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ORIGINAL PAPER

Candidate gene identification of an aluminum-activated organic acid transporter gene at the *Alt4* locus for aluminum tolerance in rye (*Secale cereale L.*)

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Abstract Among cereal crops, rye is one of the most tolerant species to aluminum. A candidate gene approach was used to determine the likely molecular identity of an Al tolerance locus (*Alt4*). Using PCR primers designed from a wheat aluminum tolerance gene encoding an aluminum-activated malate transporter (*TaALMT1*), a rye gene (*ScALMT1*) was amplified, cloned and sequenced. Subsequently, the *ScALMT1* gene of rye was found to be located on *7RS* by PCR amplification using the wheat–rye addition lines. SNP polymorphisms for this gene were detected among the parents of three F_2 populations that segregate for the *Alt4* locus. A map of the rye chromosome *7R*, including the *Alt4* locus *ScALMT1* and several molecular markers, was constructed showing a complete co-segregation between *Alt4* and *ScALMT1*. Furthermore, expression experiments were carried out to clarify the function of this candidate gene. Briefly, the *ScALMT1* gene was found to be primarily expressed in the root apex and upregulated when aluminum was present in the medium. Five-fold differences in the expression were found between the Al tolerant and the Al non-tolerant genotypes. Additionally, much higher expression was detected in the rye genotypes than the moderately tolerant "Chinese Spring" wheat cultivar. These results suggest that the *Alt4* locus encodes an aluminum-activated organic acid transporter gene that could be utilized to increase Al tolerance in Al sensitive plant species. Finally, *TaALMT1* homologous sequences were identified in different grasses and in the dicotyledonous plant *Phaseolus vulgaris*. Our data support the hypothesis of the existence of a common mechanism of Al tolerance encoded by a gene located in the homoeologous group four of cereals.

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Introduction

Aluminum (mainly existing as Al^{3+}, the trivalent cationic form) is very toxic to many crops (Foy et al. 1978; Rao et al. 1993; Kochian 1995). This light metal is the main factor that limits productivity in acidic soils that comprise approximately 30–40% of the world's arable soils and up to 70% of potentially arable land (Aniol et al. 1980; Haug 1984). The major symptom of Al toxicity is the inhibition of root growth (Delhaize and Ryan 1995; Ma and Furukawa 2003) by destroying the root apex (Ryan et al. 1993).

While liming of soil can ameliorate some Al toxicity, it is expensive and ineffective in the subsoil. For this reason, the development of new crop varieties tolerant to Al seems to be the best solution to that problem. To date, several plant mechanisms to tolerate Al stress have been proposed (Kochian et al. 2002; Samac and Tesfaye 2003; Kochian et al. 2004). One of the mechanisms explaining Al tolerance is the exudation of organic acids (malate, citrate and oxalate) that chelate Al in the rhizosphere (Miyasaka et al. 1991; Delhaize et al. 1993; Ma et al. 2000, 2001; Li et al. 2000).

Molecular strategies have been used to detect genes implicated in the plant response to Al. Several genes have been found to be activated by Al in tolerant and susceptible genotypes in species like wheat (Snowden and Gardner 1993; Richards and Gardner 1994; Cruz-Ortega et al. 1997; Hamel et al. 1998), rice (Yu et al. 1998), tobacco (Ezaki et al. 1995, 1996), *Arabidopsis* (Richards et al. 1998), pea (Brosche and Strid 1999; Savenstrand et al. 2000) and rye (Rodriguez-Milla et al. 2002). There are also some examples of genes that are expressed in Al tolerant genotypes but not in the susceptible ones in different species: soybean (Ermolayev et al. 2003), wheat (Hamilton et al. 2001; Sasaki et al. 2004) and *Arabidopsis* (Sivaguru et al. 2003; Larsen et al. 2005; Hoekenga et al. 2006). Among them, particular attention should be placed on the wheat *TaALMT1* gene because it encodes an aluminum-activated malate transporter and its Al tolerance function has been demonstrated (Delhaize et al. 2004; Sasaki et al. 2004). Recently, this gene was completely sequenced and located on the 4DL chromosome arm (Raman et al. 2005). In any case, all these genes are good candidates to be studied in other crops to find mechanisms of Al tolerance in plants.

Analysis of populations segregating for Al tolerance has generated a worthy amount of information to identify loci controlling tolerance in plants. In wheat Al tolerance segregates as a single dominant locus (Delhaize et al. 1993; Somers and Gustafson 1995; Riede and Anderson 1996; Basu et al. 1997; Raman et al. 2005). More recently, the Alt_{BH} tolerance locus was linked to some molecular markers and located on *4DL* (Riede and Anderson 1996; Luo and Dvorak 1996; Rodriguez-Milla and Gustafson 2001). In barley a dominant gene located on the *4HL* chromosome arm has been found (Stolen and Andersen 1978; Tang et al. 2000; Raman et al. 2002). In maize tolerance genes located on chromosomes 2, 6 and 10 have been described (Brondani and Paiva 1996; Sibov et al. 1999). In *Arabidopsis* semidominant Mendelian loci have been found on chromosomes 1 and 4 (Larsen et al. 1996), 1998), and different QTLs that explain 40% of total variation have been described in *Arabidopsis* and sorghum (Kobayashi and Koyama 2002; Hoekenga et al. 2003; Magalhaes et al. 2004). In rice, 11 QTLs of Al tolerance have been located (Nguyen et al. 2002, 2003). Finally, a single locus, $Alt_{SB,}$ was found to control Al tolerance in two highly Al tolerant sorghum cultivars (Magalhaes et al. 2004).

Cereal crops exhibit variation in Al tolerance, and rye (*Secale cereale* L.) is the most tolerant cereal (Mugwira et al. 1978; Little 1988; Aniol and Madej 1996), whereas barley is the most Al-sensitive (Foy 1983). Rye can be used as a source of resistance genes for wheat (*Triticum* ssp.) through wheat–rye introgression and as a component of triticale (x*Triticosecale* Wittmack). Therefore, the knowledge of the mechanism and the genes that control Al tolerance in rye will provide fundamental information than can be used to increase Al tolerance in other cereals. Using wheat–rye addition lines, at least three different Al tolerance genes have been discovered in rye: *Alt1*, *Alt2* and *Alt3* located on *6RS, 3R* and *4RL*, respectively (Aniol and Gustafson 1984; Ma et al. 2000; Aniol 2004). The analysis of segregating populations has detected three independent and dominant loci that confer Al resistance in rye located on *4RL* (*Alt3*), *6RS* (*Alt1*) and *7RS* (*Alt4*) (Gallego and Benito 1997; Gallego et al. 1998a, b; Miftahudin et al. 2002; Matos et al. 2005).

The secretion of organic acids to the rhizosphere seems to be the main mechanism of Al tolerance in rye (Li et al. 2000). We hypothesized that at least a part of the Al tolerance behavior in rye is conditioned by a gene that is part of an organic acid exudation system. The main objective of the present work was to investigate whether a homolog of the Al tolerance *TaALMT1* gene of wheat is a good candidate to be the *Alt4* locus located on the *7RS* chromosome arm of rye.

Materials and methods

Plant material

To map the *Alt4* rye locus three F_2 families from crosses between Al tolerant rye plants (cv. "Ailés") and Al non-tolerant rye plants ("Riodeva" inbred line), segregating 3 Al tolerant:1 Al non-tolerant, were used: AR1-6⊗, AR1-25⊗ and AR6-5⊗. These F_2 populations were obtained by selfing three different F_1 plants originated from different crosses between a tolerant plant from the "Ailés" (A) rye cultivar and an individual from the non-tolerant inbred line "Riodeva" (R).

To assign loci to rye or barley chromosomes, hexaploid wheat, cv "Chinese Spring" (CS), rye *Secale cereale* L., cv. "Imperial" (I), barley *Hordeum vulgare* L., cv. "Betzes" (B), amphiploids wheat–rye and wheat–barley and the wheat–rye and wheat–barley disomic addition lines and the two ditelosomic wheat–rye addition lines *7RS* and *7RL* were used.

The *TaALMT1* wheat marker was located using the 'Chinese Spring' nullitetrasomics and ditelocentrics available for homoeologous group four.

Aluminum tolerance screening test

The Al tolerance test was carried out using the nutrient-culture, modified-pulse method (Aniol 1984). Seeds were sterilized for 10 min with $HgCl_2$ (0.1%), well rinsed with water and germinated overnight on filter paper in Petri dishes. Sprouted seeds were transferred the next day onto polyethylene nets fixed in Lucite frames. Styrofoam blocks were attached to the frames with rubber bands and floated on the surface of the vigorously aerated nutrient solution. Containers with the nutrient solution were placed in a water bath at 25°C under 16 h per day illumination. The nutrient solution consisted of: 0.4 mM $CaCl_2$, 0.65 mM KNO_3, 0.25 mM $MgCl_2{\cdot}6H_2O$, 0.01 mM $(NH_4)_2SO_4$ and 0.04 mM NH_4NO_3. Four days after sowing, seedlings were incubated for 24 h in nutrient solution with aluminum in the form of $AlKSO_4{\cdot}12\ H_2O$ at the concentration of 150 µM. After each exposure to Al, seedlings were removed from Al-containing solution, thoroughly washed for 2–3 min in running tap water. Then, roots were stained with 0.1% aqueous solution of Eriochrome cyanine R for 10 min. After staining the excess dye was removed by washing under tap water. Subsequently, seedlings were transferred to Al-free nutrient solution for 48 h. Additional root growth after these 2 days was easily assessed. In seedlings where the aluminum treatment did not destroy the root apical meristem, the root segment growing after Al-treatment was white (unstained) and contrasted to the heavily stained root part exposed to aluminum. When the apical meristem was damaged, root tips did not show any regrowth after 48 h in Al-free medium, remaining intensely stained. During all stages of growth, and particularly during Al-treatment the pH of the nutrient solution was adjusted at 4.0 with 1 N HCl. The nutrient solution was changed daily.

For mapping purposes, aluminum tolerance was determined as a dominant trait in the F_2 generation.

DNA extraction

Young leaves from plants were frozen in liquid nitrogen and stored at −80°C. The extraction was carried out using a small-scale DNA isolation method (Dneasy Plant Mini Kit, Qiagen).

PCR

Amplifications from genomic DNA were done in reactions of 10 µl containing 60 ng template DNA, 100 µM of each dNTP, 5 pmoles of each primer, $MgCl_2$ 2 mM, 0.25 µl of *Taq* DNA polymerase, and 1× reaction buffer (Tris–HCl 100 mM pH 8.3, KCl 100 mM). Amplifications were carried out in a PTC-100 thermal cycler (MJ Research) with the following program: a preliminary step of 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 59–69°C (depending on the pair of primers, Table 1) and 2 min at 72°C and a final step of 10 min at 72°C. PCR reactions were stored at 4°C until electrophoresis on 2% agarose gels stained with ethidium bromide. Several pairs of primers were designed based on the sequences of the *TaALMT1* wheat gene (Sasaki et al. 2004).

Cloning and sequencing of products

After PCR, bands of the approximate expected sizes were excised from 2% agarose TAE gels, purified with a Bioclean kit (Biotools), and cloned using a TA cloning kit (Invitrogen, Inc.). Cloned PCR products were sequenced at the Universidad Complutense facility using an automatic sequencer model 3730 (Applied Biosystems).

Sequence analyses

DNA sequences were analyzed using the Chromas Lite 1.0 program (Technelysium Pty Ltd). To confirm product identity, the GenBank BLAST application was used to compare DNA sequences, and the amino acid sequences predicted from them, with corresponding NCBI sequences (http://www.ncbi.nlm.nih.gov/).

The program Genscan (http://www.genes.mit.edu/GENSCAN.html) was initially used for predicting the locations and exon–intron boundaries of the *ScALMT1* gene in genomic sequences of rye.

Table 1 Sequences of the primer pairs utilized in this study

Primer	Sequence (5′→3′)	Ta (°C)
ScALMT1-1F[a]	ATGGATATTGATCACGGCAGAGAG	60
ScALMT1-1R	CCAGACGGGGAAGAGGAAGACGGT	
ScALMT1-2F	ACCAGCGGTTCTACACCATAGTTG	60
ScALMT1-2R	ATTGGCTCCATGGGTGTCGAAATC	
ScALMT1-3F	GTCATGGCCAGTTCAGATTTCGAC	59
ScALMT1-3R[a]	TTACAAAATAACCACGTCAGGCAAAGG	
ScALMT1-4F	GCTCGCGCTGGTGTCCGTCGTCTA	69
ScALMT1-4R	GCCAGCCACCAACGTCGCCAAGG	

Ta annealing temperature

[a] Primers reported by Sasaki et al. (2004) to amplify the complete cDNA of the *ALMT1* wheat gene

MegAlign Software (DNAStar Inc.) was utilized to align sequences using the CLUSTAL V method (Higgins and Sharp 1988). In some cases, after the alignment was completed, a neighbor-joining method was employed to reconstruct phylogeny for the putative alignment (MEGA version 2.1, Kumar et al. 2001). Bootstrap resampling of 1,000 replicates was performed to test the robustness of the dendrograms.

In order to predict the secondary structure of the putative protein and to interpret the effect of the amino acid differences among the different wheat and rye *ALMT1* variants, the computer programs shown in Table 2 were utilized.

Genetic mapping

Linkage analyses were performed on F_2 segregation data using the JoinMap 3.0 computer program (Van Ooijen and Voorrips 2001). Two loci were considered linked if the LOD score was greater than 3 and if the two-point distance was less than 45 cM. Genetic distances were calculated using the Kosambi function (Kosambi 1944).

RNA extraction and RT-PCR

Al tolerant (cv. "Ailés"), non-tolerant ("Riodeva" inbred line) rye plants and the moderately Al tolerant wheat cultivar "Chinese Spring" were grown in the described Al tolerance test conditions with 150 μM Al for five different times (not exposed, 30, 60, 360 and 1,200 min). Root apices, non-apical parts of the roots and leaves were removed and immediately frozen in liquid nitrogen and stored at −80°C.

Total RNA was extracted from 20 different samples per genotype and per exposure time. RNA quality was checked by gel electrophoresis and then quantified with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA (1 μg) was reverse transcribed using random hexamer primers and 200 units of MMLV reverse transcriptase (Advantage™ RT-for-PCR Kit, Clontech Laboratories, Inc.) in the presence of RNase-free DNase, under conditions specified by the supplier.

Quantitative PCR

Briefly, based on the complete sequence of the *ScALMT1* rye gene one pair of PCR primers (FWD 5′-GCGGCTTTGTTGCAAGTGA-3′ and REV 5′-TCAACCAAGTCCGCGAGAAG-3′) and one TaqMan® probe (5′-6-FAM-ATGGCAGTCACCGCAA-MGB-3′) were designed using the Primer Express® Software Version 2.0 (Applied Biosystems, USA). The PCR reactions contained TaqMan® universal PCR master mix (Applied Biosystems), 6.0 pmol of each primer, 5.0 pmol of FAM-tagged probe (Applied Biosystems), and cDNA made from ~100 ng of RNA. The PCR was carried out using the Applied Biosystems 7900 HT Fast Real-Time PCR System, under the following conditions: one step at 95°C for 10 min and 40 cycles with steps at 95°C for 15 s and 60°C for 1 min.

A completely conserved region among ribosomal 18S gene sequences from rye, wheat, barley and rice were used to design the following primers and probe: FWD 5′-TCAACGAGGAATGCCTAGTAAGC-3′, REV 5′-ACAAAGGGCAGGGACGTAGTC-3′, probe 5′-6-FAM- AGTCATCAGCTCGCG-MGB-3′. This was to provide an internal constitutively expressed control that would be useful not only for this experiment but also in future expression studies on such species.

All PCR samples and controls were prepared in duplicates using 0.2 ml MicroAmp Optical reaction tubes and MicroAmp Optical tube caps (Applied Biosystems, USA), and all 96-well plates included two standard curves (target gene and endogenous control) in order to apply the "relative standard curve method" supported by Applied Biosystems, to analyze the data. In this approach, unknown sample values are interpolated from the standard curves. Duplicated control reactions for every sample without reverse transcription were included to ensure that PCR products were not due to amplification of contaminant genomic

Table 2 Software programs utilized to deduce the structure of *ScALMT1* proteins

Program	Http address	Reference
MEMSAT	http://www.bioinf.cs.ucl.ac.uk/psipred/	Jones (1999)
TMAP	http://www.mbb.ki.se/tmap/index.html	Persson and Argos (1997)
TopPred2	http://www.bioweb.pasteur.fr/seqanal/interfaces/toppred.html	Claros and Vonheijne (1994)
HMMTOP	http://www.enzim.hu/hmmtop/	Tusnady and Simon (1998)
PHDhtm	http://www.embl-heidelberg.de/predictprotein/	Rost (1996)
DAS	http://www.enzim.hu/DAS/DAS.html	Cserzo et al. (2002)
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/	Krogh et al. (2001)

DNA. For comparison purposes, expression level at each time point is expressed as the fold-increase over the level obtained in the non-tolerant genotype without exposure to aluminum.

Results

Identification in rye of sequences related to *TaALMT1*

The pair of primers previously reported by Sasaki et al. (2004) to amplify the Al tolerance wheat *TaALMT1* gene (AB081803 and AB081804) were used in rye. After failing to amplify the complete gene using one set of primers, several primer pairs were designed, and positive results were achieved by amplifying the gene in three different parts with the *ScALMT1*-1, *ScALMT1*-2 and *ScALMT1*-3 primer pairs (Table 1). Then, the amplified fragments were sequenced from the "Riodeva" inbred line (Al-non-tolerant) to confirm the identity. The three sequences were assembled into one that matched with the *TaALMT1* gene sequences of wheat (91% similarity at the nucleotide level). The ends of the transcript were not determined experimentally. This new gene in rye was named *ScALMT1* and its sequence was sent to the NCBI data bank (DQ158087). Afterwards, the same strategy was conducted to obtain the *ScALMT1* sequence in the Al-tolerant cultivar "Ailés" (DQ158086). The genes detected in "Ailés" and "Riodeva" were 3,898 and 3,855 bp long, respectively, and were 98.0% identical.

The program Genscan (http://www.genes.mit.edu/GENSCAN.html) for predicting the locations and exon–intron structures was used on the *ScALMT1* genomic sequences of "Ailés" and "Riodeva" and showed a total of five introns with sizes ranging from 109 to 1,423 bp (Fig. 1). The predicted transcription unit was 1,359 bp long and the deduced protein consisted of 253 residues with a predicted molecular mass of 49.4 and 49.6 kDa and a theoretical pI of 6.64 and 7.14, respectively. The cDNAs obtained to carry out the subsequent expression experiments were sequenced confirming the length of exons, introns and transcription units in the Al tolerant and Al non-tolerant genotypes. The predicted proteins each have six hydrophobic putative transmembrane regions (TMHMM software). A search of the NCBI protein-database (http://www.ncbi.nlm.nih.gov/) with the *ScALMT1* genomic sequence revealed 86% amino acid sequence identity to the *TaALMT1* wheat gene and 63.3% to a rice gene (CAD40928) that encodes a putative protein of unknown function. At least 17 additional putative proteins encoded by expressed sequence tags (ESTs) from rice and *Arabidopsis thaliana* showed from 38.1 to 35.2% identity to *ScALMT1*.

The deduced protein sequences of the *ScALMT1* genes from the Al tolerant cultivar "Ailés", the Al non-tolerant inbred line "Riodeva", and the sequences previously deduced from wheat were compared (Fig. 2). The rye proteins showed forty residue substitutions and seven amino acid deletions with respect to the wheat proteins. The different programs did not detect any structural changes in the proteins due to these amino acid substitutions and deletions with possible functional implications. On the other hand, the deduced rye proteins differed among themselves at six amino acid residues. Five out of the six changes are substitutions of amino acids with the same or very similar chemical properties. However, the substitution of a glycine in the Al tolerant genotype with an arginine in the Al non-tolerant one in position 73 could have important structural implications. Only one program (PSIPRED) out of the seven programs predicted a secondary structure change from a strand (with the Gly) to a helix (with the Arg).

To assign the *ScALMT1* gene to a rye chromosome arm, amplifications with primers *ScALMT1*-4F and *ScALMT1*-4R in the disomic and ditelosomic wheat Chinese Spring and rye Imperial addition lines were carried out (Fig. 3). The amplification product was present on the *7R* and *7RS* lines and absent in the rest of them, including *7RL*.

Fig. 1 Schematic diagram of the structure of *ScALMT1* gene. Exons are represented by *solid boxes* and the *triangles* indicate the position of the five intercalated introns. *Numbers* indicate the length in base pairs of each sequence. The approximate position of the primers used for amplifying the *ALMT1* gene (Table 1) and for quantitative PCR are indicated

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Rye Als         MDIDHGREIDGEMVSTIASCGLLLHSLLA-------GFARKVGGAAREDPRRVAHSLKVG
Rye Alt         MDIDHGREIDGEMVSTIASCGLLLHSLLA-------GFARKVGGAAREDPRRVAHSLKVG
Wheat Als       MDIDHGRESDGEMVGTIASCGLLLHSLLAGLGRRAAGFARKVGGAAREDPRRVAHSLKVG
Wheat Alt       MDIDHGRESDGEMVGTIASCGLLLHSLLAGLGRRAAGFARKVGGAAREDPRRVAHSLKVG
                ******** ***** **************       ************************

Rye Als         LALALVSAVYFVTPLFNGLRVSAIWAVLTVVVVMEFTVGATLSKGLNRALATLVAGCIAV
Rye Alt         LALALVSAVYFVTPLFNGLGVSAIWAVLTVVVVMEFTVGATLSKGLNRALATLVAGCIAV
Wheat Als       LALALVSVVYFVTPLFNGLGVSAIWAVLTVVVVMEYTVGATLSKGLNRALATLVAGCIAV
Wheat Alt       LALALVSVVYFVTPLFNGLGVSAIWAVLTVVVVMEYTVGATLSKGLNRALATLVAGCIAV
                ******* *********** *************** ************************

Rye Als         GAHQLAELAERCSDQGEPVMLTVLVFFVASAATFLRFIPEIKAKYDYGVTIFILTFGLVA
Rye Alt         GAHQLAELTERCSDQGEPVMLTVLVFFVASAATFLRFIPEIKAKYDYGVTIFILTFGLVA
Wheat Als       GAHQLAELAERCGDQGEPIVLTVLVFFVASAATFLRFIPEIKAKYDYGVTIFILTFGLVA
Wheat Alt       GAHQLAELAERCGDQGEPIMLTVLVFFVASAATFLRFIPEIKAKYDYGVTIFILTFGLVA
                ******** *** *****  ****************************************

Rye Als         VSSYRVEELIQLAHQRFYTIVVGVFICLCTTVFLFPVWAGEDVHKLASSNLGKLAQFIEG
Rye Alt         VSSYRVEELIQLAHQRFYTIVVGVFICLCTTVFLFPVWAGEDVHKLASSNLGKLAQFIEG
Wheat Als       VSSYRVEELIQLAHQRFYTIAVGVFICLCTTVFLFPVWAGEDVHKLASGNLDKLAQFIEG
Wheat Alt       VSSYRVEELIQLAHQRFYTIAVGVFICLCTTVFLFPVWAGEDVHKLASGNLDKLAQFIEG
                ******************* ************************** ** ********

Rye Als         METNCFGENNIAINLEGKDFLQVYKSVLNSKATEDSLCTFARWEPRHGQFRFRHPWSQYQ
Rye Alt         METNCFGENNIAINLEGKDFLQVYKSVLNSKATEDSLCTFARWEPRHGQFRFRHPWSQYQ
Wheat Als       MEFNCFGENSVANNFGGKDSPQMHKSVLNSKATEDSLCTFAKWEPRHGQFRFRHPWSQYQ
Wheat Alt       MEFNCFGENSVANNFGGKDFPQMHKSVLNSKATEDSLCTFAKWEPRHGQFRFRHPWSQYQ
                ** ******  * *  ***  *  ***************** ******************

Rye Als         KLGTLCRQCASSMEALASYVITTTKTQYPAAANPELSFKVRKTCREMSTHSAKVLRGLEM
Rye Alt         KLGTLCRQCASSMEALASYVITTTKTQYPAAANPELSFKVRKTCHEMSTHSAKVLRGLEM
Wheat Als       KLGTLCRQCASSMEALASYVITTSKTQCPAAANPELSCKVRKTCGEMSLHSSKVLRDLAM
Wheat Alt       KLGTLCRQCASSMEALASYVITTSKTQCPAAANPELSCKVRKTCGEMSLHSSKVLRDLAM
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Rye Als         AIRTMTVPYLANNTVVVAMKVAERLRSELEENAALLQVMHMAVTAMLLADLVDRVKEITE
Rye Alt         AIRTMTVPYLANNTVVVAMKAAERLRSELEDNAALLQVMHMAVTATLLADLVDRVKEITE
Wheat Als       ATRTMTVPSPVNITMATAVKAAESLRSELAENTALLQVMHVAVTATLLADLVDRVKEIAE
Wheat Alt       ATRTMTVPSPVNITMATAVKAAESLRSELAENTALLQVMHVAVTATLLADLVDRVKEIAE
                * ******   * *   * * ** *****  * ******* **** ************ *

Rye Als         CVDVLARLAHFKNPEDAKYAIVGALTRGIDDPLPDVVIL
Rye Alt         CVDVLARLAHFKNPEDAKYAIVGALTRGIDDPLPDVVIL
Wheat Als       CVDVLARLAHFKNPEDTKNVVVSTVSRGIDEPLPDVVIL
Wheat Alt       CVDVLARLAHFKNPEDTKNVVVSTVSRGIDEPLPDVVIL
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Fig. 2 CLUSTAL W alignment of the *ALMT1* proteins deduced from the nucleotide sequences of the rye and wheat Al tolerant (Alt) and Al sensitive (Als) alleles. The wheat *TaALMT1* sequences were obtained from the NCBI database, AB081803 and AB081804 (Sasaki et al. 2004). Gaps (represented by *hyphens*) were introduced into the sequences to maximize the alignment. *Conserved amino acids

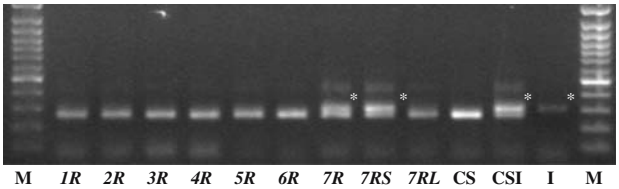

Fig. 3 Chromosomal location of the rye ScALMT1 gene. Amplification of rye genomic DNA using *ScALMT1*-4F and *ScALMT1*-4R primers. *M* Molecular-weight marker (100 bp ladder), *1R* to *7R*: wheat "Chinese Spring"–rye "Imperial" disomic addition lines *1R* to *7R*, *7RS*: ditelosomic wheat "CS"–rye "I" addition line *7RS*, *7RL*: ditelosomic wheat "CS"–rye "I" addition line *7RL*, CS: wheat "CS", CSI: wheat "CS"–rye "I" amphiploid, I: rye "I"

Segregation and mapping of *ScALMT1* locus

In order to detect variability that can be mapped, sequencing experiments were carried out in the parents of the F_2 progenies that were segregating for the *Alt4* locus. From the different polymorphisms detected, a SNP that was distinguishable with the restriction enzyme *Bsp*HI was selected.

To establish whether *ScALMT1* is associated with the *Alt4* locus in rye, we analyzed three F_2 populations segregating for this locus. Genomic DNA isolated from F_2 plants was analyzed using a cleaved amplified

polymorphic sequence (CAPS) marker assay based on the restriction with *Bsp*HI enzyme. The *ScALMT1* polymorphism showed complete cosegregation with the Al tolerant phenotypes. No recombination between the Al tolerance locus *Alt4* and the *ScALMT1* locus was found in the 381 F_2 plants analyzed in these populations (Fig. 4).

A set of markers previously mapped in rye was also included to refine the map position of *Alt4* and *ScALMT1*. Figure 5 shows the map constructed including intermicrosatellites (SCIMs), RAPDs, SCARs, microsatellites (SCMs), the *Alt4* locus and the *ScALMT1* gene. In total, the map spans 84 cM and consists of 26 loci. The *Alt4* locus and the *ScALMT1* gene mapped at the same position and they were flanked by the markers SCIM812$_{626}$ and OPQ4$_{578}$.

Expression of the *ScALMT1* gene

We investigated the level of transcripts of the *ScALMT1* gene in rye when exposed to aluminum in acidic conditions. Total RNA was extracted from root apices, non-apical parts of the roots and leaves of Al tolerant cv. "Ailés" and Al non-tolerant "Riodeva" inbred line and then analyzed by real-time PCR. A gene-specific probe and primers were designed from a conserved exonic region between "Ailés" and "Riodeva".

Firstly, we observed that the transcripts of the *ScALMT1* gene were induced by the presence of Al in the hydroponic medium, were more highly expressed in the root apices than in the non-apical parts of the roots and in the leaves (data not shown), and were more abundant in the Al tolerant genotype than the Al sensitive one (Fig. 6). The expression of the gene is not confined to the root apices but is more abundant there than in the non-apical parts of the roots (data not shown). Gene induction in Al tolerant rye seems to start during the first hour of Al exposure and keeps increasing until at least 20 h after exposure. However, induction in the Al non-tolerant inbred line does not seem to start until at least 6 h after the Al treatment. Very little expression of this gene was detected without Al even in the Al tolerant rye cultivar. The *ScALMT1* gene transcripts in root apices of Al tolerant rye ("Ailés") increases ~20 times between time zero and 20 h after Al exposure. On the other hand, gene expression at the same moment (20 h) was found to be increased just ~5 times in the root apex of the Al non-tolerant line ("Riodeva"). Five-fold differences in the expression were found between the Al tolerant and the Al non-tolerant genotypes after 20 h of exposure.

Transcripts from leaves of rye genotypes show a very weak expression (data not shown). The highest

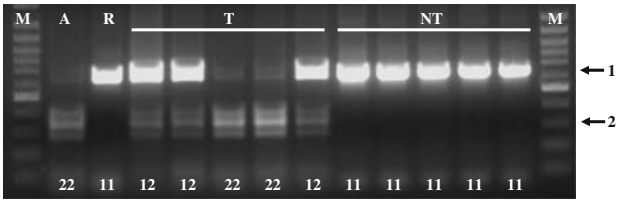

Fig. 4 Segregation of the *ScALMT1* gene in ten plants of the F_2 population AR1-6. Genomic DNA isolated from F_2 plants was analyzed using the CAPS marker assay based on the amplification using ScALMT1-3F and ScALMT1-3R primers and ulterior restriction with *Bsp*HI enzyme. *M* Molecular-weight marker (100 bp ladder), A: "Ailés" Al tolerant F_2 parent, R: "Riodeva" Al non-tolerant F_2 parent, T: F_2 Al tolerant plant, NT: F_2 Al non-tolerant plant. *Arrows* show the segregating alleles (*1* and *2*). *1* Undigested PCR product. *2* Fragments that appear after digestion with *Bsp*HI. The different homozygotes and heterozygotes are indicated at the *bottom* of the figure

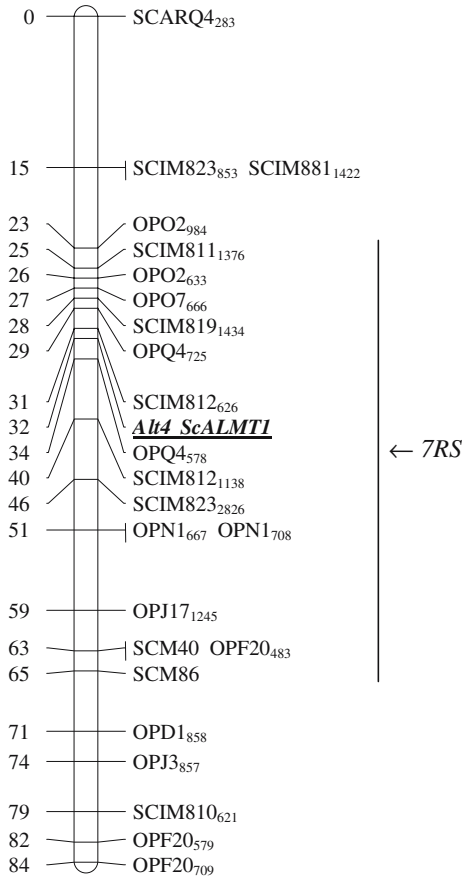

Fig. 5 Linkage map of chromosome *7R* showing the *Alt4* locus, the *ScALMT1* gene and several markers previously mapped (Matos et al. 2005). The centromere is located close to SCM40 (Korzun et al. 2001). The markers SCM86 and SCIM811$_{1376}$ had been located to *7RS* in a previous study (Matos et al. 2005). Map distances are in Morgans (cM)

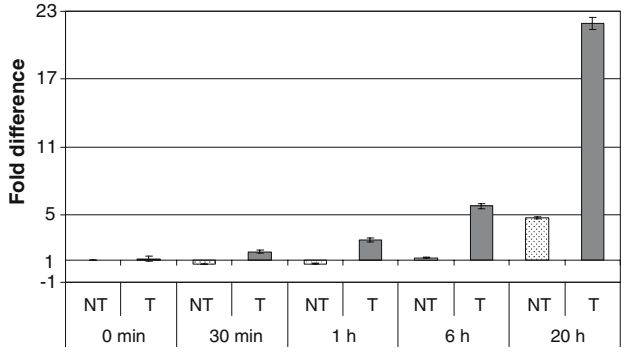

Fig. 6 Real-time RT-PCR results showing temporal expression patterns of root tip cDNA transcripts of *ScALMT1* gene from tolerant (*T*) and non-tolerant (*NT*) genotypes over a 20-h period. Change (*fold difference*) at each time point is expressed as the relative difference in expression compared to the non-tolerant genotype without exposure to aluminum. The *columns* represent the mean of two replicates per time point and the *error bars* show the range of values

level of expression detected in leaves ("Ailés" at 20 h of Al exposure) was less than the expression observed in the non-tolerant roots without Al treatment. The maximum level of leaf expression ("Ailés" at 20 h of Al exposure) is 125 times less than that detected in its own root apex at the same time. In summary, at all time-points, expression in leaves was many fold lower than in roots.

Chinese Spring is a moderately Al tolerant wheat cultivar. Quantitative PCR experiments were carried out to compare the levels of expression in this wheat cultivar and the rye genotypes utilized in the present work (data not shown). We found in Chinese Spring that the *TaALMT1* wheat gene is constitutively expressed, and it is not induced by Al. That is, no significant differences in the expression were found at different times after exposure to Al. On the other hand, 8-fold and 35-fold differences in the expression was found in the Al sensitive and Al tolerant genotypes, respectively, compared to Chinese Spring.

PCR detection of the *ALMT1* gene in different cereal species

The *ALMT1* primers (Table 1) were utilized to carry out amplifications from different cereal genomes. PCR amplifications from the available ditelocentric and nulli-tetrasomic lines for group four of wheat were performed. The *ScALMT1*-3 primer pair produced different amplification patterns, assigning the *TaALMT1* locus to the *4DL* chromosome arm of wheat. Additionally, the fragments amplified in the Nulli 4D Tetra 4A and Nulli 4D Tetra 4B lines were cloned and sequenced and two different sequences homologous to *TaALMT1* were detected (DQ158090 and DQ158091, respectively). Using the ALMT1 primers, another two homologous fragments with 94.3–96% identity to the rye one were detected in barley (DQ158088 and DQ158089). Chromosomal location experiments were attempted by amplification from the corresponding wheat–barley addition lines but no size differences between the barley and the wheat genomes were found. Then, after using eight different restriction enzymes to digest the PCR products (*Eco*RI, *Hap*II, *Hha*I, *Hae*III, *Hpa*I, *Mse*I, *Pst*I, *Afa*I) a *Hha*I restriction site was detected in wheat that was absent in barley, allowing the detection of a DNA fragment on the *4H* chromosome (Fig. 7). This fragment was sequenced to confirm that it was homologous to *ScALMT1*. Finally, the *ScALMT1*-3 pair of primers was used to amplify and sequence DNA fragments from *Triticum urartu* (DQ158093), *Aegilops speltoides* (DQ158092), *Avena sativa* (DQ322703), *Saccharum officinarum* (DQ322704), *Zea mays* (DQ322705) and *Phaseolus vulgaris* (DQ322702). These each showed at least 72.3% similarity to the wheat sequences.

Discussion

In the research described here, a candidate-gene approach was used to begin to determine the molecular identity of the *Alt4* locus (previously located on *7RS*). The segregation pattern of the *ScALMT1* candidate gene, detected on the basis of a SNP polymorphism, was determined in three mapping populations and completely cosegregated with the Al tolerance phenotype. We thought that the Al tolerance gene *TaALMT1* described in wheat (Sasaki et al. 2004) could be the *Alt4* gene in rye because the *4DL* arm in wheat containing the *TaALMT1* gene (Raman et al. 2003, 2005) is partly homoeologous to the *7RS* chromosome arm in rye (Naranjo and Fernandez-Rueda 1991; Devos et al. 1993). Subsequently, we found that this gene is primarily expressed in the root tip of rye just

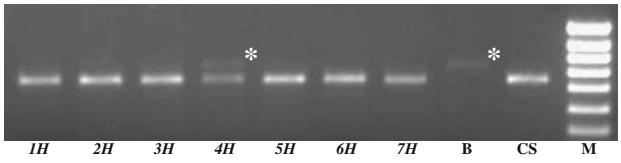

Fig. 7 Chromosomal location of the barley *hvALMT1* gene. Amplification of genomic DNA using *ScALMT1*-3F and *ScALMT1*-3R primers and ulterior restriction with *Hha*I enzyme. *1H* to *7H*: wheat "Chinese Spring"–barley "Betzes" disomic addition lines *1H* to *7H*, *B*: barley "B", CS: wheat "CS", *M* molecular-weight marker (100 bp ladder)

like in the wheat *TaALMT1* gene (Sasaki et al. 2004), and that there is an increased expression in tolerant versus sensitive genotypes.

The structure of the *ScALMT1* genomic region reveals that two of the introns are larger than expected in a plant gene, that is something around 150–350 bp on average (Wendel et al. 2002). Probably, this feature or some secondary structure prevented the amplification of the *ScALMT1* gene in just one piece. Lack of large differences in the size of the introns (less than 37 bp) between tolerant and non-tolerant genotypes does not point to an obvious involvement in the introns in determining functional allelic variation in *ScALMT1*. *ScALMT1* has a similar structure to the wheat *TaALMT1* gene (Raman et al. 2005). At least two alleles of *ScALMT1* are present in rye (*ScALMT1*-1 and *ScALMT1*-2). Although six amino acid substitutions between the two alleles were found, most of the protein analysis programs found no structural transformations of statistical significance. Only the PSIPRED software predicted a conformational change due to a transition from a Gly to an Arg. Glycine can influence the conformation of polypeptides since this amino acid can allow the polypeptide to adopt conformations which are sterically forbidden in other amino acids. However, more data are needed to investigate a potential functional significance of this difference. Also, both the wheat and rye proteins had six putative transmembrane regions. The same plasma membrane localization recently demonstrated for the wheat malate channel (Yamaguchi et al. 2005) should be expected for the rye gene. The Arabidopsis *AtALMT1* gene, which encodes a malate transporter as well, has been identified as one of the genes critical for A1 tolerance in *Arabidopsis* (Hoekenga et al. 2006). In our opinion, the minimal sequence differences detected indicate that both wheat and rye alleles are functionally capable of organic acid transport to the rhizosphere. However, a few amino acid changes can modify the function/substrate of a transport protein. Rye releases citrate from its roots as well as malate (Li et al. 2000) so the possibility that the rye *ScALMT1* gene facilitates citrate release instead of malate release has to be considered. Experimental works are being done in order to measure the malate and citrate efflux from the roots of the two rye lines (Al tolerant cv. "Ailés" and Al non-tolerant "Riodeva" inbred line) to address this question.

Gene expression experiments have demonstrated that the *ScALMT1* transcripts are primarily expressed in the root apex and are induced by aluminum. Numerous authors have presented data suggesting that the secretion of organic acids to the rhizosphere is a common mechanism of Al tolerance in plants (Ryan et al. 1995; Delhaize et al. 1993; Zheng et al. 1998). Two patterns of organic acid secretion have been identified. In pattern I, no discernible delay is observed between the addition of Al and the onset of organic acid release. In wheat the secretion of malate was detectable within 15–30 min after exposure to Al (Delhaize et al. 1993). In pattern II, organic acid secretion is delayed for several hours after exposure to Al. In rye, citrate and malate efflux increases steadily during a 10-h period (Li et al. 2000). These observations could be linked to a different pattern of mRNA expression between these species. Constitutive expression of the *TaALMT1* gene, similar to malate secretion behavior, has been reported in wheat (Sasaki et al. 2004). We also found in Chinese Spring that the *TaALMT1* homologous wheat gene is constitutively expressed, and it is not induced by Al (data not shown). In our work, time-course experiments suggest that this different organic acid secretion behavior between wheat and rye may be due to a different pattern of gene expression of homologous organic acid transporter genes. Further work will be needed to elucidate the reason for this different behavior between wheat and rye.

Our sequencing, mapping and expression results support the hypothesis that the first isolation of a gene of agronomic interest in rye has probably been reached. Cereal crops exhibit variation in Al tolerance, and rye (*Secale cereale* L.) is the most tolerant cereal (Aniol and Madej 1996; Kim et al. 2001), whereas barley is the most sensitive (Foy 1983). Therefore, the understanding of the mechanism and the genes that control Al tolerance in rye is important and will provide fundamental information than can be used to increase Al tolerance in other cereals. The introduction of the *TaALMT1* gene of wheat into barley cultivars increases their tolerance to aluminum (Delhaize et al. 2004). The cv. Chinese Spring has previously been shown to exhibit a *TaALMT1* transcript expression level which is approximately half that of the Al tolerant genotypes (ET8 line and Atlas 66 cultivar) (Raman et al. 2005). We have shown that the Al tolerant "Ailés" rye cultivar is expressing this gene at higher levels than the Chinese Spring wheat cultivar. These results would indicate that the *ScALMT1* rye gene might be a source of greater Al tolerance. This fact should be taken with great care because equal expression of the reference gene in wheat and rye has been assumed, and the comparison of the expression of the 18S reference gene has not be done yet. Taking into account this very high level of Al tolerance and gene expression in rye, we will investigate whether the use of the *ScALMT1* gene can further increase plant production in acidic soils.

There are previous data showing that the wheat *4DL* arm is homoeologous to the barley *4HL* and to the rye *7RS* and *5RL* chromosome arms, and is not homoeologous to the rye *4RL* arm (Naranjo and Fernandez-Rueda 1991; Devos et al. 1993; Naranjo et al. 1997). Moreover, Gale and Devos (1998) have obtained a consensus grass comparative map and have pointed out that rice chromosome 3 is homoeologous to wheat chromosome 4. A conserved aluminum tolerance locus in the homoeology group four of triticeae species has been proposed (Rodriguez-Milla and Gustafson 2001; Raman et al. 2002; Nguyen et al. 2003). To date, genes for aluminum tolerance on *4DL* (Rodriguez-Milla and Gustafson 2001), *4HL* (Raman et al. 2002), chromosome 3 of rice (Nguyen et al. 2002, 2003) and chromosome *7RS* (Matos et al. 2005) have been mapped.

Our results support the hypothesis of the existence of a common mechanism of Al tolerance encoded by a gene located in the homoeologous group four of cereals. We have isolated the second member of the family of the Al-activated organic acid transporters in cereals (*ScALMT1*) and found complete co-segregation between the *ScALMT1* genotype and the phenotype of tolerance to aluminum. We have shown that the *ALMT1* genes of wheat, rye and barley are located on the *4DL*, *7RS*, and *4H* chromosomes, respectively. The wheat chromosomal location result confirmed those obtained by different authors (Ma et al. 2005; Raman et al. 2005). Our chromosomal location of the putative *HvALMT1* gene on *4H* chromosome suggests that the barley Al tolerance locus (Alp) previously located on *4HL* arm (Raman et al. 2002) could be such gene. Additionally, we have also detected the putative *ALMT1* sequence in eight grass genomes and even in one dicotyledoneous species. In conclusion, it is very likely that in the homeology group four of grasses, there is a mechanism of aluminum tolerance of different magnitudes depending on the species based on organic acid efflux from the soil via the *ALMT1* gene.

A map of the *7R* chromosome including the *ScALMT1* gene and the *Alt4* locus has been constructed. In rye there are several examples of the use of molecular markers to detect traits of interest (Borner et al. 1999; Miedaner et al. 2000; Korzun et al. 2001; Miftahudin et al. 2002, 2004, 2005). Although there are also some genetic maps, the rye genome has not been mapped to a saturation level (Korzun et al. 2001; Ma et al. 2001; Hackauf and Wehling 2002; Bednarek et al. 2003; Khlestkina et al. 2005). Also, with the genome size of rye reaching approximately 8,000 Mb (Bennett and Smith 1976), a positional cloning is very difficult to achieve in this cereal species. In fact, to date there is no map-based cloned gene in this species. Therefore, as we have shown here, a candidate gene approach is a logical choice in some cases.

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