 2012, Tovkach *et al.* 2013, Contreras *et al.* 2014). This fact may be a sign that the *ScMATE1* gene is involved in the Al-tolerance of ryes since roots are the main target for Al toxicity and this region is expected to express the genes contributing in Al resistance.

Silva-Navas *et al.* (2012) found in one F_2 that an allele of the *ScMATE1* contributes to Al-tolerance. In addition, they detected that this gene is clearly Al-inducible in Imperial, Riodeva, 2672/4, and Petkus and that it is not induced in Ailés, all cultivated ryes. Our results concerning *ScMATE1* induction in Riodeva (Fig. 3) agree with results obtained by those authors. The *ScMATE1* gene is clearly induced in Lamego (20 fd) and Montalegre (13.5 fd) (Fig. 3), poorly induced in *S. sylvestre* (2.10 fd) and *S. segetale* (1.73 fd), and not induced in *S. ancestrale* (1.18 fd), *S. strictum* (1.15 fd) and *S. vavilovii* (1.19 fd) (Fig. 4). However, a different allele of *ScMATE1* does not contribute to Al-tolerance in the rye lines analyzed by Collins *et al.* (2008). Another allele from an inbred line is induced by Fe deficiency rather than the presence of Al, as Yokosho *et al.* (2010) detected; these authors suggested that *ScMATE1* is involved in the citrate efflux into the xylem important for Fe translocation. Different works with different species indicate that the induction or non-induction of a gene is not necessarily indicative of its implication in the Al-tolerance. The *TaALMT1* gene of wheat, related to Al-tolerance, is constitutively expressed in roots of Al-tolerant and Al-sensitive lines (Sasaki *et al.* 2004, Delhaize *et al.* 2007).

There is not a direct relation between Al-tolerance and the expression of the *ScMATE1* gene as there is not the relation between the expression of this gene and the citrate exudation. Citrate exudation was relatively high and constitutive in the sensitive Riodeva, but the expression of *ScMATE1* was higher in Al presence, indicating that this gene is not the only one involved in citrate exudation. The tolerant Imperial and *S. segetale* showed an inducible citrate exudation, being coincident with an increase in *ScMATE1* expression. This leads us to suggest that the *ScMATE1* gene is involved in the Al-tolerance in these two ryes. Both ryes exuded little amount of citrate but was highly induced by Al, and, as previously stated, citric acid is efficient at minor quantities (Ma *et al.* 1997). Conversely, the tolerant *S. ancestrale* and *S. vavilovii* had an inducible citrate secretion, but the *ScMATE1* gene was not induced, indicating that this gene is not the only one involved in it. The constitutive expression of the gene can interfere with the Al-tolerance of these wild ryes. The tolerant *S. strictum* had a constitutive citrate exudation pattern, with no alteration in expression of the *ScMATE1* gene, whereas the sensitive *S. sylvestre* had an inducible citrate exudation and a low increment in the *ScMATE1* gene expression. *S. strictum* exhibited the highest amount of citrate exudates both with and without Al, which could be related to its tolerance to Al stress.

The data obtained indicate that there exists a great variability in the expression of *ScMATE1* gene within *S. cereale*, as among different *Secale* species/subspecies. This could be related with the high variability found for this gene. In *S. cereale* plants, this gene showed a larger expression than in wild species. This could be due to an adaptation of the cultivated ryes to acid soils that allows the evolution to genotypes with better Al-tolerance. Lamego and Montalegre are landraces that come from the Northeast Portugal and the Imperial cultivar derived from North America where, in both cases, acid soils are abundant.

It was found that Al-induced expression of Al resistance genes was positively regulated by a transcription factor member of the C2H2-type zinc-finger family, sensitive to proton rhizotoxicity 1 (AtSTOP1) in *Arabidopsis* (Iuchi *et al*. 2007) and an Al resistance transcription factor (OsART1) in rice (Yamaji *et al*. 2009). Although *AtSTOP1* is not induced by Al, it is very important in the Al-tolerance, since when it is not expressed in the *AtSTOP1* mutant, the Al-tolerance of the plant decreases drastically. In turn, *OsART1* is constitutively expressed in the roots, and its expression is not affected by Al treatment. The expression of the Al-tolerance genes *OsFRDL2* and *AtMATE* are regulated by OsART1 and AtSTOP1, respectively (Liu *et al.* 2009, Yamamoto *et al.* 2016),

Variations in the promoter and downstream regions of *MATE* genes could be associated with enhanced Al-tolerance. The insertion of multiple miniature inverted-repeat transposable elements (MITEs) in the sorghum *SbMATE* promoter (Magalhães *et al.* 2007) and a 1-kb insertion in the upstream of the *HvMATE1* coding region in barley (Fujii *et al.* 2012) has been correlated with Al-tolerance. In addition, insertion of MITEs in the downstream region of *ScMATE1* gene has been described in rye, but their implication in tolerance has not been demonstrated (Silva-Navas *et al.* 2012).

There are at least three ways to recognize if a gene is

implicated in Al-tolerance: one is the observation of an increase in Al-tolerance of transgenic plants; the second, to have a QTL or a major locus with Mendelian inheritance for Al-tolerance co-segregating with the candidate gene in a cross; and at last, to possess a knockout mutant. Silva-Navas *et al.* (2012) have detected a QTL for Al-tolerance co-segregating with *ScMATE1* gene.

Our results, together with previous data obtained in rye by other authors, suggest that the *ScMATE1* gene is involved in cultivated rye and wild rye Al-tolerance, however, not all alleles of *ScMATE1* contribute to Al-tolerance like in other species.

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