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Adaptation to acidic soil is achieved by increased numbers of *cis*-acting elements regulating *ALMT1* expression in *Holcus lanatus*

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SUMMARY

Yorkshire fog (*Holcus lanatus*), which belongs to the Poaceae family and is a close relative of the agronomic crop oat (*Avena sativa*), is a widely adaptable grass species that is able to grow on highly acidic soils with high levels of Al, but the mechanism underlying the high Al tolerance is unknown. Here, we characterized two accessions of *H. lanatus* collected from an acid plot (soil pH 3.6, HL–A) and a neutral plot (pH 7.1, HL–N) in terms of Al tolerance, organic acid anion secretion and related gene expression. In response to Al (pH 4.5), the HL–A roots secreted approximately twice as much malate as the HL–N roots, but there was no difference in citrate secretion. Cloning of the gene *HlALMT1* responsible for malate secretion showed that the encoded amino acid sequence did not differ between two accessions, but the expression level in the outer cell layers of the HL–A roots was twice as high as in the HL–N roots. This difference was not due to the genomic copy number, but was due to the number of *cis*-acting elements for an Al-responsive transcription factor (HlART1) in the promoter region of *HlALMT1*, as demonstrated by both a yeast one-hybrid assay and a transient assay in tobacco protoplasts. Furthermore, introduction of *HlALMT1* driven by the HL–A promoter into rice resulted in significantly more Al-induced malate secretion than introduction of *HlALMT1* driven by the HL–N promoter. These findings indicate that the adaptation of *H. lanatus* to acidic soils may be achieved by increasing number of *cis*-acting elements for ART1 in the promoter region of the *HlALMT1* gene, enhancing the expression of *HlALMT1* and the secretion of malate.

Keywords: adaptation, aluminum tolerance, malate, *ALMT1*, *Holcus lanatus*.

INTRODUCTION

Soluble ionic aluminum in acid soils (mainly Al^{3+}) rapidly inhibits root elongation at the micromolar level, subsequently affecting the uptake of water and nutrients and resulting in low crop productivity on such soils (Kochian, 1995; Ma, 2007; Ryan *et al.*, 2011). However, some plants have evolved mechanisms to detoxify Al both internally and externally. There is a wide variation in Al tolerance between plant species and cultivars within a species. Several mechanisms for the variation in Al tolerance have been proposed (Kochian, 1995), among which variation in the secretion of organic acid anions, such as citrate, malate or oxalate, from roots appears to be the most common mechanism in both monocots and dicots (Ryan *et al.*, 2001; Kochian *et al.*, 2004; Ma, 2005, 2007; Magalhaes, 2006). These organic acid anions are able to chelate toxic Al ions, thereby detoxifying Al externally.

Genes responsible for Al-induced organic acid anion secretion have been identified in various plant species. They belong to two gene families: *ALMT* (aluminum-activated malate transporters) and *MATE/AACT* (multi-drug and toxic compound extrusion/aluminum-activated citrate transporters) (Ryan *et al.*, 2011; Delhaize *et al.*, 2012). The *ALMT1* gene has been identified in wheat (*Triticum aestivum*) (Sasaki *et al.*, 2004), Arabidopsis (*Arabidopsis thaliana*) (Hoekenga *et al.*, 2006), oilseed rape (*Brassica napus*) (Ligaba *et al.*, 2006), rye (*Secale cereale*) (Collins *et al.*, 2008), maize (*Zea mays*) (Krill *et al.*, 2010) and soybean (*Glycine max*) (Liang *et al.*, 2013), although the expression

patterns differ among plant species. The proteins encoded by *ALMT1* genes transport malate and are localized to the plasma membranes of root cells. In contrast, *MATE1/AACT1* genes have been identified in barley (*Hordeum vulgare*) (Furukawa *et al.*, 2007), sorghum (*Sorghum bicolor*) (Magalhaes *et al.*, 2007), Arabidopsis (*Arabidopsis thaliana*) (Liu *et al.*, 2009), rye (*Secale cereale*) (Yokosho *et al.*, 2010), maize (*Zea mays*) (Maron *et al.*, 2010), rice bean (*Vigna umbellata*) (Yang *et al.*, 2011), rice (*Oryza sativa*) (Yokosho *et al.*, 2011) and wheat (Tovkach *et al.*, 2013). The proteins encoded by the *MATE1/AACT1* genes are also localized to the plasma membranes, but transport citrate. High expression levels of these genes are associated with high Al tolerance in wheat and barley (Sasaki *et al.*, 2006; Furukawa *et al.*, 2007). Furthermore, recent studies have shown that alterations of the promoter regions are responsible for enhanced expression of *ALMT1* (Sasaki *et al.*, 2006; Ryan *et al.*, 2010) and *MATE1/AACT1* (Magalhaes *et al.*, 2007; Fujii *et al.*, 2012; Tovkach *et al.*, 2013).

In addition to Al-tolerance genes related to organic acid anion secretion, other Al-tolerance genes have been identified, especially in rice. Identification of a transcription factor for Al tolerance, ART1, revealed that multiple genes are involved in the high Al tolerance in rice (Yamaji *et al.*, 2009). Functional analyses of several downstream genes regulated by ART1 showed that they are involved in both internal and external detoxification of Al at various cellular sites, such as the cell wall, plasma membrane and tonoplast (Huang *et al.*, 2009, 2012; Xia *et al.*, 2010; Yokosho *et al.*, 2011; Chen *et al.*, 2012).

Compared with the progress made in important crops and model plant species, little is known about the molecular mechanisms of Al tolerance in plant species growing in their natural habitats. Yorkshire fog (*Holcus lanatus*), which belongs to the Poaceae family and whose closest relative is the agronomic crop oat (*Avena sativa*), is a highly adaptable grass species found in wide environmental conditions (Grime *et al.*, 1988). For example, *H. lanatus* was found in most plots in the long-term Park Grass Experiment at Rothamsted Research, which vary greatly in nutrient availability and soil pH (from 3.6–7.5) (Silvertown *et al.*, 2006). In the plots that are highly acidic due to long-term applications of ammonium sulfate, *H. lanatus* is the dominant species (>90% of the total above-ground plant biomass in the plot), presumably due to its strong tolerance to Al toxicity. However, it is also found in plots receiving the same fertilizer treatment but limed to the neutral range of soil pH, where its biomass production decreases to <10% of the total due to competition from other plant species. In an effort to understand how *H. lanatus* adapts to highly acidic soils, we compared two accessions of plants from an acidic plot (pH 3.6, designated HL–A) and a limed plot (pH 7.1, designated HL–N) with regard to their Al tolerance, organic acid anion secretion and expression of related genes. We found that the high Al tolerance in the accession from the acidic plot results from an increased number of *cis*-acting elements for the transcription factor HlART1 in *HlALMT1* gene, resulting in increased expression of *HlALMT1* and higher secretion of malate from the roots.

RESULTS

Comparison of tolerance to low pH and Al between two accessions of *Holcus lanatus*

We first compared the root elongation at various pHs between HL–A from an acidic plot and HL–N from a neutral plot. However, there was no difference in the root elongation between the two accessions at pHs ranging from 3.5 to 6.5 (Figure 1a). We then compared the Al tolerance between two accessions. In the presence of 50 μM Al, root elongation of HL–N was inhibited to a great extent than that of HL-A at each time point (Figure 1b). The Al tolerance of HL–A was as high as for a *japonica* rice cultivar, Nipponbare, the most Al-tolerant species among small-grain cereal crops (Figure S1). Eriochrome cyanine R staining showed that HL–N accumulated more Al in the root tips than HL–A (Figure 1c). Consistent with this result, the Al content in the cell sap and cell wall of the root tips (0–1 cm) was also significantly higher in HL–N than in HL–A at each Al concentration (Figure 1d,e), indicating that an exclusion mechanism is involved in the higher Al tolerance of HL–A.

HL–A secreted more malate than HL–N in response to Al

Next we investigated whether Al-induced secretion of organic acid anions is involved in higher Al tolerance in the HL–A accession. Both accessions secreted malate and citrate from the roots in response to Al (Figure 2a), but the amount of citrate secreted was small and did not differ significantly between the two accessions. In contrast, HL–A secreted approximately twice as much malate as HL–N (Figure 2a). The malate secretion induced by Al in HL–A (Figure 2) was comparable to that in rye and wheat, which utilize organic acid anion secretion for Al detoxification (Li *et al.*, 2000). A dose–response experiment showed that the amount of malate secreted increased with increasing Al concentrations in both accessions, but HL–A secreted more malate than HL–N at all Al concentrations tested (Figure 2b). Furthermore, the secretion was only induced by Al, but not by Cd or La, indicating that the organic acid anion secretion is specific to Al (Figure 2c).

Cloning of a gene responsible for malate secretion, *HlALMT1*

Al-induced secretion of malate has been reported to be mediated by ALMT1 (Sasaki *et al.*, 2004; Hoekenga *et al.*, 2006; Ligaba *et al.*, 2006). Based on sequence information for *ALMT1* genes in other species, we performed RT–PCR to amplify the highly conserved region and used 5′ and 3′

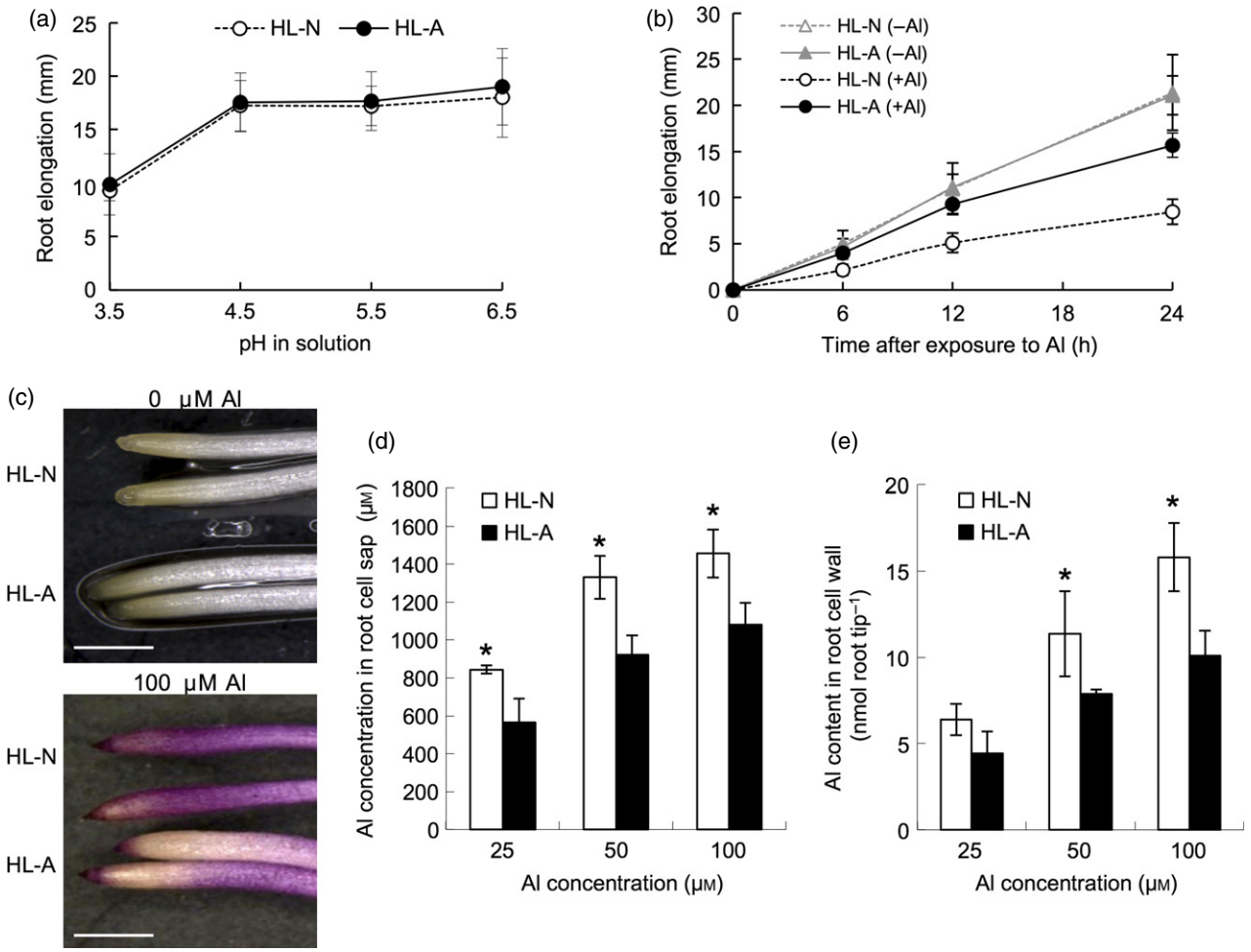

Figure 1. Physiological analysis of tolerance to low pH and Al in two *Holcus lanatus* accessions.
(a,b) Response to various pHs (a) and Al (b). Seedlings were exposed to a buffered solution at various pHs for 24 h or a solution containing 50 μM Al for 0, 6, 12, 18 and 24 h. The root length was measured before and after the treatment. Values are means ± SD (n = 10).
(c) Eriochrome cyanine R staining. Roots exposed to 0 and 100 μM Al for 24 h were stained in 0.1% Eriochrome cyanine R for 15 min. Scale bar = 10 mm.
(d,e) Al concentration in the cell sap (d) and Al content in the cell wall (e) of root tips (0–1 cm). Seedlings of both accessions were exposed to a solution containing 25, 50 and 100 μM Al for 24 h. The root tips were excised and fractionated into cell sap and cell wall. The Al concentration was determined by atomic absorption spectrophotometer (AAS). Values are means ± SD (n = 3). The asterisks indicate a significant difference between accessions ($P < 0.05$ by Tukey's test).

RACE to obtain the full-length cDNA of the putative malate transporter gene in both accessions of *H. lanatus*. The isolated gene (designated *HlALMT1*) consists of a 1532 bp long cDNA, including a 1389 bp ORF. There are five single nucleotide polymorphisms between HL–A and HL–N (Figure S2a), but the encoded peptides (463 amino acids) were the same (Figure S2a). HlALMT1 shared 81, 43, 44 and 42% identity, respectively, with TaALMT1, GmALMT1, BnALMT1 and AtALMT1 (Figure S2b).

Transport activity of HlAMLT1

To investigate the transport activity of HlALMT1 for malate efflux, we expressed this gene in *Xenopus* oocytes and injected the oocytes with ^{14}C-labeled malate. Measurement of the ^{14}C radioactivity showed that oocytes expressing *HlALMT1* had a significantly higher efflux activity for malate than the control in the absence of Al (Figure 3a), indicating that HlALMT1 is able to transport malate out of the cells.

To examine whether HlALMT1 is activated by Al, we performed two-electrode voltage clamp analysis. The results showed that the current in oocytes expressing *HlALMT1* was much higher in the presence of Al than that in the absence of Al (Figure 3b). This result indicate that HlALMT1 is activated by Al.

Subcellular localization of HlAMLT1

The subcellular localization of HlALMT1 was investigated by transiently expressing *GFP–HlALMT1* or *HlALMT1–GFP* together with the *DsRed* gene encoding red fluorescence protein in onion epidermal cells. DsRed was used as a marker which is localized at cytoplasm and nucleus. When

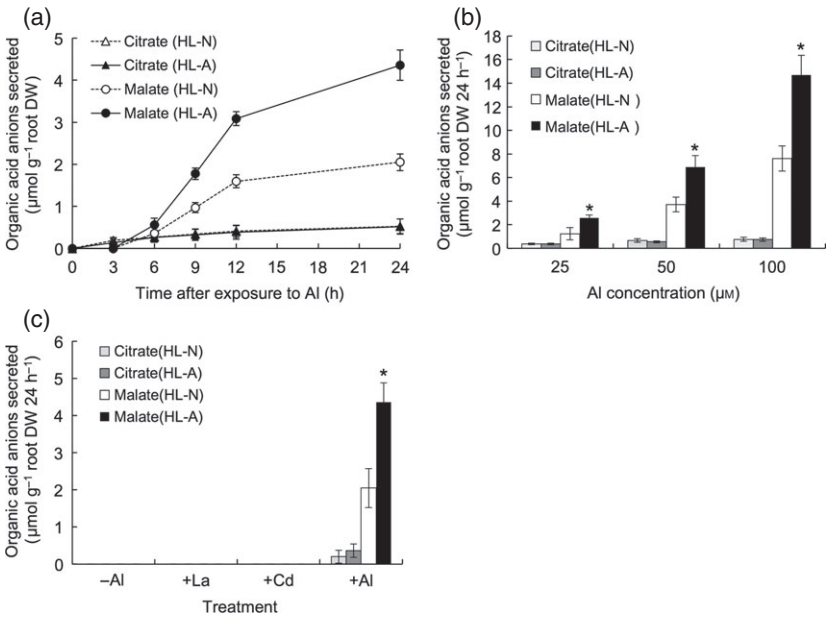

Figure 2. Time- and dose-dependent Al-induced secretion of organic acid anions in two *Holcus lanatus* accessions.
(a) Time-dependent secretion of organic acid anions. Seedlings were exposed to a solution containing 50 μM Al for 0, 3, 6, 9, 12 and 24 h.
(b) Dose–response. Seedlings were exposed to a solution containing 25, 50 or 100 μM Al for 24 h.
(c) Metal specificity. Seedlings were exposed to a solution containing 50 μM of Al, La or Cd for 24 h.
Root exudates were collected after each treatment, and the levels of organic acid anions were determined by an enzymatic method. Values are means ± SD (n = 3). (b, c) The asterisks indicate a significant difference between accessions (P < 0.05 by Tukey's test).

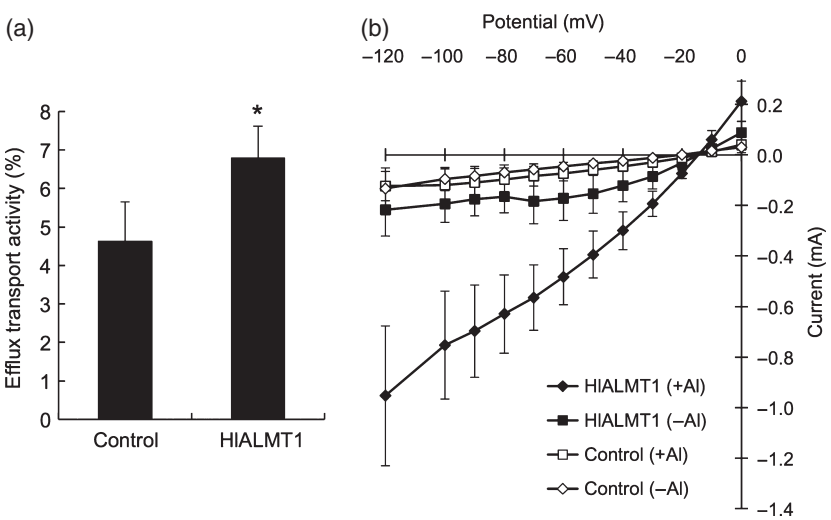

Figure 3. Transport activity of HIALMT1 in *Xenopus* oocytes.
(a) Basal transport activity for malate efflux in *Xenopus* oocytes. *HIALMT1* cRNA or water (control) was injected into *Xenopus* oocytes. After 1–day cultivation, the oocytes were injected with ^{14}C-labeled malate. The release of ^{14}C-labeled malate from the oocytes in the absence of Al was determined after 1 h. The percentage of total malate injected is shown. Values are means ± SD (n = 3–4). The asterisk indicates a significant difference compared with control (P < 0.05 by Tukey's test).
(b) Al-activated transport activity for malate efflux. Malate was injected into oocytes with or without *HIALMT1* expression, and the inward current was recorded at membrane potentials between -120 and 0 mV in the presence and absence of 100 μM Al. Values are means ± SD (n = 5–9).

GFP alone was expressed, fluorescence was observed in the cytosol and nucleus, and completely overlapped with the DsRed signal (Figure 4, top panels). By contrast, the green fluorescence of both GFP–HIALMT1 (Figure 4, middle panels) and HIALMT1–GFP (Figure 4, bottom panels) was only observed at the cell periphery distinct from the DsRed signal. These results indicate that HIALMT1 is localized to the plasma membrane.

Expression pattern of *HIALMT1* in *H. lanatus*

Expression of *HIALMT1* was hardly detected in both roots and shoots of two accessions in the absence of Al (Figure 5a). However, expression in the roots but not in the shoots was greatly induced by Al in both accessions (Figure 5a). Spatial analysis showed that *HIALMT1* expression in both the root tips (0–1 cm) and the mature regions (1–3 cm) was up-regulated by Al (Figure 5a). A time-course experiment showed that this induction occurred after 3 h exposure to Al (Figure 5b). The expression level of *HIALMT1* was significantly higher in HL–A than in HL–N after between 6 and 24 h of Al exposure (Figure 5b). The expression of *HIALMT1* increased with increasing Al concentrations in both accessions, but the expression level in HL–A was higher than that in HL–N at each Al concentration (Figure 5c).

To investigate the tissue specificity of *HIALMT1* expression, we used laser microdissection to separate the root tip tissues into outer and inner parts (Figure 5d,e). Expression of *HIALMT1* in the outer tissues was higher than that in the inner tissues in both accessions (Figure 5f). There was no

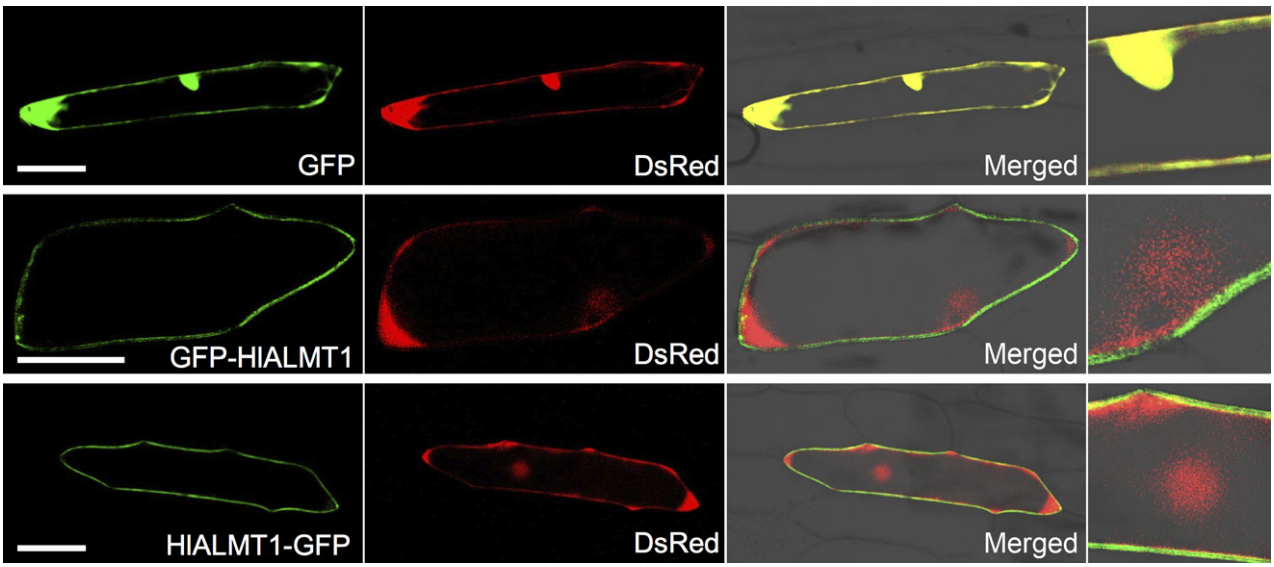

Figure 4. Subcellular localization of HIALMT1.
Constructs expressing GFP alone (top panels) or fusions between *HIALMT1* and green fluorescent protein gene (GFP) at the N–terminus (middle panels) or the C–terminus (bottom panels) were introduced into onion epidermal cells together with construct expressing DsRed. Scale bars = 100 μm.

difference in the expression level in the inner tissues between the two accessions, but expression in the outer tissues was much higher in HL–A than HL–N (Figure 5f). These results indicate that the difference in *HIALMT1* expression between the two accessions arises from expression in the outer tissues.

Copy number of the *HIALMT1* gene

The differential expression of *HIALMT1* in the two accessions may be due to a difference in the genomic copy number of *HIALMT1*. However, this possibility was ruled out because the two accessions have the same number of genomic copies of *HIALMT1* [normalized against that for a silicon transporter gene *Lsi1* (*low silicon rice 1*; *Os02 g0745100*)] (Figure 6a) (Ma *et al.*, 2006).

We then compared the absolute mRNA level of *HIALMT1*. The transcript copy number calculated from the standard curves using C_T values was three times higher in HL–A than in HL–N (Figure 6b). These results indicate that the expression levels of *HIALMT1* in the two accessions may be attributed to the transcript copy number but not the genomic copy number.

Comparison of the promoter region of *HIALMT1* in the two accessions

A possible mechanism for the differential expression of *HIALMT1* in the two accessions may be different promoter activities. To test this possibility, we isolated and compared the promoter region (−2 kb) between the two accessions. Although there was no difference in the amino acid sequence of *HIALMT1* between the two accessions (Figure S2a), variations in the promoter region were found (Figure S3). However, these differences are not due to large deletions or insertions in the promoter region. In Arabidopsis, expression of *AtMATE1* and *AtALMT1* is regulated by a transcription factor, STOP1 (Liu *et al.*, 2009; Sawaki *et al.*, 2009). In rice, expression of *OsFRDL4* (*MATE*) is regulated by ART1, a homolog of STOP1 (Yamaji *et al.*, 2009), and its core *cis*-acting element sequence has recently been identified as [GGN(T/g/a/C)V(C/A/g)S(C/G)] (Tsutsui *et al.*, 2011). Searches for the high-affinity ART1 *cis*-acting element (GGTCCT, GGCCCT, GGTACT, GGTCGT, GGGCCT, GGACCT or GGTGCT, the terminal T is unchanged) in the promoter region of *HIALMT1* revealed that five elements are present in HL–A but only three in HL–N (Figure S3). The difference in the *cis*-acting element number between the two accessions is caused by single nucleotide substitutions.

Cloning and expression analysis of *HIART1*

To examine whether the difference in the *cis*-acting element number explains the different expression levels of *HIALMT1*, we first cloned an *ART1* homolog (*HIART1*) in *H. lanatus* based on the conserved sequences of *ART1* and *ART1*-like genes in rice and Arabidopsis. A cDNA fragment of 360 bp was amplified, and a 1697 bp full-length *HIART1* gene including a 1443 bp ORF was obtained from the cDNA of the two accessions of *H. lanatus* roots using 5′ and 3′ RACE. There was no difference in the nucleotide sequence of *HIART1* between the two accessions (Figure S4a). *HIART1* encodes a peptide of 481 amino acids (Figure S4a), and the encoded protein shares 51.8 and 46% iden-

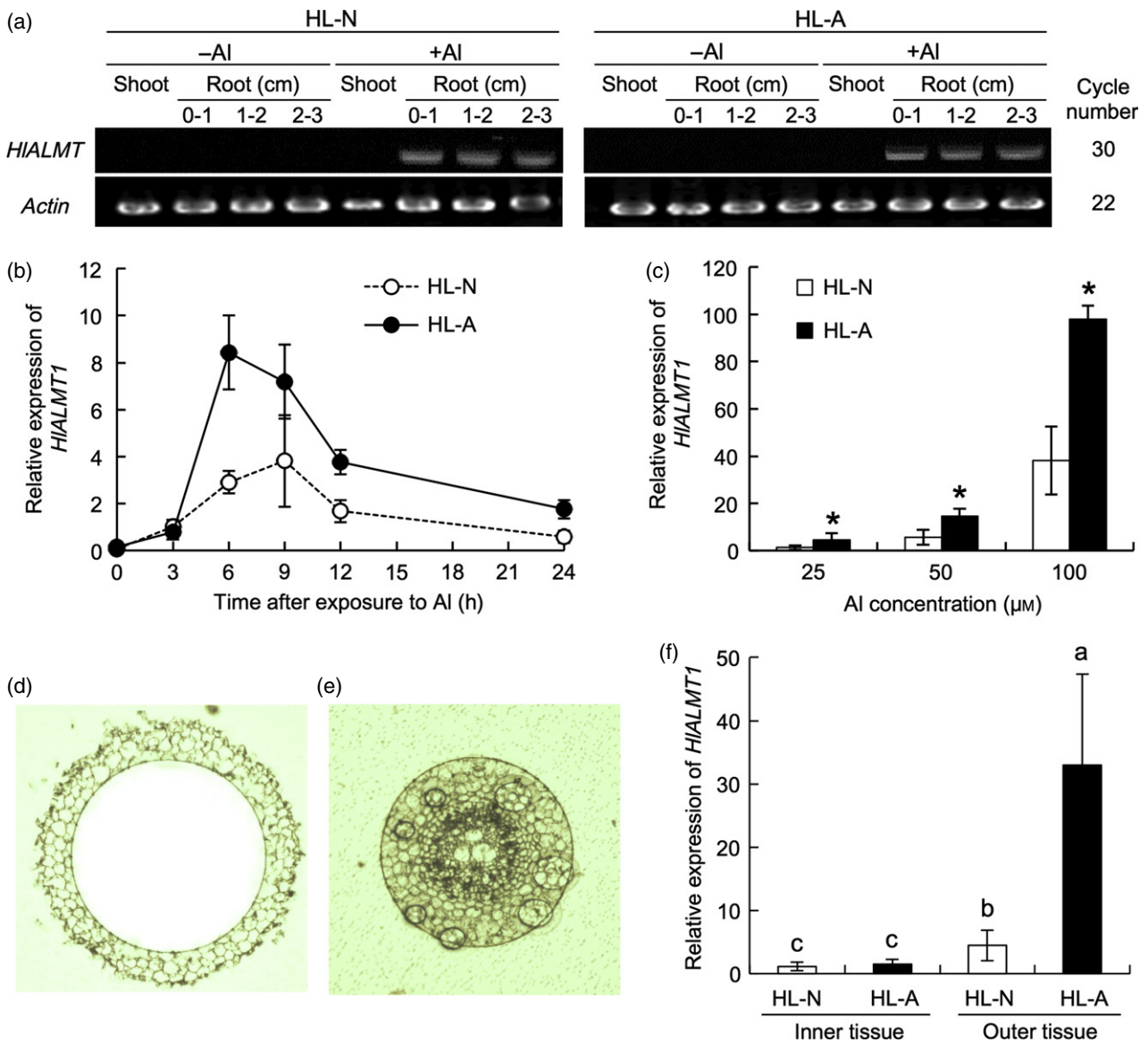

Figure 5. Expression pattern of *HlALMT1* in *H. lanatus.*

(a) Organ-dependent expression. Expression of *HlALMT1* in the various root segments and shoots in the two accessions treated with or without Al. The PCR cycle number is shown on the right.

(b) Time-dependent expression of *HlALMT1*. Seedlings were exposed to a solution containing 50 μM Al for 0, 3, 6, 9, 12 and 24 h. The expression level relative to HL–N at 3 h is shown.

(c) Dose-dependent expression of *HlALMT1*. Seedlings were exposed to a solution containing 25, 50 and 100 μM Al for 24 h. The expression level relative to HL–N at 25 μM is shown.

(d,e) Outer tissues (d) and inner tissues (e) of the roots (7.5–12.5 mm) after laser microdissection.

(f) Tissue specificity of *HlALMT1* expression. The expression level relative to the inner tissues of HL–N is shown.

Actin was used as an internal standard. Values are means ± SD (n = 3). The asterisk in (c) and means with different letters in (f) indicate significant differences between accessions ($P < 0.05$ by Tukey's test).

tity, respectively, with rice ART1 and Arabidopsis STOP1 (Figure S4b). HlART1 has four conserved C2H2 domains (Figure S4a), suggesting a role in the regulation of gene transcription.

The expression level of *HlART1* was similar in the roots of HL–A and HL–N, and was not affected by various concentrations of Al in either accession (Figure 7).

Promoter binding and transcriptional activation assay

To investigate whether HlART1 interacts with the promoter of *HlALMT1*, we performed a yeast one-hybrid assay. In the absence of 3–amino-1,2,4–triazole (3AT, an inhibitor of HIS3 protein which catalyses histidine biosynthesis), there was no difference in growth among the transformed yeast

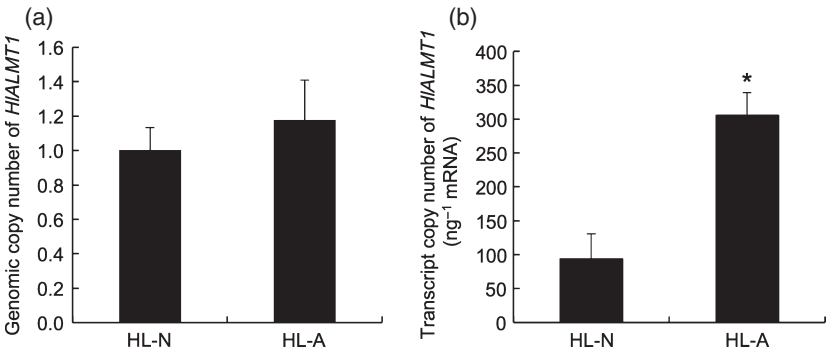

Figure 6. Genomic and transcript copy number of *HlALMT1* in *H. lanatus.*
(a) Genomic copy number in the two accessions. The values were normalized to the C_T values of *Lsi1*.
(b) Transcript copy number. The seedlings were exposed to 50 μM Al for 24 h prior to determination of the absolute expression level of *HlALMT1* in the root tips (0–1 cm) by quantitative real-time RT–PCR. The transcript copy number was calculated from standard curves using C_T values.
Values are means ± SD (n = 3). The asterisk indicates a significant difference compared with HL–N ($P < 0.05$ by Tukey's test).

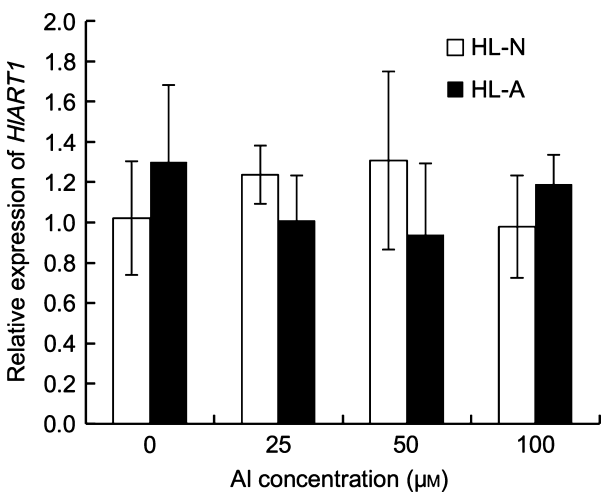

Figure 7. Expression pattern of *HlART1.*
Seedlings of both accessions were exposed to a solution containing 0, 25, 50 or 100 μM Al for 24 h, and then the root tip (0–1 cm) were excised for expression analysis. The expression level relative to HL–N (–Al) is shown. *Actin* was used as an internal standard. Values are means ± SD (n = 3).

cells (Figure 8a). However, in the presence of 20 or 50 mM 3AT, the *HlART1*-expressing cells showed better growth than pGADT7-expressing (control vector) cells (Figure 8a). Furthermore, growth of yeast cells containing the HL–A promoter was better than that of those containing the HL–N promoter. The results for rice ART1, which was included as a positive control, were similar to those for HlART1, suggesting that both rice ART1 and HlART1 probably bind to the same *cis*-acting element in the promoter region of *HlALMT1*. These results suggest that HlART1 interacts with the promoter region of *HlALMT1*, and that the number of *cis*-acting elements is involved in regulation of the expression level of *HlALMT1*.

To quantify the promoter activity for the two accessions, we performed a transient assay in tobacco leaf protoplasts. The *HlALMT1* promoter from HL–A or HL–N was fused to the CaMV 35S minimal (–46) promoter (Fang *et al.*, 1989) and GFP as a reporter gene, and then transformed into tobacco (*Nicotiana tabacum*) mesophyll protoplasts together with the constructs expressing *HlART1* (effector gene) and *DsRed* (internal control) (Figure 8b). The expression of *HlART1* normalized against that of *DsRed* was similar in all transformed protoplasts (Figure 8c). In contrast, expression of the reporter gene GFP normalized against that of *DsRed* was higher in protoplasts expressing constructs carrying *HlALMT1* promoters from either accession than those carrying the CaMV 35S minimal promoter (control) when introduced together with HlART1, indicating HlART1-dependent activity of *HlALMT1* promoter. Furthermore, the GFP expression was twice as high in protoplasts carrying the HL–A promoter than in those carrying the HL–N promoter (Figure 8d). These results show that the *HlALMT1* promoter from HL–A has a higher activity than that from HL–N.

Functional analysis of the *HlALMT1* promoter in transgenic rice

To further examine the role of the promoter region of *HlALMT1* in the regulation of gene expression, we introduced *HlALMT1* driven by promoters from the two accessions into rice. We obtained a number of transgenic lines and selected three independent lines with a single copy of *HlALMT1* for further analysis (Figure 9a). The expression level of *HlALMT1* was higher in the lines in which expression was driven by the HL–A promoter than in those driven by the HL–N promoter (Figure 9b). Rice secretes citrate but not malate in response to Al (Ma *et al.*, 2002). There was no difference in citrate secretion between the transgenic lines and the vector control when exposed to Al (Figure 9c). However, the amount of malate secreted from the roots was higher in the transgenic lines expressing *HlALMT1* driven by the promoter from either accession

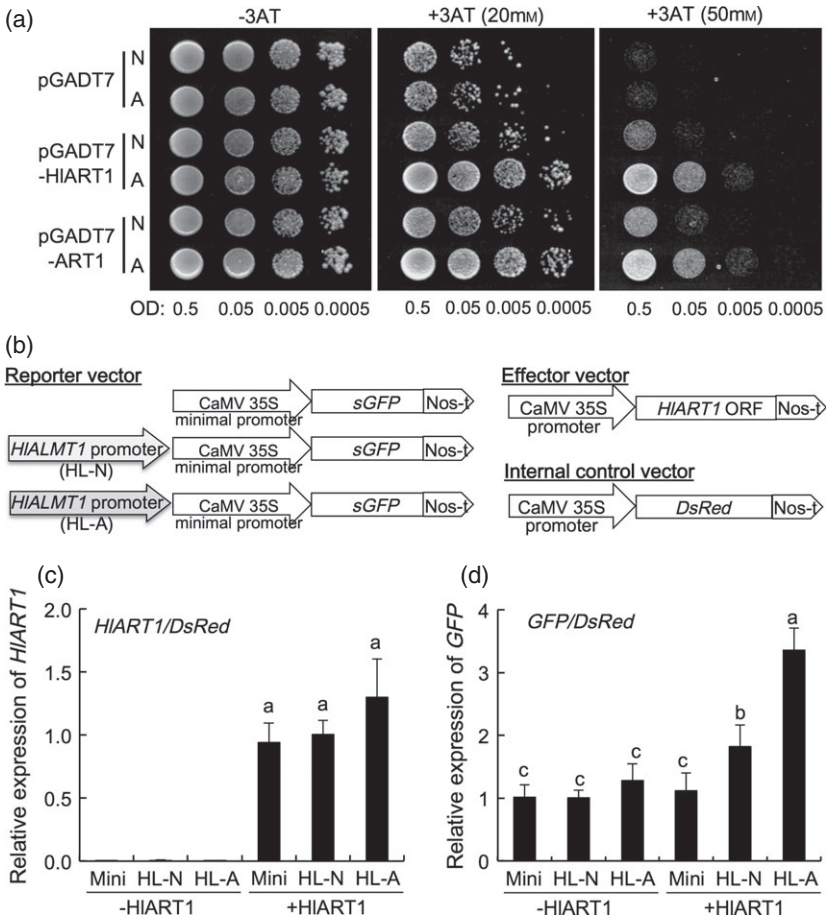

Figure 8. Yeast one-hybrid assay and promoter activity assay.

(a) Yeast one-hybrid assay. The plasmids pGADT7, pGADT7-HlART1 or pGADT7-ART1 together with plasmids N (pHIS2.1 + *HlALMT1* promoter from HL–N) or A (pHIS2.1 + *HlALMT1* promoter from HL–A) were introduced into yeast strain Y187 and cultured on synthetic complete (SC) medium without His in the presence of 0, 20 mM or 50 mM 3–amino-1,2,4–triazole (3AT, a competitor of HIS3). Four serial 1:10 dilutions (from left to right) of yeast cell suspensions starting from OD_{600} = 0.5 were spotted on plates. The yeast was allowed to grow at 30°C for 3 days.

(b–d) Promoter activity assay in tobacco protoplasts. (b) Schematic diagram of the reporter, effector and internal control plasmids used in transient expression analysis. (c,d) Expression levels of the effector gene (*HlART1*) (c) and the reporter gene (GFP) (d) driven by the CaMV 35S minimal promoter (Mini) or the *HlALMT1* promoters from HL–N and HL–A. The reporter vector, effector vector and internal control vector were co-transformed into tobacco protoplasts by the poly(ethylene glycol) method. The expression level was determined by quantitative RT–PCR. The expression level relative to that of Mini in the presence of HlART1 is shown. Values are means ± SD (n = 3). Means with different letters are significantly different (P < 0.05 by Tukey's test).

compared with the vector control (Figure 9d), indicating that *HlALMT1* functions as a transporter of malate. Moreover, the amount of malate secreted was significantly higher in the transgenic lines expressing *HlALMT1* driven by the HL–A promoter than that by the HL–N promoter (Figure 9d). The Al tolerance in the transgenic lines did not significantly change compared with vector control, probably due to the basic high Al tolerance of rice (data not shown). These results further indicate that the different expression levels of *HlALMT1* in the two accessions result from the difference in the promoter region.

DISCUSSION

Differential expression of *HlALMT1* is responsible for natural variation in Al tolerance of *Holcus lanatus*

Analysis of the HL–A accession of *Holcus lanatus*, which has adapted to acidic soil, revealed a higher Al tolerance than the HL–N accession collected from a near-neutral soil from the neighboring experimental plot (Figure 1b). Physiological studies showed that this accession accumulated less Al in the roots and secreted more malate in response to Al exposure (Figures 1d,e and 2). Malate secretion from the roots has been established as a mechanism for Al tolerance in many plant species (Ma, 2000; Ma *et al.*, 2001).

The two accessions differ in the extent of malate secretion induced by Al (Figure 2), indicating that this difference is at least one of the reasons responsible for the natural variation in Al tolerance between the two accessions, although its contribution to Al tolerance is unknown.

There are two patterns of organic acid anion release, which differ in the timing of secretion (Ma, 2000; Ma *et al.*, 2001). In pattern I, no discernible delay is observed between addition of Al and the onset of secretion, while, in pattern II, organic acid anion secretion is delayed for several hours after exposure to Al. Our results show that malate secretion in *H. lanatus* follows pattern II (Figure 2a): requiring 6 h to release malate from the roots in both accessions. This pattern is different from that in wheat (Delhaize *et al.*, 1993), but similar to that observed in Arabidopsis, maize, rye and triticale (X *Triticosecale Wittmack*) (Pellet *et al.*, 1995; Li *et al.*, 2000; Ma *et al.*, 2000; Kobayashi *et al.*, 2007), suggesting that different mechanisms for organic acid anion secretion are involved in different plant species.

The Al-induced secretion of malate is mediated by ALMT1 in wheat, Arabidopsis, oilseed rape and soybean

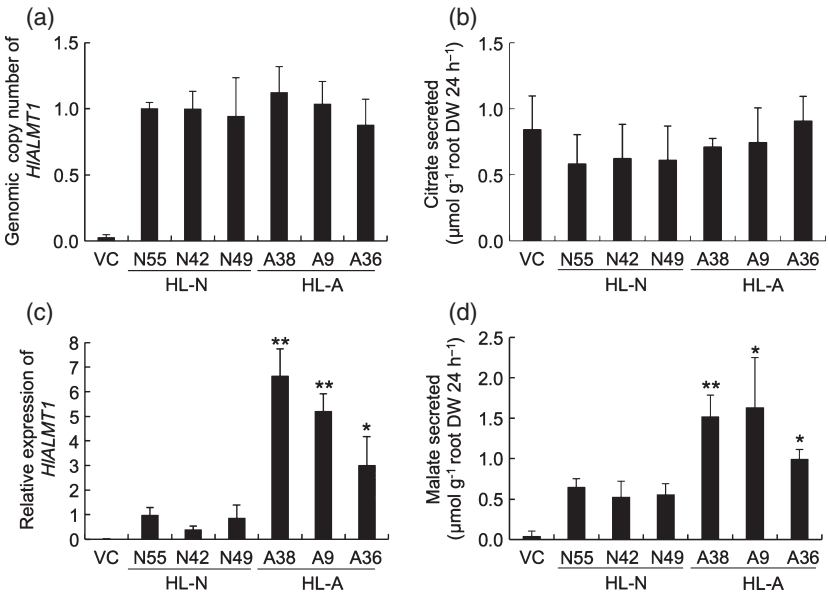

Figure 9. Functional analysis of the *HlALMT1* promoter in transgenic rice.
(a) Genomic copy number of *HlALMT1* in transgenic rice. The values were normalized using the C_T value of silicon transporter gene *Lsi1*.
(b) Expression level of *HlALMT1* in transgenic rice. Seedlings were exposed to a solution containing 50 μM Al for 24 h. The expression level relative to line N49 is shown.
(c,d) Al-induced secretion of citrate (c) and malate (d) from transgenic rice. Root exudates were collected after exposing transgenic seedlings to a solution containing 50 μM Al for 24 h. VC, vector control. Values are means ± SD ($n = 3$). The asterisks indicate a significant difference compared with the value of line N49 (*$P < 0.05$ and **$P < 0.01$ by Tukey's test).

(Sasaki *et al.*, 2004; Hoekenga *et al.*, 2006; Ligaba *et al.*, 2006; Liang *et al.*, 2013). In the present study, we isolated a homolog of ALMT1 in *H. lanatus*. HlALMT1 showed the highest similarity to TaALMT1 (Figure S2b). Like other ALMT1s, HlALMT1 is localized to the plasma membrane and mediates malate efflux when expressed in *Xenopus* oocytes (Figures 3 and 4). The expression of *HlALMT1* in *H. lanatus* was specifically induced by Al in the roots (Figure 5a) and limited to the outer cells of the roots (Figure 5f). Furthermore, expression of *HlALMT1* in rice resulted in malate efflux from the roots (Figure 9d). All these results indicate that the *HLALMT1* gene isolated in the present study is responsible for Al-induced secretion of malate in *H. lanatus*.

In addition to the induction of the *HlALMT1* gene (Figure 5), the protein encoded by *HlALMT1* was activated by Al in *Xenopus* oocytes (Figure 3). This feature is the same as for other ALMT1 from various species such as wheat (Sasaki *et al.*, 2004) and Arabidopsis (Hoekenga *et al.*, 2006). The mechanisms underlying Al-induced activation of ALMT1 is unknown, but the extracellular C–terminus is required for both the basal and Al^{3+}-dependent transport activity of TaALMT1 in wheat and AtALMT1 in Arabidopsis (Furuichi *et al.*, 2010). Further studies using site-directed mutagenesis showed that three acidic amino acids (E274, D275 and E284) in the C–terminal region are essential for Al^{3+}-activated transport activity of TaALMT1. These three amino acid residues are also present in HlALMT1 (Figure S2a), suggesting that they are conserved in different plant species.

There was no difference in the amino acid sequence of *HlALMT1* between the two accessions differing in Al tolerance and malate secretion (Figure S2a); however, HL–A showed higher expression of *HlALMT1* than HL–N in a time-dependent and dose-dependent manner after Al treatment (Figure 5b,c). Furthermore, the higher expression of *HlALMT1* in HL–A was only found in the outer root tissues (Figure 5f). This difference in the expression level is not due to genomic copy number of *HlALMT1*, but to the transcript number (Figure 6). These findings indicate that the differential expression levels of *HlALMT1* are consistent with the differential malate secretion, which may contribute to the different Al tolerance in the two accessions.

The expression level of *HlALMT1* is determined by the number of cis-acting elements for ART1

The expression level of a gene may be regulated by many factors. Recently, several studies have examined the transcriptional regulation mechanisms of genes involved in Al-induced secretion of organic acid anions. In sorghum, tourist-like miniature inverted repeat transposable elements in the promoter region of *SbMATE* was suggested to be involved in regulating expression of this gene (Magalhaes *et al.*, 2007). In wheat, tandemly repeated elements of 33–803 bp located upstream of the *TaALMT1* coding region are associated with high expression of *TaALMT1* (Sasaki *et al.*, 2006; Ryan *et al.*, 2010). It is speculated that the repeats contain regulatory elements that enhance *TaALMT1* expression when present in multiple copies (Ryan *et al.*, 2010). *Aegilops tauschii*, a diploid species, is the donor of the D genome in wheat, which is the location of *TaALMT1* . Interestingly, the tandemly repeated elements are not present in the promoter of *AetALMT1* from *A. tauschii*, suggesting that these mutations occurred recently. In addition, a 1 kb insertion in the 5′ UTR region of *HvAACT1* enhances its expression in Al-tolerant accessions of barley (Fujii *et al.*, 2012). More recently, the higher expression level of *TaMATE1B* in several Brazilian wheat lines was

found to be associated with the presence of a Sukkula-like transposable element in its promoter (Tovkach *et al.*, 2013). In the present study, we found that an Al-tolerant accession of *H. lanatus* has evolved a different mechanism to enhance expression of *HlALMT1*. It is not achieved by increasing tandemly repeated elements as in wheat, but simply by increasing the number of *cis*-acting elements for ART1 in the promoter region examined (up to 2 kb) (Figure S3). It appears that amplification of transcription factor binding sites may be the root cause for functional differences in both wheat and *Holcus*, but the scale of these changes is far more specific in *Holcus* than the variation of longer sequences that occurs in wheat. The response of the *ALMT1* gene to Al has been lost in wheat (i.e. *TaALMT1* is not induced by Al), and constitutive high expression in Al-tolerant cultivars may be achieved by re-organization of the promoter. By contrast, expression of ALMT1 in *Holcus* is induced by Al in both accessions, and this up-regulation requires ART1. Therefore, the relatively small change in the promoter in terms of the number of *cis*-acting elements may result in enhanced expression of ALMT1, which contributes to Al tolerance.

ART1 is a transcription factor for Al tolerance that was identified initially in rice (Yamaji *et al.*, 2009). It regulates the expression of at least 30 genes. As *H. lanatus* belongs to the Gramineae, like rice does, we isolated a homolog of ART1 in *H. lanatus* from both accessions (Figure S4). Both HlART1 and rice ART1 interact with the promoter region of *HlALMT1*, indicating that HlART1 functions as a transcription factor like ART1 (Figure 8a). HlART1 did not differ in amino acid sequence or expression level between the two accessions (Figure 7 and Figure S4a). Furthermore, its expression level was not affected by Al, as has been found for ART1 in rice (Figure 7) (Yamaji *et al.*, 2009). This means that the differential expression of *HlALMT1* in the two accessions is not caused by variations in *ART1* expression.

Rice ART1 binds to the core *cis*-acting element [GGN (T/g/a/C)V(C/A/g)S(C/G)] that is present in the promoter ART1 downstream genes in rice (Tsutsui *et al.*, 2011). This *cis*-acting element was also found in the promoter region of *HlALMT1* in the two accessions, but with different numbers: five in HL–A compared with three in HL–N (Figure S3). Our assays in yeast, tobacco protoplasts and transgenic rice indicate that this difference in number of the *cis*-acting elements may be responsible for the different expression levels of *HlALMT1* in the two accessions (Figures 8d and 9b).

The difference in the number of *cis*-acting elements between the two accessions is caused by single nucleotide substitutions (Figure S3). These substitutions may have occurred over 150 years as a result of adaptation to the gradual acidification in the plots receiving ammonium sulfate. Similar variation also occurred in *Anthoxanthum odoratum* (Davies and Snaydon, 1973). Populations of *A. odoratum* collected from unlimed plots in the Park Grass Experiment were more tolerant of high Al concentrations than those from limed plots. It would be interesting to examine whether similar mechanisms are involved in these variations. Changes in *cis*-regulatory elements have also been implicated in evolution of heavy metal hyper-accumulator *Arabidopsis halleri* (Hanikenne *et al.*, 2008).

In conclusion, our results show that an accession of *H. lanatus* adapted to acid soil has evolved a mechanism of Al tolerance by enhancing malate secretion mediated by a plasma membrane-localized transporter, HlALMT1. The higher *HlALMT1* expression is probably achieved by an increase in the number of *cis*-acting elements for transcription factor HlART1 in the promoter region.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Two accessions of *H. lanatus* were obtained from the Park Grass Experiment established in 1856 at Rothamsted Research (Harpenden, UK): one from an acidic soil plot (plot 9/2d, soil pH 3.6; designated HL–A) and the other from a limed plot with the same fertilizer treatment (plot 9/2a, soil pH 7.1, designated HL–N). Seeds of *H. lanatus* were soaked in deionized water for 2 h, and then germinated in a plastic Petri dish lined with filter paper saturated with distilled water in darkness at 25°C for 2 days. Seedlings were then transferred to a 3.5 liter plastic pot containing continuously aerated one-fifth strength Hoagland solution at 25°C. For the experiments described below, tillers from one plant of HL–N and HL–A each were used. The nutrient solution was changed every 2 days.

For rice (*Oryza sativa*; cv Nipponbare), germinated seeds were placed on a net floating on a 0.5 mM $CaCl_2$ solution at 25°C. After three days, the seedlings were used for Al tolerance evaluation as described below. Preparation of transgenic rice seedlings for collection of root exudates followed the method by Yokosho *et al.* (2010).

Evaluation of pH response and Al tolerance

To investigate the effect of pH on root elongation, seedlings of both HL–N and HL–A were exposed to a 0.5 mM $CaCl_2$ solution buffered with 10 mM Homo-PIPES at pH values ranging from 3.5 to 6.5 for 24 h. To compare Al sensitivity, seedlings were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 50 μM $AlCl_3$ for 0, 6, 12 and 24 h. Root length was measured with a ruler before and after the treatment, and the root elongation was calculated. To compare Al tolerance between rice and the two accessions of *H. lanatus* , the seedlings were exposed to 50 μM $AlCl_3$ for 24 h, and relative root elongation (i.e. root elongation with Al/root elongation without Al) was calculated. For Al staining, the roots were exposed to 100 μM $AlCl_3$ for 24 h, and then stained with 0.1% Eriochrome cyanine R for 15 min. The stained roots were observed under a microscope and photographed.

To determine the Al accumulation, seedlings of both HL–N and HL–A were exposed to a 0.5 mM $CaCl_2$ solution containing 25, 50 and 100 μM Al (pH 4.5). After 24 h, the roots were washed three times on ice for 5 min with 0.5 mM $CaCl_2$, and then root segments (0–1 cm) were excised. Fractionation of the cell wall and cell sap

was performed as described by Chen *et al.* (2012). The amount of Al in the fractions was determined using an atomic absorption spectrophotometer (Z–8270; Hitachi, http://www.hitachi-hitec.com/).

Collection of root exudates and organic acid determination

Seedlings of both HL–N and HL–A (1-month old) were used for root exudate collection. Before collection, the seedlings were placed in a 0.5 mM $CaCl_2$ (pH 4.5) solution overnight, and then transferred to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing various concentrations of Al (25, 50 and 100 μM) for various times (0, 3, 6, 9, 12 and 24 h). Seedlings were also exposed to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing 50 μM Cd or La. Root exudate collection and organic acid determination were performed as described by Yokosho *et al.* (2010).

Gene cloning and sequencing

Total RNA was extracted from *H. lanatus* roots using an RNeasy plant mini kit (Qiagen, http://www.qiagen.com/). One microgram of total RNA was used for first-strand cDNA synthesis using a SuperScript II kit (Invitrogen, http://www.invitrogen.com) according to the manufacturer's instructions. To clone *HlALMT1*, we first amplified a *HlALMT1* cDNA fragment of 635 bp using primers 5′-ACCGTCGTCGTCGTCATGGAGTA-3′ and 5′-TTCTGGTATTGGCTCCATGGGTG-3′ designed based on the conserved sequences of *AtALMT1* (Arabidopsis), *TaALMT1* (wheat) and *BnALMT1/2* (rape, *Brassica napus*). Then we performed 5′ and 3′ RACE (Smart RACE cDNA amplification kit; Clontech, http://www.clontech.com/) to amplify the full-length cDNA sequence of *HlALMT1*. The ORF of *HlALMT1* cDNA was amplified by RT–PCR using primers 5′-ATGGATGTTGAGCACAACAGA-3′ and 5′-ACCACTCTGCTCTGCACCAT-3′. To clone *HlART1*, we amplified an *HlART1* cDNA fragment of 360 bp using primers 5′-ACGCGAACCTGCGGATGCACATG-3′ and 5′- CGTGGGCGAAGAGCTTGTCCTT-3′ designed based on the conserved sequences of *ART1* and *ART1*-like genes in rice and Arabidopsis. The full-length cDNA of *HlART1* was generated by the RACE method as described above. The ORF of *HlART1* cDNA was amplified by RT–PCR using primers 5′-CAAATACGCGACTCTATAGAAGTT-3′ and 5′-GTACATCTGGAACTTTCCTGGTGAAAA-3′.

To clone the promoters of *HlALMT1*, self-formed adaptor PCR (SEFA-PCR; Wang *et al.*, 2007) was performed to amplify the 2 kb upstream sequence of *HlALMT1* in the *H. lanatus* genome using primers SP1 (5′-AACCGCAAAAAGGTAGCCGCCGAT-3′), SP2 (5′-GCGAGCGATATGGCTTCTCCTA-3′) and SP3 (5′-TTCTGCCAACTTATGGGNNNNNNNNNCGATGC-3′). The amplified fragments were cloned into the pGEM–T Easy vector (Promega, http://www.promega.com). The sequence was confirmed using an ABI PRISM 310 genetic analyzer and BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, http://www.appliedbiosystems.com).

Phylogenetic analysis

Peptide sequence alignment was analyzed by ClustalW using default settings (http://clustalw.ddbj.nig.ac.jp/). The phylogenetic tree was constructed from the amino acid sequences using the Tree View program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Expression pattern of *HlALMT1* and *HlART1*

For expression analysis, seedlings of *H. lanatus* were exposed to a solution containing 0, 25, 50 or 100 μM Al for various times (0, 3, 6, 9, 12 and 24 h), and the roots (including 0–1, 1–2 and 2–3 cm segments) and shoots were sampled for RNA extraction and expression level determination. Samples were immediately frozen in liquid nitrogen. RNA extraction and cDNA preparation were performed as described above. The gene expression level was determined by real-time RT–PCR using Thunderbird SYBR qPCR mix (TOYOBO, http://www.toyobo.co.jp/) on Mastercycler ep realplex (Eppendorf, http://www.eppendorf.com/). The relative expression was normalized to the expression level of the *actin* gene (internal control). The primers used for *HlALMT1* were 5′-AGAGAGCAGCGACGAGATGGTCGG-3′ and 5′-TTACTCAGCGTTGCTCCGACGG-3′. The primers used for *HlART1* were 5′-ACGCGAACCTGCGGATGCACATG-3′ and 5′-GTGGCTCTTCTCACAGTGGCT-3′. The primers used for actin were 5′-TTGGATTCTGGTGATGGTGT-3′ and 5′-GGAAGCTCGTAGCTCTTCTC-3′. The expression level of actin was unaffected by Al treatment and did not differ between the two accessions.

To investigate the tissue specificity of *HlALMT1* expression, seedlings of *H. lanatus* were exposed to a solution containing 50 μM Al for 6 h, and roots of both HL–N and HL–A were dissected at 7.5–12.5 mm from root apex as described by Takahashi *et al.* (2010). The samples were immediately immersed in a fixing solution containing ethanol/acetic acid at a 3:2 ratio. Inner and outer tissues were collected from the root tissue sections using a Veritas LCC1704 laser microdissection system (Molecular Devices, http://www.moleculardevices.com/), and used for total RNA extraction as described above. The relative expression was normalized to the expression level of the *actin* gene.

Copy number determination

To investigate *HlALMT1* copy number in the genomic DNA, we used the same primers and PCR conditions as described above, except that 100 ng genomic DNA was used as the template, instead of cDNA, for each reaction. The genomic copy number of *HlALMT1* in the two accessions was normalized on the basis of the cycle threshold (C_T) value for *Lsi1* (*low silicon rice 1*; *Os02 g0745100*). The *Lsi1* fragment was amplified using primers 5′-CGGTGGATGTGATCGGAACCA-3′ and 5′-CGTCGAACTTGTTGCTCGCCA-3′.

To estimate transcript copy number, we generated standard curves for absolute quantification of *HlALMT1*. A series of dilutions (from 1×10^{-1} to 1×10^{-6} ng) of plasmids was prepared, and subjected to real-time PCR. Amplification efficiency was calculated to be 99.1% (*HlALMT1*). The C_T values for each sample were converted into absolute copy numbers using the standard curves.

Subcellular localization

The subcellular localization was investigated by introducing 35S:GFP-HlALMT1 or 35S:HlALMT1-GFP into onion epidermal cells. The primers used for amplification and introduction of restriction sites were 5′-CTCGAGGGATCCCCCGGGATGGATGTTGAGCACAAC-3′ (sense) for both constructs, and 5′-CTCGAGCCCGGGCTCTGCACACTGAATAAC-3′ (antisense) for 35S:HlALMT1-GFP and 5′-CCCGGGATCCGCGGCCGCTTACTCTGCACACTGAAT-3′ (antisense) for 35S:GFP-HlALMT1. Gold particles (diameter 1 μm) coated with 35S:GFP-HlALMT1, 35S:HlALMT1-GFP or 35S:GFP and 35S:DsRed were introduced into onion epidermal cells by particle bombardment (PDS–1000/He particle delivery system; Bio–Rad, http://www.bio-rad.com/) using 1100 psi rupture disks. After incubation at 25°C for 18 h, the GFP signal was observed using an LSM700 laser scanning microscope (Zeiss, http://www.zeiss.com/).

Malate transport activity assay in *Xenopus oocytes*

The ORF of the *HlALMT1* cDNA fragment was amplified using the primers 5′-CTCGAGGGATCCCCCGGGATGGATGTTGAGCACAAC-3 and 5′-CCCGGGATCCGCGGCCGCTTACTCTGCACACTGAAT-3′. The fragment that contained the ORF was inserted into a *Xenopus laevis* oocyte expression vector, pXbG–ev1 (Preston *et al.*, 1992). cRNA preparation, injection and radioactivity measurement were performed as described previously (Yokosho *et al.*, 2011).

For electrophysiological studies, *HlALMT1* cRNA- or water-injected oocytes were incubated in modified Barth's saline at 18°C. After a 1 day incubation, 50 nl of 25 mM sodium citrate was injected into the oocytes, which were then incubated for 0.5–2 h in ND96 buffer containing 0 or 100 μM Al at pH 4.5 (Furuichi *et al.*, 2010). The net current across the oocyte membrane was measured using the two-electrode voltage clamp system with amplifier (MEZ-7200 and CEZ-1200, Nihon Kohden, http://www.nihonkohden.co.jp/) at various membrane voltages. The electrical potential difference across the membrane was clamped from 0 to -120 mV. Five to nine replicates were used for each measurement.

Yeast one-hybrid assay

The yeast one-hybrid assay was performed using a MATCHMAKER one-hybrid library construction and screening kit (Clontech). The ORF of *HlART1* was amplified by PCR and cloned in-frame after the transcriptional activation domain of the yeast GAL4 transcription factor (without DNA binding domain) in pGADT7 (http://biochem.web.utah.edu/hill/links/pGADT7-map.pdf) (pGADT7-HlART1). The primers used for amplification and introduction of restriction sites were 5′-ACTGTCGACATGGACC GCGGCAAGAAT-3′ and 5′- TAATGCGGCCGCCGGTACATCT GAAATTAT-3′. pGADT7-ART1 was constructed previously by Yamaji *et al.* (2009). The promoter regions of *HlALMT1* (-1986 to -139 bp from the start codon in HL–N and −1981 to −128 bp from the start codon in HL–A) were amplified from the genomic DNA of HL–A and HL–N, and cloned upstream of the HIS3 reporter gene in the pHIS2.1 vector (http://download.bioon.com.cn/upload/month_0809/20080922_63fb16fe8e498a5934734R4C51uR0n7W.attach.pdf). The primers used for amplification and introduction of restriction sites were 5′-TAGAATTCGCGCAGTGGCAACCTGG-3′ and 5′-TATACGCGTTAGGCTGGCAGACAAACA-3′. The plasmids pGADT7, pGADT7-HlART1 or pGADT7-ART1, together with plasmids N (pHIS2.1+ *HlALMT1* promoter from HL–N) or A (pHIS2.1 + *HlALMT1* promoter from HL–A), were introduced into yeast strain Y187 and cultured on synthetic complete medium without His containing 0, 20 or 50 mM 3–amino-1,2,4–triazole (3AT, a competitor of HIS3) at 30°C according to the manufacturer's instructions (Clontech). The yeast growth was photographed after 3 days.

Transient assay in tobacco protoplasts

Tobacco plants were cultivated hydroponically as described previously (Tsutsui *et al.*, 2011). Young and fresh leaves were cut into small pieces with a razor and protoplasts were isolated as described by Tsutsui *et al.* (2011). For transient assays in tobacco protoplasts, GFP was used as a reporter gene. The promoter regions of *HlALMT1* (-1986 to -1 bp from the start codon in HL–N and −1981 to −1 bp from the start codon in HL–A) were amplified from the genomic DNA, and cloned upstream of a region comprising the CaMV 35S minimal promoter (−46) + GFP + the NOS terminator in pBluescript vector (Stratagene, La Jolla, CA, USA). The primers used for amplification and introduction of restriction sites were 5′-CGTCTAGAGCGCAGTGGCAACCTGG-3′ (forward), 5′-CGTCTAGAAGCAGAAGTGCAGAACCA-3′ (reverse for HL–N) and 5′-CGTCTAGAGGCAGAACCAATGGTGGC-3′ (reverse for HL–A). To construct the effector vector, the ORF of *HlART1* was amplified by PCR and cloned between the CaMV 35S promoter and the NOS terminator in pBluescript vector using primers 5′-ACTGTCGAC ATGGACCGCGGCAAGAAT-3′ and 5′-TAATGCGGCCGCCGGTAC ATCTGAAATTAT-3′. *DsRed* was used as an internal standard. The internal control vector used was as constructed by Tsutsui *et al.* (2011). The reporter vector, effector vector and internal control vector were co-transformed into tobacco protoplasts by the poly (ethylene glycol) method as described by Tsutsui *et al.* (2011). The transformed protoplasts were incubated in Murashige and Skoog medium (0.22% w/v Murashige and Skoog salts, 400 mM mannitol, 10 mM MES/KOH, pH 5.4) in the dark for 17 h at 22°C, followed by exposure to 200 μM $AlCl_3$. After 6 h, the protoplasts were collected by centrifugation at 100 ***g*** for 5 min, and samples of the pellet were frozen in liquid nitrogen for RNA extraction. Quantitative real-time RT–PCR was performed using specific primers 5′-AG GAGCGCACCATCTTCTTCAA-3′ and 5′-GCTGTTGTAGTTGTACTC CAGC-3′ for GFP, and 5′-GGACAACACCGAGGACGTCATC-3′ and 5′-CGCCCTTGGTCACCTGCAGCTT-3′ for *DsRed*.

Generation of transgenic rice

The *HlALMT1* promoter regions from HL–N (−1986 bp) and HL–A (−1981 bp) were amplified from genomic DNA, and fused with the *HlALMT1* ORF by overlap PCR. These fragments containing *HlALMT1* promoters and the *HlALMT1* ORF were ligated into pPZP vector (Fuse *et al.*, 2001) and then transformed into *Agrobacterium tumefaciens* strain EHA101. The primers used for amplification and introduction of restriction sites were 5′-ATGGGCCCGCGCAGTGGCAACCTGGA-3′ and 5′-GCTCTAGACGCTCTCTAGACAGCTGG-3′. To transform these plasmids into rice, callus was generated from mature embryos of rice cultivar Nipponbare for *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994).

The genomic copy number of *HlALMT1*, expression of *HlALMT1* in the roots of transgenic lines, and levels of citrate and malate in the root exudates were determined using enzymatic method according to Yokosho *et al.* (2010).

Accession numbers

Sequence data for the sequences referred to in this paper may be found in the GenBank/EMBL databases under accession numbers AB792703 for *HlALMT1* (HL–N), AB792704 for *HlAlMT1* (HL–A), AB792707 for *HlART1*, AB792705 for HL–N promoter and AB792706 for HL–N promoter.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Comparison of Al tolerance between rice and *H. lanatus*.

Figure S2. Sequence of *HlALMT1* in the two accessions of *H. lanatus*.

Figure S3. Alignment of the promoter region from HL–A and HL–N accessions of *H. lanatus*.

Figure S4. Phylogenetic analysis of HlART1.

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