

Functional characterization of plasma membrane-localized organic acid transporter (CsALMT1) involved in aluminum tolerance in *Camelina sativa* L.

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Abstract Aluminum (Al) toxicity is a major limiting factor that inhibits root elongation and decreases crop production in acidic soils. The symptoms of inhibited root growth include a reduced uptake of nutrients because the roots become stubby and brittle. The release of organic anions from roots can protect a plant from Al toxicity. The mechanism relies on the efflux of organic anions, such as malate or citrate, which protect roots by chelating the Al³⁺. In this study, homologs of *TaALMT1*, a *Camelina* gene that encodes an aluminum-activated malate transporter, were investigated. The expression of this gene was induced by Al in the root, but not in the shoots. Using green fluorescent protein (GFP) fusion constructs and Western-blot analysis, we observed that CsALMT1 was localized in the plasma membrane. Also, to determine the degree to which Al tolerance was affected by malate secretion in *Camelina* root, we generated *CsALMT1* overexpressing plants. *CsALMT1* overexpressing transgenic plants showed a higher root elongation rate than the wild-type plant. Damaged cell staining analysis by hematoxylin under 25 μM Al treatment for 2, 4, and 6 h showed a pattern of

less damage in *CsALMT1* transgenic plants than in wild-type plant, especially in the root elongation zone. Furthermore, the rate of increase of secretion of organic acid in overexpressed plants after Al treatment was higher than that in the wild-type plant. In addition, in the Al-specific dye morin staining on root protoplast under 50 μM Al treatment, less Al accumulation was observed in the *CsALMT1* transgenic plants than in the wild-type plant. The Al contents in the roots of the transgenic plants were at a lower level than those in the wild-type plant. These results show that the overexpression of *CsALMT1* improves Al tolerance by increasing the release of malate from the root to the soil and, thereby, detoxifies the Al³⁺.

Keywords Aluminum · Plasma membrane · *Camelina* · Malate · CsALMT1

Abbreviation

HS Hoagland solution

Introduction

At soil pH values below 5.0, soluble aluminum (Al) in the soil exists as a toxic trivalent cation that affects plant growth and function (Kochian et al. 2004). One of the most significant symptoms of Al toxicity in the soil inhibited root growth and root elongation via impaired cell division and cell elongation. The root apex is a primary target and plays a central role in the mechanism of Al toxicity and tolerance (Ryan et al. 1993; Kochian 1995; Ahn et al. 2001). However, many plant species have developed mechanisms to detoxify Al, both internally and externally. Internal detoxification involves the sequestration of Al into the plant's vacuoles and chelation with an organic acid.

W. Park and H.S. Kim equally contributed to experimental conception, design, and writing of the article.

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The most documented and generally known mechanism of external detoxification in both monocotyledons and dicotyledons is the secretion of organic acid anions, such as malate, citrate, and oxalate, from the cytoplasm of root cells (Sasaki et al. 2004; Kochian et al. 2005; Ma 2007). These organic anions can be released through an anion transporter and can chelate toxic Al^{3+} into a nonphytotoxic Al form in the rhizosphere. This external tolerance of Al toxicity is a general mechanism in many species (Ryan and Delhaize 2010). The first tolerance gene, designated as an aluminum-activated malate transporter (TaALMT1), was isolated from wheat (*Triticum aestivum*) by Sasaki et al. (2004). Other members of Al-activated secretion of malate (ALMT1) have been identified in *Arabidopsis thaliana* (AtALMT1), rapeseed (BnALMT1), barley (HvALMT1), and maize (ZmALMT1) (Hoekenga et al. 2006; Ligaba et al. 2006; Gruber et al. 2010; Pineros et al. 2008).

Patterns of organic acid anion secretion were summarized by Ma (2000), and can be grouped into two patterns depending on the timing of secretion. In pattern I, the secretion of organic acid anions immediately occurs when the plant is exposed to Al, as observed in wheat (Delhaize et al. 1993) and buckwheat (Zheng et al. 2005). Pattern II exhibits a significant lag phase until the onset of organic acid anion release, as in *Cassia tora* (Ma et al. 1997b), *Sorghum bicolor* (Magalhaes et al. 2007), and *Zea mays* (Maron et al. 2008). These results indicate that the genetically engineered protein that modulates the organic acid anion release in the plasma membrane is an effective way to enhance Al tolerance in plants. Although the function of ALMT1 from other plant species has been well studied, the regulatory role of *CsALMT1* (Genbank accession number: KF021618) from Camelina in malate exudation remains unknown.

Camelina sativa L. is an emerging bioenergy crop that can be grown under harsh soil conditions (Knorzer 1978; Putnam et al. 1993; Vollmann et al. 1996; Zubr 1997; Park et al. 2014). Furthermore, Camelina ALMT1 (*CsALMT1*), which is a homolog of the Arabidopsis ALMT1, was isolated. This gene may mediate Al tolerance via malate exudation in Camelina roots under Al treatment. Therefore, overexpression of *CsALMT1* in Camelina plants may improve the Al tolerance. In this study, we conducted tests to investigate the effect of *CsALMT1* on the detoxification of aluminum toxicity by comparing with Camelina wild-type and *CsALMT1* overexpressing transgenic plants.

Materials and methods

Plant material and growth conditions

Camelina (*Camelina sativa* L.) seeds were sown on nylon mesh at 25 °C under dark conditions for 2 days with half-

strength Hoagland solution [contained 1.25 mM KNO_3 , 1.5 mM $\text{Ca}(\text{NO}_3)_2$, 0.75 mM MgSO_4 , 0.5 mM KH_2PO_4 , 75 μM FeEDTA, 50 μM H_3BO_3 , 10 μM MnCl_2 , 2 μM ZnSO_4 , 1.5 μM CuSO_4 , and 0.75 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$]. After germination, seedlings were grown under cycles of 16 h light/8 h dark for 3 days. The Hoagland solution (HS) was changed every day. To create a hydroponic culture for several experiments, 4-day-old seedlings were transferred to a modified HS adjusted to pH 4.5 without phosphate at least 12 h prior to treatment. To analyze the seedling growth rate under Al stresses, plants were grown vertically on 125 × 125 × 20 mm polystyrene Petri dishes containing half-strength nutrient medium (without phosphate) containing 1.25 mM KNO_3 , 1.5 mM $\text{Ca}(\text{NO}_3)_2$, 0.75 mM MgSO_4 , 75 μM FeEDTA, 50 μM H_3BO_3 , 10 μM MnCl_2 , 2 μM ZnSO_4 , 1.5 μM CuSO_4 , and 0.075 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ containing 0.8% (w/v) phytoagar (Duchefa Biochemie) after sterilization with 75% ethanol for 10 min. Al was supplemented to the corresponding media at the concentrations indicated in Fig. 4.

RNA extraction and Cloning of *CsALMT1* from cDNA

The roots of 5-day-old seedlings treated with 50 μM Al were harvested from Camelina, homogenized in liquid nitrogen, and RNA extracted using Trizol (Invitrogen, Carlsbad, CA) and an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity of RNA was measured using a spectrophotometer. RNA was treated with RNase-free DNase-I for 45 min (Ambion, Austin, TX) followed by DNase-I removal according to the manufacturer's instructions. Total RNA was synthesized to cDNA using a PrimeScript 1st strand cDNA Synthesis Kit (Takara).

The degenerate primers (5'-CAARGTSGGMCTKG CKCTYG-3' and 5'-CYTSYCCRGCCCARACVGG-3') were designed according to the conserved domains of the Aluminum-activated Malate Transporter 1 (ALMT1s) from Arabidopsis (*Arabidopsis thaliana*), maize (*Zea mays*), and wheat (*Triticum aestivum*). A fragment of 475 bp was PCR amplified using the degenerate primers from the cDNA. cDNA was synthesized using a SMARTer[™] RACE cDNA Amplification Kit (Clontech). *CsALMT1* was amplified using Rapid Amplification of cDNA Ends (RACE) PCR followed by primers (5'-GTCCTTTCCCAAGTGTGGCC CAACAG-3', 5'-GGTCA -TGTTGGTCTTTGTGCAAGC TGC-3'). All the PCR products were cloned into the pGEM T-Easy vector (Promega, Madison, WI, USA) for sequence analysis.

Northern-blot hybridization

For Northern-blot hybridization, 10 μg of RNA was denatured in formamide and formaldehyde, and separated

by electrophoresis on 1.2% (w/v) agarose gels. A full-length gene-containing Open Reading Frame (ORF) region of the *CsALMT1* probe was used. RNA was transferred to a nylon membrane (Amersham HybondTM-N⁺, GE Healthcare) and a cDNA probe was radioactively labeled as [α -³²P]dCTP using the Prime- α -Gene-Labeling System (Invitrogen). Northern blot was hybridized at 60 °C in 10 ml hybridization buffer for 18 h and washed at 65 °C four times with 0.1 \times SSC, 0.1% SDS for 15 min. Hybridization was detected using a phosphorimager (Typhoon FLA7000, Fujifilm-Life Science).

Characterization of *CsALMT1* expression in different tissues using the qRT-PCR

5-day-old and 6-week-old plants were used for the *ALMT1* expression pattern analysis of different tissues such as the flower, silique, stem, leaf, total root, basal root, and root tip. For comparative quantification of the *CsALMT1* gene, real-time quantitative PCR (qRT-PCR) was performed in a Bio-Rad CFX96 Real-Time PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The 20- μ L PCR mixture contained 4 μ L of diluted cDNA, 10 μ L of SYBR Premix (Takara), 2 μ L of a mix of the primer pair, and 4 μ L of water. The real-time PCR condition was followed by a three-step protocol with the melting curve. The data were analyzed using the Bio-Rad CFX manager (version 1.0) after normalization to *CsActin*. Triplicate PCR and three biological replicates were analyzed. The primer pairs used for this experiment were as follows: *CsALMT1* 5'-CCGTCAGTGTGCCTACAGAA-3' (forward) and 5'-TGCAGGCAGATTGTGAGTTC-3' (reverse), *CsActin* 5'-ACAATTTCCCGCTCTGCTGTTGTG-3' (forward) and 5'-AGGGTTTCTCTCTTCCACATGCCA-3' (reverse).

Subcellular localization of CsALMT1::GFP fusion protein

To determine the subcellular localization of the *CsALMT1*::GFP fusion protein, a green fluorescent protein (GFP) gene was fused in-frame to the C-terminus of the *CsALMT1* gene and was subsequently expressed under the control of the CaMV 35S promoter. The *CsALMT1*::GFP fusion protein was transiently introduced to tobacco leaves. After 48 h of fusion protein infiltration, the fluorescence was observed by Laser Confocal Scanning Microscope (TCS NT, Leica Instruments).

Isolation of the plasma membrane vesicles and Western-blot analysis

Plasma membrane (PM) vesicles were prepared at exactly 4 °C following the method by Palmgren et al. (1990). PM

was isolated from 2-month-old tobacco leaves with the addition of *CsALMT1* fusion protein. The leaves were ground in an ice-cold homogenization buffer containing 50 mM MOPs-BTP (pH 7.5), 330 mM sucrose, 5 mM EDTA, 5 mM dithiothreitol (DTT), 0.5 mM PMSF, 0.2% (w/v) bovine serum albumin (BSA), 0.2% (w/v) casein, and 0.5% polyvinylpyrrolidone (PVP). The homogenate was filtered through four layers of cheesecloth and centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was centrifuged at 21,000 rpm for 45 min, and the resulting precipitate was resuspended in suspension buffer consisting of 330 mM sucrose, 5 mM potassium phosphate (KH₂PO₄; pH 7.8), 5 mM KCl, 1 mM DDT, and 0.1 mM EDTA. The homogenate was loaded into a two-phase system containing 6.5% (w/w) Dextran T-500, 6.5% (w/w) polyethylene glycol (PEG)-3350, 250 mM sucrose, 4 mM KCl, and 5 mM KH₂PO₄. After the batch procedure, the resulting upper phase and low phase were collected and mixed with a dilution buffer containing 5 mM 3-(*N*-morpholino) propanesulphonic acid-Bis-tris Propane (MOPs-BTP) (pH 7.5), 330 mM sucrose, and 5 mM KCl, and then centrifuged at 100,000g for 60 min. The resulting pellet was resuspended in the dilution buffer. PM samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) consisting of a 5.6% (w/v) stacking gel and a 12% (w/v) separating gel. For Western-blot analysis, the SDS-PAGE resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and blocked in a phosphate buffered saline-Tween (PBST) buffer. The PVDF membrane was incubated at room temperature for 1 h with rabbit polyclonal GFP antibody (1:10,000, Invitrogen) and rabbit polyclonal PM H⁺-ATPase. After rinsing in PBST buffer, each membrane was incubated for 1 h with a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG as secondary antibody (Promega, Madison, WI, USA). The signal detection was performed with enhanced chemiluminescence (ECL) solution (ECL Western-blotting substrate, Promega) using G-Box iCHEMi XL (Syngene, Cambridge, UK).

Hematoxylin staining of Al-treated roots

5-day-old intact seedlings were grown in HS that included 25 μ M Al. After 0, 2, 4, and 6 h exposure to Al, the seedlings were removed from the HS and stained for the visual detection of Al using the method proposed by Polle et al. (1978). The seedlings were washed with distilled water (DW) for about 5 min and stained with 0.2% hematoxylin solution (sigma) for 10 min. The seedlings were washed again for 15 min with DW to remove excess stain and were then photographed.

Organic acid assay

The 4-day-old seedlings were transferred to a modified HS adjusted to pH 4.5 without phosphate at least 12 h prior to treatment. The 5-day-old seedlings were subjected to the same solution condition containing 25 μM AlCl_3 (pH 4.5) and the root exudates were collected every 2 h (0, 2, 4, 6, and 12 h after Al treatment) for organic acid analysis. The collected solutions containing the root exudates were passed through a cation exchange column (16 \times 14 mm) filled with 5 g of Amberlite IR-120B (H^+ form) resin, and then through an anion-exchange column (16 \times 14 mm) filled with 2.5 g of Bio-Rad AG 1-X8 resin in a cold room. Organic acids retained in the anion-exchange resin were eluted with 2 M HCl, and the eluate was concentrated using a rotary evaporator at 40 °C. The residue was redissolved in 1 ml of water, and the concentrations of organic acids were analyzed by HPLC equipped with an ion-exclusion column (Shim-pack SCR-102 H, 8.0 mm \times 30 cm, Shimadzu, Kyoto, Japan) as described in detail by Ma et al. (1997a) and Yang et al. (2001).

Vector construction and *Camelina* transformation

Two recombination sites, attB1 and attB2 at 5'-ends, were added to the forward and reverse primer, respectively. The PCR products were purified and cloned into the pGEM-T Easy vector (Promega, Madison, WI), followed by sequencing. Products were cloned in pDONR207 using the Gateway cloning system (Invitrogen). The binary vector pCB302-3 (Xiang et al. 1999) contains the promoter and terminator of the CaMV 35S promoter to overexpress *CsALMT1* cDNA in *Camelina*. pCB302-3 was modified to be compatible with the Gateway system as described by the manufacturer. The construct was transformed into the *Agrobacterium tumefaciens* strain GV3101, which was used for *Camelina* transformation conducted via vacuum infiltration (Bechtold and Pelletier 1998). Antibiotic selection was performed in *E. coli* and *Agrobacterium tumefaciens* with kanamycin (50 mg/ml in MS medium containing 1% agar) and in transgenic plants with DL-phosphinotricin (PPT, 0.03% was sprayed on seedlings) (Duchefa, Harlem, The Netherlands).

Protoplast isolation

Protoplasts were isolated from antibiotic-selected transgenic plants and wild-type *Camelina* roots. The roots were excised using a blade and digested in 10 ml of enzyme solution. The enzyme solution was prepared before the roots were excised. The enzyme solution consisted of Mannitol 0.72 g (final 1.5%), 0.2 M MES 1 ml (final 20 mm), Cellulase 150 mg (final 1.5%), Macerozyme 40 mg (final 0.4%), 1 M CaCl_2

100 μl (final 10 mM), 10% BSA 100 μl (final 0.4%), and DW to a final volume of 10 ml, all of which were filtered (0.45 μm) into a 50-ml falcon tube. Digested roots were subjected to vacuum for 30 min and incubated for 3 h (at room temperature (RT), in the dark, and covered with aluminum foil). During incubation, WI and W5 solutions were prepared. The WI solution (50 ml) consisted of 4.5 g Mannitol (final 2 mM), 0.2 M MES (pH 5.7) 1 ml (final 4 mM), 2 M KCl 0.5 ml (final 20 mM), and DW added to a final volume of 50 ml adjusted to pH 4.5 (for Al treatment). W5 consisted of 0.2 M MES (pH 5.7) 0.5 ml (final 2 mM), 1 M CaCl_2 6.25 ml (final 125 mM), 1 N NaCl 7.7 ml (final 154 mM), 2 M KCl 125 μl (final 5 mM), and DW added to a final volume of 50 ml. After incubating for 3 h, 10 ml (1 \times vol.) of W5 buffer was added to the enzyme solution and mixed by swirling. The solution was filtered through a mesh into a 50-ml conical tube and centrifuged at 500 rpm for 4 min. The supernatant was then removed. 4 ml of W5 was added for re-suspension, which was incubated on ice for 30 min. After incubation, W5 was removed without touching the pellet, which was resuspended with 3–4 ml of WI buffer adjusted to pH 4.5.

Al accumulation in root protoplasts

The Al accumulation experiments were performed using morin. Morin reagent was dissolved in 0.1 M dimethyl sulfoxide (DMSO). Isolated root protoplasts were incubated with 50 μM morin for 1 h, and the 50 μM Al was then applied for 40 min. The green fluorescence signal occurred due to Al-morin complexes. The signal intensity was determined using Image J software (Schneider et al. 2012).

Determination of Al contents in roots

Three-week-old plants were treated with P 1/2 HS (pH 4.5) containing 25 μM Al. After 0, 2, 4, and 6 h treatments, the roots were washed with DW. Each of the individual excised roots was transferred to a test tube (16 \times 124 mm) and dried at 80 °C for 48 h. The dried samples were weighed and digested with 12 N HCl at 200 °C overnight. The digested samples were diluted with 2 N HCl and then analyzed using inductively coupled plasma mass spectroscopy (IRIS-AP; Thermo Scientific, Pittsburgh, PA).

Statistical analysis

All biological data were analyzed using the Dunnett's test after one-way ANOVA. Statistical analyses were carried out using SPSS version 20 software (IBM, Chicago, IL, USA). Statistical differences among data for different results were considered statistically significant at $P \leq 0.05$.

Results

Isolation of *CsALMT1* gene from *Camelina sativa* L

A *CsALMT1* fragment (475 bp) was amplified from cDNA using degenerate primers designed according to the conserved domains of ALMT1 s from *AtALMT1* (Hoekenga et al. 2006), *TaALMT1-1* (Sasaki et al. 2004), and *ZmALMT1* (Pineros et al. 2008). *CsALMT1* (Gene accession number: KF021618) was amplified using RACE PCR, and the coding region of a full gene was 1482 bp in length and the proteins comprised 493 amino acid residues with five transmembrane domains according to SOUSI software (Fig S1). CLUSTAL 2.1 Multiple Sequence Alignments indicated that *CsALMT1* was 86% similar to the *At1g08430* (*AtALMT1*) in *Arabidopsis*. However, it was 36% similar to the *TaALMT1-1* in wheat and 37% similar to the *ZmALMT1* in the maize (Fig. 1). Fig. S1b presents a phylogenetic tree that shows the difference in homology between monocotyledons and dicotyledons.

Expression of *CsALMT1* gene affected by Al

To determine the expression pattern of *CsALMT1*, it is investigated under various Al doses (0, 10, 25, 50, 75, and 150 μ M Al) and time periods (0, 1, 3, 6, 12, and 24 h). Northern-blot analysis was used to analyze *CsALMT1* expression patterns in *Camelina* seedling. *CsALMT1* has Al-dependent expression (Fig. 2a). Very low levels of expression were observed within 10 μ M Al concentration. However, the transcript levels of *ALMT1* greatly increased at concentrations greater than 25 μ M Al and then decreased to relatively low levels by 150 μ M Al treatment. We also determined the expression pattern of *CsALMT1* according to Al treatment time. *CsALMT1* gene transcription level remained unchanged up to 1 h after Al treatment, but increased from 3 h after Al treatment, maintaining a steady state until 24 h of treatment. Furthermore, we tested the expression specificity of *CsALMT1*, which is expressed only in the roots, not in the shoots, at both vegetative and reproductive stages (Fig. 2b). These results suggested that *CsALMT1* has Al-dependent and root-specific expression. Also, we determined the suitable experimental conditions of Al concentration and treatment time to identify *CsALMT1* function.

Subcellular localization of *CsALMT1* protein in tobacco

To examine the subcellular localization of *CsALMT1*, a fluorescent protein (GFP) was fused to the C-terminus of the full-length *CsALMT1*. *CsALMT1::GFP* was transiently introduced into tobacco leaf. The *CsALMT1::GFP* fusion

green fluorescence was observed in the plasma membrane (Fig. 3a). Similar results have been observed in many ALMT1 s (Pineros et al. 2008; Gruber et al. 2010). We also performed Western-blot analysis with the GFP antibody to further substantiate the subcellular localization of *CsALMT1*. The transiently introduced plasma membrane of tobacco leaf was isolated for the Western blot. *CsALMT1* fusion protein was detected in the membrane fraction (upper phase), but not in the control (Fig. 3b). The H^+ -ATPase antibody was used as a positive control. These results indicate that *CsALMT1* is localized in the plasma membrane.

Overexpression of *CsALMT1* in *Camelina* improved Al tolerance

To test whether *CsALMT1* can be used to develop plants with enhanced Al tolerance, we generated transgenic *Camelina* expressing a 35S promoter, *CsALMT1*, using a gateway cloning system. *CsALMT1* transcript levels of two *CsALMT1* independent transgenic T2 plants (OX1 and OX2) and the wild-type plant were determined by RT-PCR using a *CsALMT1*-specific primer (Fig. 4a). To evaluate the Al tolerance of *CsALMT1* transgenic plants, seeds of wild-type and two transgenic plants were germinated and grown on agar plates containing 10 and 25 μ M Al for 4 days. No differences were observed in the germination between the wild-type and transgenic plants in treatments compared to the control. The root elongation rate was measured for 4 days after treatment. The growth of the transgenic plants was superior to the wild-type plants under Al stress. In particular, the transgenic plants showed enhanced Al tolerance in 25 μ M Al concentration. However, in the control medium, the growth of each plant was similar (Fig. 4b). When the roots exhibited the first signs of Al toxicity, root growth was inhibited as the concentration of Al ions increases and root elongation was clearly inhibited when 25 μ M Al was applied (Fig. 4c).

Pattern of *Camelina sativa* L. root cell disruption under Al stress

Camelina seedlings were exposed to 25 μ M Al concentration with different durations (0, 2, 4, and 6 h). Al treatments were performed in hydroponic culture without phosphate. To compare the Al-induced root cell disruption, hematoxylin was used to stain the Al-treated roots of *Camelina*. No differences were observed in the germination between the wild-type and transgenic plants in normal condition. However, the difference in the degree of staining between the wild-type and the *CsALMT1* transgenic plants gradually increased in the roots after 2 h of Al treatment. The swollen section in the root of the wild-type plant was

CsALMT1	1	ME-----RVRDITVKEGMRVGNEDPRRI
AtALMT1	1	ME-----KVREIVREGRVGNEDPRRI
TaALMT1-1	1	MDIDHGREG---SDGEMVGTI--ASCGLLLHSLLAGLGRRAAGFARKVGGAAAREDPRRV
ZmALMT1	1	MEIDEMESGVVNGSGGGGGGSFRRRARCWELLCSA-AG---KVVGFARKLGRIRARDPRRV
CsALMT1	23	IHAFAKVGGLALVLSVSYFYYPQFPFPFTDYFGINAMWAVMTVVVVFVFEFSGVATLGKGLNRGV
AtALMT1	23	IHAFAKVGGLALVLSVSYFYYPQFPFPFTDYFGINAMWAVMTVVVVFVFEFSGVATLGKGLNRGV
TaALMT1-1	54	AHSLKVGLALALVSVVYEVTPLE--FNGL-GVSAIWAVALTVVVVMEYTVGATLSKGLNRAL
ZmALMT1	57	AHSLKVGLALTLVSVLYYVRPL--FNNG-GVSTMWAVLTVVVVMEYTVGGTLSKGLNRAC
CsALMT1	83	ATLVAGGLGIGAHQLARFS--GATVEPILLVMLVVFVQAALSSTFVRFFFPWVKKFFDYAIL
AtALMT1	83	ATLVAGGLGIGAHQLARLS--GATVEPILLVMLVVFVQAALSSTFVRFFFPWVKTKFDYAIL
TaALMT1-1	111	ATLVAGCIAVGAHQLAELAERCGDQGEPIMLTVLVVFFVASAATFLRFIPEIKAKYDYGV
ZmALMT1	114	GTLAAGFIAVGAHKVAVL--CGDKAEPVLLAVFVFLSSAATFSRFIPEVKARYDYGV
CsALMT1	140	IFILTFALISLSGFRDEEILDIAESRISTVIVIGGISCVFISIFVCPVWAGQDLHSLLASN
AtALMT1	140	IFILTFALISLSGFRDEEIMDLAESRISTVIVIGGVSCILISIFVCPVWAGQDLHSLLASN
TaALMT1-1	171	IFILTFGLVAVSSYRVBELIQLAHQRFYTIAVGVFICLCTTVFLFPVWAGEDVHKLASGN
ZmALMT1	171	IFILTFSLVAVSSYRVDELIRLAHQRFSTIVVGVGTCLCTTVFVFPVWAGEDLHRLAIGN
CsALMT1	200	FDTLAHFLQDFGDEYFEARVEAGDYKVVVEKRGRLDRFKSVLDSKSEEEALANVAEWEPP
AtALMT1	200	FDTLASHFLQDFGDEYFEAR-EKGDYKVVVEKRRKNLERYKSVLDSKSDDEALANVAEWEPP
TaALMT1-1	231	LDKLAQFIEGMEFNCFGENSVANNFGG---KDFPQMHKSVLNSKATEDSLCTFAKWEPR
ZmALMT1	231	LNKLAEFFEGLSECFRENATFENLEA---KPFLQVYKSVLNSKATEDSLCNFAKWEPC
CsALMT1	260	HGKFRFRHPWKQYLAVGALLRQCA YRIDALDSYI--NSDFQIPM---DIKKKLEEPLRR
AtALMT1	259	HGQFRFRHPWKQYVAVGALLRQCA YRIDALNSYI--NSDFQIPV---DIKKKLEETPLRR
TaALMT1-1	287	HGQFRFRHPWSQYQKLGTLCRQCA SSMALASYVITTSKTQCPAAANPELSCKVRKTCGE
ZmALMT1	287	HGKFRFRHPWSQYQKLGALSROCA SSMALASYVITLRTTEYPEA-RPELRSEVTRACRQ
CsALMT1	314	MSSESGKSMKEVSISSLKKM- IKSSSSDVHVLNSQSAACKALSTLLKSGIINDVEPLQMISL
AtALMT1	313	MSSESGNSMKEMSISSLKKM- IKSSSSDTHVNSQAACKSLSTLLKSGIINDVEPLQMISL
TaALMT1-1	347	MSLHSSKVLRLDIAMATRTMTPV-SPVNTMATAVKAAESL---RSELAENTALQVMHV
ZmALMT1	346	MSLHSAKALRELSAAMRTMAVPPSPANAHMSAAAKAKDL---RVEL-EDADLAQAMHV
CsALMT1	373	LTAVSLLIDI VNLTEKISESLHELASAARFKNMMRPTVY--SDSKSIGRAMPKESHAGD
AtALMT1	372	MTTVSMLIDI VNLTEKISESVHELASAARFKNMMRPTVLYEKSDSGSIGRAMPIDSHEDH
TaALMT1-1	402	AVTATLLADLVDRVKEIAECVDVLA RLAFKN-----
ZmALMT1	401	AVVASLLSDLVTKAKQITESTVGTLARLARFVK-----
CsALMT1	430	HVVTVLHDVDT S-SNNADNSLGESSQNPCHHIAINIVDDYSNHE--DGEIHHTSLLLED
AtALMT1	432	HVVTVLHDVDNDRSNNVDSDRGGSSQDSCHHVAIKIVDDNSNHEKHEDGGEIHVHTL----
TaALMT1-1	434	-----PEDTKNVVSVTVSRGIDEPLPDVVIL*-----
ZmALMT1	433	-----NNDHENADDKDIA-AID-----AVS*-----
CsALMT1	486	VKRNDRLH*
AtALMT1	488	--SNGHLQ*
TaALMT1-1		-----
ZmALMT1		-----

Fig. 1 Multiple sequence alignment of the ALMT1 gene from Wheat (TaALMT1-1), Camelina (CsALMT1), Arabidopsis (AtALMT1), and Maize (ZmALMT1). Alignments were carried out using CLUSTAL (<http://clustalw.genome.jp>). Identical amino acids and similar amino

acids are indicated by dark shading and light shading, respectively, using BOXSHADE software (http://www.ch.embnet.org/software/BOX_form.html). The two plants indicate the position of degenerate primers

observed, which is one of the symptoms of damage by Al stress. The difference of staining color formation in the root was distinct from the Al treatment for 4 h between the wild-type and the *CsALMT1* transgenic plants. The blue color was more intense in the root of the wild-type plant than in the *CsALMT1* transgenic plants. These results indicate that Al stress causes more damage to the cells in

the vicinity of the growth point of wild-type roots than in *CsALMT1* transgenic plants (Fig. 5).

Time period of malate secretion

Because several plants with aluminum tolerance are well known for their increased secretion of organic acids

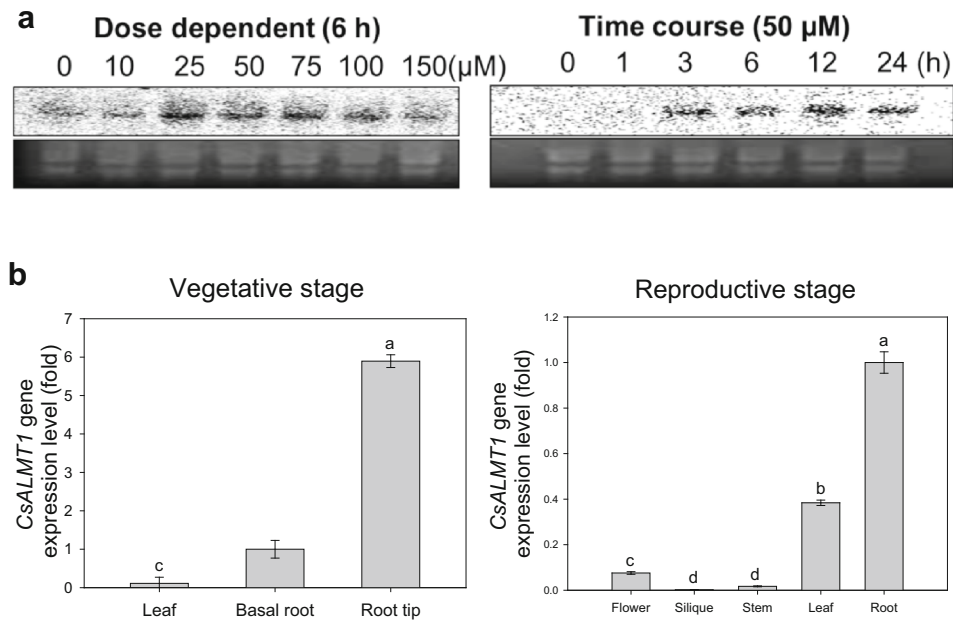


Fig. 2 Analysis of *CsALMT1* expression in Camelina. **a** Northern blot analysis of *CsALMT1* expression in Camelina seedlings by dose and time course with AlCl_3 . Plants were exposed to 1/2 HS (Hoagland solution) without phosphate (pH 4.5). **b** Analysis of *CsALMT1* expression in vegetative and reproductive stage of Camelina using quantitative real-time PCR. Total RNA was isolated from the leaves, the basal root (1–4 cm), and the root tip (0–1 cm) at vegetative stage

and from the flower, silique, stem, leaf, and root at the reproductive stage. Expression (fold) was calculated by relative comparison with the expression of the *CsALMT1* gene in the basal root (vegetative stage) or root (reproductive stage). *CsActin* was used as an internal control for normalization. Values are the averages of three replicates \pm SE. Different lowercase letters in the figure indicate significant difference at $P < 0.05$ by Tukey's test

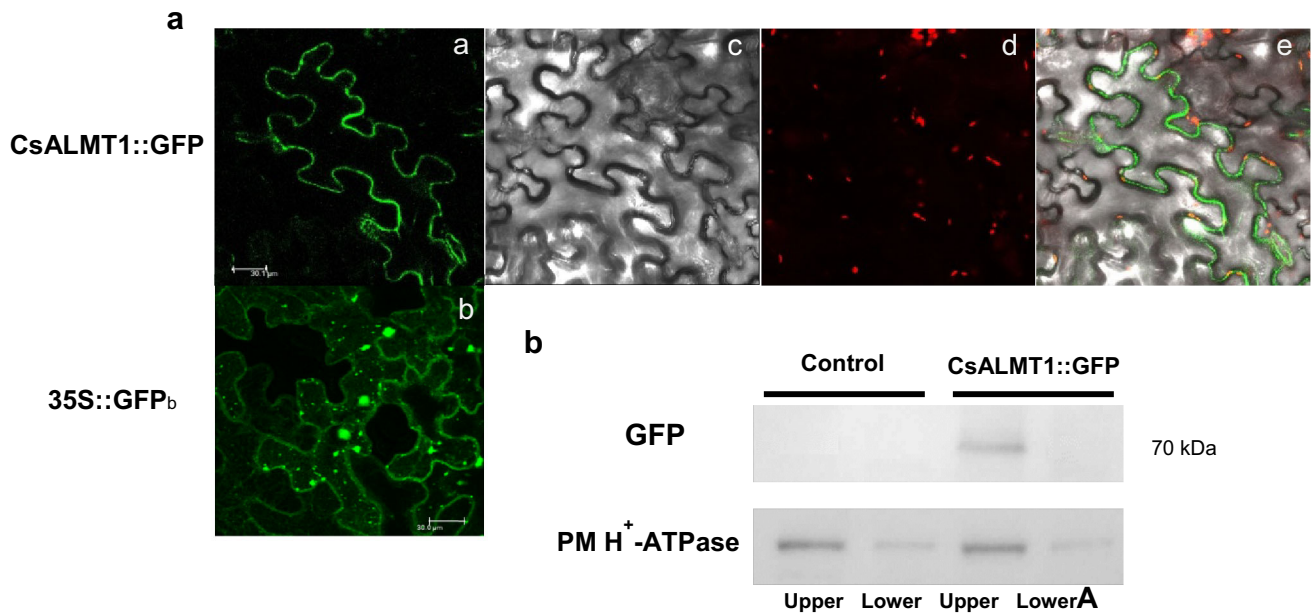


Fig. 3 Subcellular localization and western blot of *CsALMT1::GFP* fusion proteins in Tobacco. **a** Laser confocal laser scanning microscope images of tobacco cells expressing *CsALMT1::GFP* fusions and GFP proteins [**a** and **b** GFP-fluorescence (green), **c** bright-field image, **d** merged images show bright-field image, chlorophyll fluorescence (red), and GFP-fluorescence (green)]. Scale bars

represent 30 μ m. **b** Western blot of *CsALMT1::GFP* in tobacco leaf. Plasma membranes were isolated from leaves using two-phase partitioning method. Upper phase and lower phase were separated by SDS-PAGE followed by analysis with anti-GFP and anti- H^+ -ATPase (positive control for plasma membrane control)

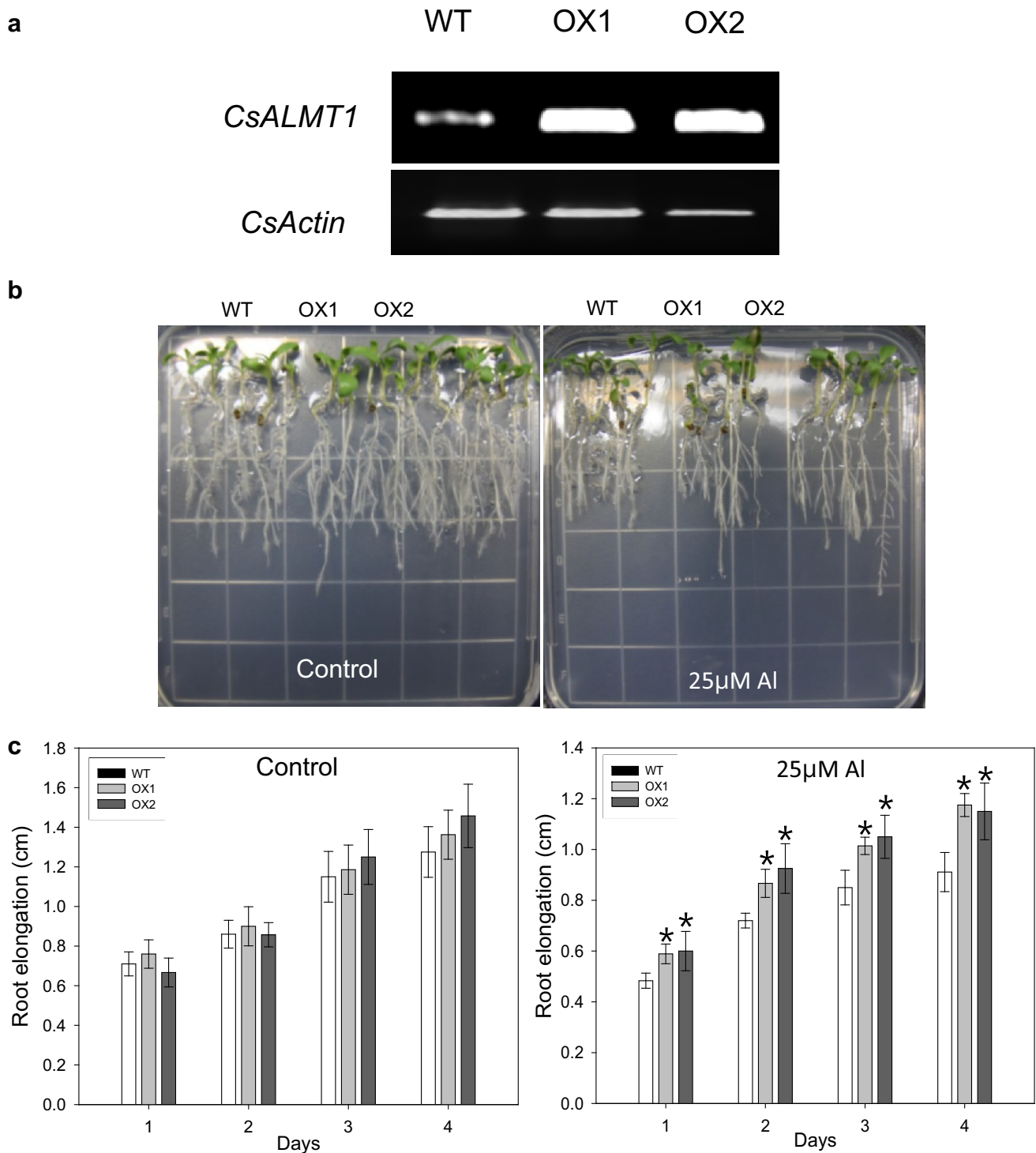


Fig. 4 Effect of Al treatments on root growth in wild-type and *CsALMT1*-overexpressing plants. **a** *CsALMT1*-overexpressing plants identified by RT-PCR using *CsALMT1*-specific primer. One-week-old seedlings were transferred in agarose medium containing Al adjusted to pH 4.5 without phosphate. **b** Photographs of Camelina wild-type and two *CsALMT1*-overexpressing lines grown on agar plates with or

without 25 μ M Al concentration for 4 days. **c** Root elongation of the plants measured daily during 4 days after transferring to Al-mediums. Data represent mean \pm SD ($n = 30$). Asterisks in the figure indicate significant difference from wild type subjected to the same treatment time at $*P < 0.05$ by Dunnett's test

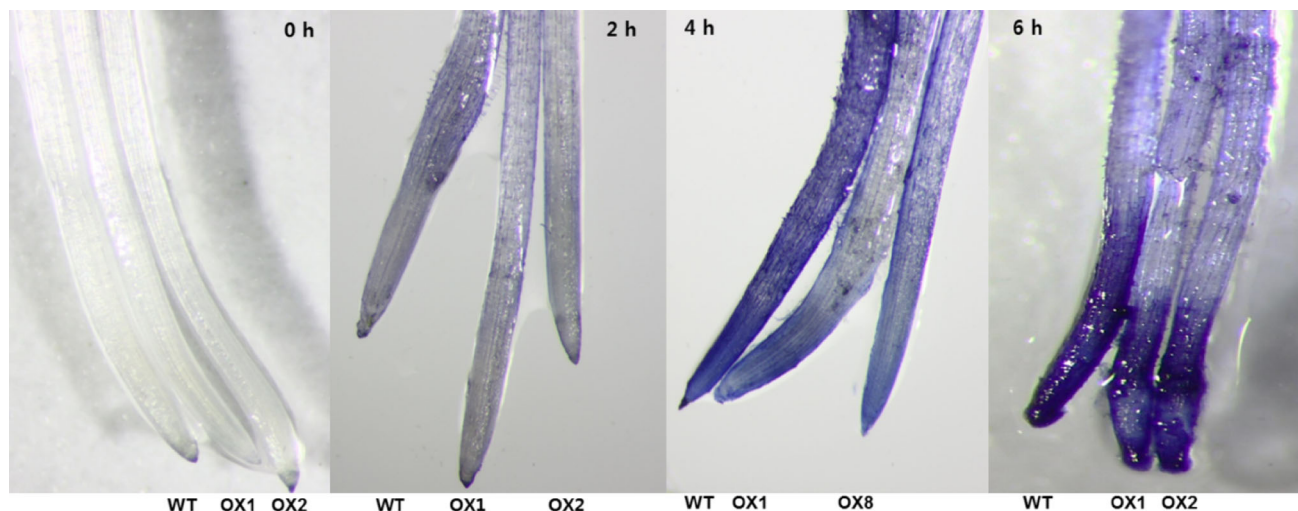


Fig. 5 Hematoxylin staining in roots of wild-type and *CsALMT1*-overexpressing plants. Camelina seedlings were grown for 4 days in normal condition. The plants were stained with 0.2% hematoxylin for

10 min after 0, 2, 4, and 6 h Al (25 μ M) treatment periods without phosphate (pH 4.5)

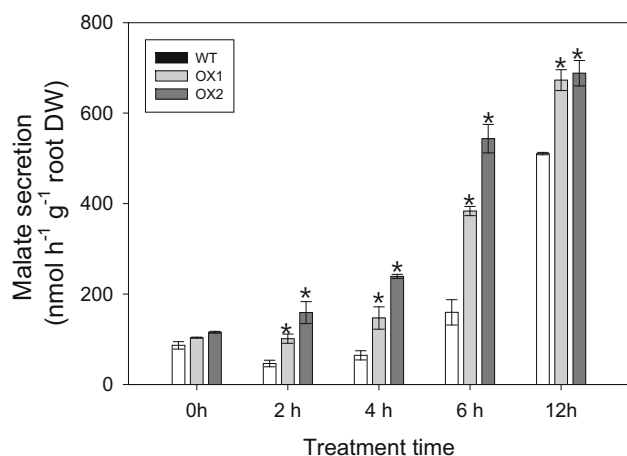


Fig. 6 Time course of malate secretion from roots of camelina wild-type and *CsALMT1* overexpressing transgenic plants. Root exudates were collected at 0, 2, 4, 6, and 12 h after 25 μ M Al treatment at pH 4.5. Organic acids were analyzed by HPLC. Asterisks in the figure indicate significant difference from wild type subjected to the same treatment time at $*P < 0.05$ by Dunnett's test

under Al stress, we analyzed the malate secretion in root exudates of the wild-type and *CsALMT1* transgenic plants collected at 2, 4, and 6 h after initiation of Al treatment. A small amount of secretion of malate in the root exudate of the wild-type plant was maintained steadily up to 4 h after Al treatment, and then increased after 6 h Al treatment by about 1.5-fold compared to the initial secretion amount (Fig. 6). However, *CsALMT1* transgenic plants secreted larger amounts of malate than the wild-type plant from the beginning of Al treatment (2 h), and continued to increase significantly up to 6 h after Al treatment (Fig. 6).

Comparison of Al absorption in roots of wild-type and *CsALMT1* transgenic plants

Protoplasts of the roots of wild-type and *CsALMT1* transgenic plants were isolated to compare the Al absorption of the root protoplasts. 50 μ M Al was treated for 40 min after morin staining for 1 h. Then, measurements of the green fluorescence signal made by the Al-morin complexes indicated that the signal intensity was brighter in wild-type protoplasts than in the *CsALMT1* overexpressing plants after 40 min (Fig. 7). This indicates that wild-type protoplasts absorbed more Al than the *CsALMT1* overexpressing protoplasts. Subsequently, a comparison between the wild-type and *CsALMT1* transgenic plants in Al content was performed. Plants were treated with 25 μ M Al, measured at the roots, and sampled at 0, 2, 4, and 6 h. The result confirmed that the roots of the wild-type plant absorbed more Al than the roots of the *CsALMT1* transgenic plants at all times (Fig. 8).

Discussion

Al-induced organic acid secretion is a major Al tolerance mechanism in both monocotyledons and dicotyledons (Sasaki et al. 2004; Kochian et al. 2004, 2005; Ligaba et al. 2006; Hoekenga et al. 2006; Ma 2007; Gruber et al. 2010). *CsALMT1* was cloned and characterized in this study. *CsALMT1* amino acid sequence was 86% similar to *AtALMT1*, but it was 36–37% similar to *TaALMT1-1* or *ZmALMT1* amino acid sequences (Fig. 1). These differences in homology may arise from the differences between monocotyledons and dicotyledons (Fig. S1B). In this study,

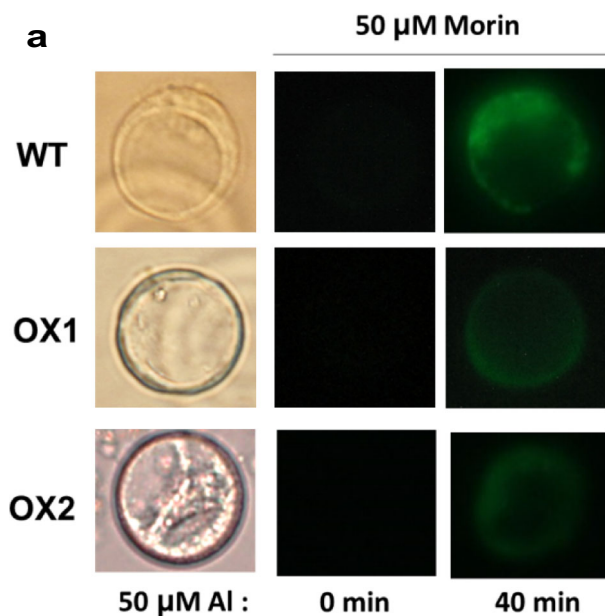
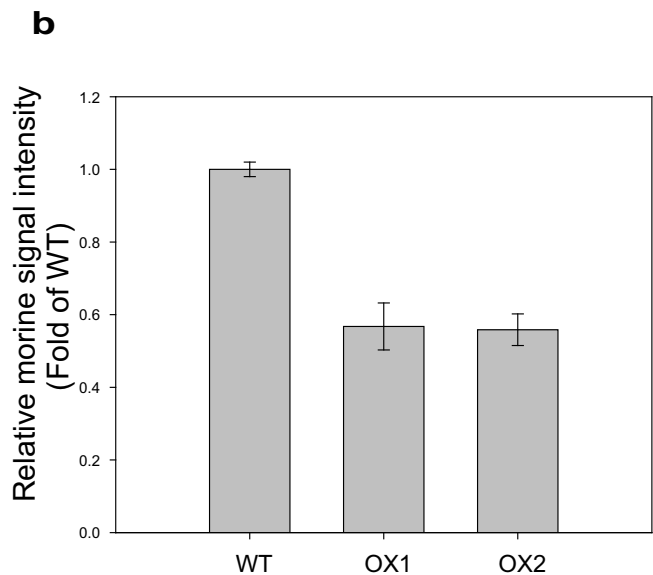


Fig. 7 Aluminum accumulation in Camelina root protoplast. Root protoplasts from the wild-type and *CsALMT1*-overexpressing plants were incubated with 50 μM morin for 1 h before 50 μM Al treatment.



Then, 50 μM Al was applied for 40 min. **a** Green fluorescence signal occurring due to Al-morin complexes. **b** Signal intensities were quantitated by ImageJ

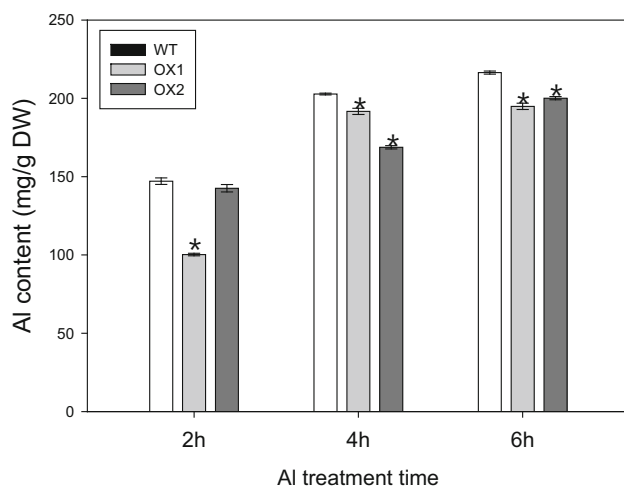


Fig. 8 Al contents in roots of the wild-type and *CsALMT1* transgenic lines. 3-week-old plants were grown hydroponically and treated with 25 μM Al for 2, 4, and 6 h without phosphate (pH4.5). Data represent mean \pm SD ($n = 3$). Asterisks in the figure indicate significant difference from wild type at $*P < 0.05$ by Dunnett's test

CsALMT1 expression was increased with the Al concentration and treatment time (Fig. 2). This is similar to *BnALMT1* (Ligaba et al. 2006), *AtALMT1* (Hoekenga et al. 2006), and *ZmALMT1* (Pinos et al. 2008) but contrasts with *TaALMT1* (Sasaki et al. 2004) and *HvALMT1* (Gruber et al. 2010) that were unaffected by Al treatment. However, these differences in *ALMT1* expression patterns observed from the various plants with or without Al treatment could not be correlated with the differences in homology between monocotyledons and dicotyledons.

Seven members of the ALMT family were characterized for two groups, where one group was characterized by little or no response to Al^{3+} and the other was strongly activated by Al^{3+} , which includes *CsALMT1*. The first group includes *HvALMT1* and *ZmALMT1*, and the second group includes *AtALMT1*, *BnALMT1*, *BnALMT2*, *TaALMT1* (Gruber et al. 2010), and *CsALMT1*. Furthermore, *CsALMT1* has a root-specific expression at both the vegetative and reproductive stages (Fig. 2b). The ALMT1 family of membrane proteins consists of 5–8 predicted transmembrane domains (Sasaki et al. 2004; Delhaize et al. 2007). The *CsALMT1* has five putative transmembrane regions, which indicates that it is likely to be membrane-bound (Fig. S1). This result shows that *CsALMT1* is probably localized to the plasma membrane. As expected, the *CsALMT1::GFP* fusion protein was localized to the plasma membrane, which was transiently introduced into tobacco leaf (Fig. 3a). This was also confirmed using Western blot, in which protein is localized in the plasma membrane (Fig. 3b).

The first sign of Al toxicity is the inhibition of root growth and cell division (Clarkson 1966; Klimashevski and Dedov 1975). In the present study, analysis of the root elongation and hematoxylin staining was used as an index for the Al tolerance in Camelina wild-type and *CsALMT1* overexpressing plants (Figs. 4, 5, respectively). The results show that Camelina root elongation was clearly inhibited when 25 μM Al was applied because the Camelina seedling roots were very soft (Fig. 4). Interestingly, hematoxylin staining showed that the roots of the wild-type plant

were more damaged than the roots of the *CsALMT1* overexpressing plants. The root apex has been recognized as a primary site of Al toxicity in plants (Ryan et al. 1993; Kochian 1995; Ahn et al. 2001). However, in the case of Camelina, Al-induced injury occurred in the entire root, not only in the root apex, with 25 μM Al treatment (Fig. 5). This result indicates that the all the root cell membranes of Camelina were disrupted simultaneously.

The patterns of organic acid anion secretion can be grouped into two patterns (Ma 2000). In pattern I, the secretion of organic acid anions immediately occurs when exposed to Al. Pattern II exhibits a significant lag phase until the onset of organic acid anion release. We propose a hypothetical model of Al-activated malate secretion from Camelina root, which is typical of pattern II. Al^{3+} interacts with a receptor on the plasma membrane to initiate a signal transduction pathway. In Arabidopsis, Al^{3+} and/or protons interact with a receptor on the plasma membrane to initiate a signal transduction pathway. A protein kinase phosphorylate sensitive to proton rhizotoxicity1 (STOP1) is used to convert the STOP1 from an inactive to active form (Iuchi et al. 2007; Sawaki et al. 2009). This activated transcription factor in Camelina may interact with sequences in the promoter of *CsALMT1* s. Then, *CsALMT1* s mRNA translates into the protein CsALMT1 s that are located on the plasma membrane of root cells, where they may transport malate to the external medium. The secreted malate binds and detoxifies Al^{3+} to protect the root. This result is similar to the regulation of the citrate secretion step in the rice bean (Liu et al. 2013). We demonstrated the simple regulation of malate secretion in response to Al exposure in the Camelina root. A lag phase of 0–4 h occurs for the secretion of malate from the Camelina root in response to Al treatment (Fig. 6), which is typical of pattern II organic acid release, as proposed by Ma (2000). A high level of malate secretion required almost 6 h of Al treatment. Interestingly, *CsALMT1* overexpressing plants can secrete a substantial amount of malate secretion as pattern I, unlike wild-type plant (Fig. 6). This result suggested that high malate secretion enhanced the Al tolerance of the *CsALMT1* overexpressing line. In addition, oxalic acid is an important organic acid in Al detoxifying mechanisms, both internally and externally (Zheng et al. 1998; Ma et al. 1998). Therefore, further study is required on the regulation of oxalic acid secretion in response to Al exposure in the *CsALMT1* overexpressing line.

The roots undergo Al toxicity both externally and internally. When the root absorbs Al^{3+} , internal toxicity occurs. To identify the amount of Al absorbed by the Camelina root, 25 μM Al was applied for 1, 3, 6, 12, and 24 h. The Al contents in 1 cm root segments from the root tip were compared with the basal root (2–3 cm). The accumulation of Al did not differ significantly in each root

segment but increased slightly in the root tips after 12 h of treatment (data not shown). Al is rarely translocated from the root to shoots (data not shown). Furthermore, the root protoplasts from the wild-type Camelina absorbed more Al than in the *CsALMT1* overexpressing plants (Fig. 7), which eventually led to lower aluminum content in the roots of the *CsALMT1* overexpressing plants (Fig. 8). These results suggest that when *CsALMT1* transgenic plants are stressed by Al, *CsALMT1* as a malate transporter can contribute to induce the malate secretion and enhance the Al detoxification function by limiting Al absorption.

In conclusion, we isolated *CsALMT1* from Camelina, which has Al tolerance enabled by malate secretion. The function of *CsALMT1* was investigated using *CsALMT1* overexpressing plants. *CsALMT1* transgenic plants have Al tolerance compared with wild-type plants. This genetically induced plant changes in *CsALMT1* expression, which may lead to increased malate exudation in the Camelina roots. Therefore, we suggest that *CsALMT1* might be a malate transporter localized to the root plasma membrane that plays a critical role in Camelina Al tolerance.

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Author contribution In this study, WP and H-S Kim contributed to the experimental conception, design, and writing the article. Dr. S-J Ahn participated in designing the study and revised the manuscript. All authors contributed to the complete article.

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