

The barley *MATE* gene, *HvAACT1*, increases citrate efflux and Al³⁺ tolerance when expressed in wheat and barley

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• **Background and Aims** Aluminium is toxic in acid soils because the soluble Al³⁺ inhibits root growth. A mechanism of Al³⁺ tolerance discovered in many plant species involves the release of organic anions from root apices. The Al³⁺-activated release of citrate from the root apices of Al³⁺-tolerant genotypes of barley is controlled by a *MATE* gene named *HvAACT1* that encodes a citrate transport protein located on the plasma membrane. The aim of this study was to investigate whether expressing *HvAACT1* with a constitutive promoter in barley and wheat can increase citrate efflux and Al³⁺ tolerance of these important cereal species.

• **Methods** *HvAACT1* was over-expressed in wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) using the maize ubiquitin promoter. Root apices of transgenic and control lines were analysed for *HvAACT1* expression and organic acid efflux. The Al³⁺ tolerance of transgenic and control lines was assessed in both hydroponic solution and acid soil.

• **Key Results and Conclusions** Increased *HvAACT1* expression in both cereal species was associated with increased citrate efflux from root apices and enhanced Al³⁺ tolerance, thus demonstrating that biotechnology can complement traditional breeding practices to increase the Al³⁺ tolerance of important crop plants.

Key words: Acid soil, aluminium, resistance, tolerance, wheat, transgenic, barley, *Hordeum vulgare*, *Triticum aestivum*.

INTRODUCTION

Approximately 30 % of total arable lands are acidic (von Uexkull and Mutert, 1995; Ma and Ryan, 2010). The two main geographical regions of acid soils are the humid northern temperate zone and the humid tropics (von Uexkull and Mutert, 1995). Acid soils limit crop yields due to nutrient deficiencies and mineral toxicities. Non-adapted plants grown on acid soils typically have smaller root systems because high concentrations of soluble aluminium (Al³⁺) inhibit root elongation (Munns, 1965). Al³⁺ can begin to inhibit root growth of wheat (*Triticum aestivum*) within minutes or hours in simple hydroponic solutions (Ryan *et al.*, 1992). Longer exposures result in thickened roots, damaged root caps and lesions in the epidermal and cortical tissues near the apices (Foy, 1984). Al³⁺ absorbed by plants can rapidly trigger other stress responses such as oxidative stress and callose production (Sivaguru *et al.*, 2000; Yamamoto *et al.*, 2001; Horst *et al.*, 2010) as well as interfering with cytosolic Ca²⁺ homeostasis, cell wall metabolism and nutrient uptake (Pineros and Tester, 1993; Gassmann and Schroeder, 1994).

Variation in Al³⁺ tolerance occurs within many crop species including maize (*Zea mays*), wheat, barley (*Hordeum vulgare*), rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*) (Foy, 1988; Furlani *et al.*, 1987; Foy *et al.*, 1993; Koyama *et al.*, 2003; Kochian *et al.*, 2004; Magalhaes *et al.*, 2007; Ryan *et al.*, 2011). This variation provides opportunities for breeders to develop new cultivars better suited to acid soils. Barley is among the most Al³⁺-sensitive cereal crops and, although genotypic variation exists within this species, Al³⁺-tolerant genotypes are more

sensitive than Al³⁺-tolerant wheat plants. A seedling-based screen of diverse barley genotypes identified the cultivars Kearney and 'Golden Promise' as being Al³⁺ sensitive and 'Dayton' and several Japanese cultivars as being among the most tolerant (Moroni *et al.*, 2010). Hexaploid wheat has a greater variation in Al³⁺ tolerance than barley, with differences in root growth that can vary by >10-fold in short-term growth assays or in field screens (Rengel and Jurkic, 1992; Bona *et al.*, 1993; Ryan *et al.*, 1995; Pinto-Carnide and Guedes-Pinto, 1999; Garvin and Carver, 2003; Tang *et al.*, 2003; Raman *et al.*, 2008; Dai *et al.*, 2009).

Al³⁺ tolerance mechanisms in wheat and barley rely on the release of organic anions from root apices (Ma *et al.*, 2001; Ryan *et al.*, 2001; Delhaize *et al.*, 2007). Malate and citrate are the major organic anions released from wheat and barley, respectively (Ma *et al.*, 2001; Ryan *et al.*, 2001). Once these anions are released from root cells they bind the Al³⁺ and exclude it from the sensitive root apices. Efflux is largely restricted to the root apices and in most cases is activated by exposure to Al³⁺.

The Al³⁺ tolerance genes controlling organic anion efflux from roots have been isolated from several crop species (Zhou *et al.*, 2011; Delhaize *et al.*, 2012a). The pattern that has emerged to date indicates that genes controlling malate efflux belong to the *ALMT* (aluminium-activated malate transporter) family of genes and genes controlling citrate efflux belong to the *MATE* (multidrug and toxic compound extrusion) family. The first Al³⁺ tolerance gene cloned from wheat, *TaALMT1*, was identified by transcript analysis (Sasaki *et al.*, 2004), and

the first *MATE* genes involved in Al^{3+} tolerance were identified in sorghum (*SbMATE*) (Magalhaes *et al.*, 2007) and barley (*HvAACT1*) by map-based cloning (Furukawa *et al.*, 2007). Both *HvAACT1* and *TaALMT1* are constitutively expressed in roots, and Al^{3+} -tolerant cultivars show significantly greater expression in the root apices than sensitive cultivars (Sasaki *et al.*, 2004; Furukawa *et al.*, 2007). In contrast, Al^{3+} induces *SbMATE* expression in sorghum, with tolerant genotypes attaining a higher expression level than sensitive genotypes (Magalhaes *et al.*, 2007).

Raising the productivity of marginal land will be essential for increasing the food requirement of a growing population. Land management practices such as liming and the use of Al^{3+} -tolerant germplasm are complementary strategies for managing acid soils. Transgenic strategies have the potential to enable crops normally sensitive to acid soils such as barley to be grown effectively on a wide range of acid soils (Ryan *et al.*, 2011). A diverse range of genes have been expressed in plants to enhance their Al^{3+} tolerance. In view of the importance of organic anion efflux in tolerance mechanisms, most of these studies have targeted genes involved in organic anion synthesis and organic anion transport. For example, the wheat *TaALMT1* gene enhanced the Al^{3+} tolerance of barley, wheat and *Arabidopsis thaliana* (Delhaize *et al.*, 2004; Pereira *et al.*, 2010; Ryan *et al.*, 2011). Expression of *TaALMT1* in barley was found to confer Al^{3+} -activated malate efflux and a significant increase in Al^{3+} tolerance (Delhaize *et al.*, 2004). The Al^{3+} tolerance of some transgenic wheat lines in both hydroponic and soil experiments was similar to levels measured in naturally Al^{3+} -tolerant wheat lines such as ET8 (Pereira *et al.*, 2010). *MATE* transporters have also been transformed into model species such as tobacco and *Arabidopsis* to increase Al^{3+} tolerance (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007), but have not been extensively assessed in crop species such as barley (Fujii *et al.*, 2012).

The aim of this study was to investigate whether expressing *HvAACT1* with a constitutive promoter in barley and wheat can increase citrate efflux and Al^{3+} tolerance of these important cereal species.

MATERIALS AND METHODS

Genotypes

'Golden Promise', an Al^{3+} -sensitive cultivar of barley, *Hordeum vulgare*, was transformed with *HvAACT1*. The Al^{3+} -tolerant cultivar 'Dayton' was used as a control for assessing citrate efflux and Al^{3+} tolerance. The two cultivars of wheat (*Triticum aestivum*) transformed with the *HvAACT1* gene were the Al^{3+} -sensitive cultivar 'Bob White 26' ('BW26') and the Al^{3+} -tolerant cultivar 'Fielder'. The Al^{3+} -tolerant wheat cultivar 'Carazinho' was included as a positive control in several experiments because it possesses both Al^{3+} -activated malate efflux and constitutive citrate efflux from root apices (Ryan *et al.*, 2009).

Barley transformation

HvAACT1 cDNA was obtained from JianFeng Ma (Okayama University, Japan) and cloned into pWBvec8 (Wang *et al.*, 1998) which uses the maize ubiquitin promoter to drive transgene

expression. The plasmid was introduced into *Agrobacterium* (strain AGL-1) by electroporation. The *Agrobacterium* was then used to transform barley ('Golden Promise') as described by Tingay *et al.* (1997). Genomic DNA was isolated from the 119 primary transgenic (T_0) plants, and polymerase chain reaction (PCR) was used to confirm the presence of the transgene. The primers used (forward: 5'-TGCAGCATCTATTCATATGC-3'; reverse: 5'-AGAGGTAGAGCCCCGTCGT-3') did not amplify the endogenous *HvAACT1* gene of barley because the forward primer targets the maize ubiquitin intron included in the construct. Primary T_0 and segregating T_1 lines were grown for several days in hydroponics and tested for citrate efflux and Al^{3+} tolerance. A sub-set of these lines was chosen to generate T_2 seed for further analysis. The transgenic plants (either homozygous or hemizygous) and null plants within these families were identified by PCR or by an agar-based antibiotic resistance screen using leaf segments (Wang *et al.*, 2007).

Wheat transformation

Two wheat cultivars were transformed with *HvAACT1* using the biolistic method as described by Pereira *et al.* (2010). *HvAACT1* cDNA was ligated into the pWubi_tml vector where expression of the transgene is driven by the maize ubiquitin promoter (Wang *et al.*, 1998). Embryos were co-bombarded with the pCMneoSTLS2 vector which contained a geneticin (G418) resistance gene to enable selection of transgenic plants. A total of 56 T_0 wheat plants generated from 'BW26' and one line from 'Fielder' were used to generate T_1 seed (segregating generation). Citrate efflux was measured from 10–15 plants in each of the 57 T_1 wheat families (data not shown). Lines with the highest citrate efflux were used to generate T_2 seed. Putative homozygous T_2 lines and null lines were identified by PCR. A single 'Fielder' line named *Ta*(Fielder): $T1_8$ was also grown for T_2 seed, but no null lines could be identified from this family so the parental line 'Fielder' was used as a control.

Quantitative real-time PCR

Root apices (5 mm) were collected and the total RNA was extracted using an RNeasy Mini kit (Qiagen). First-strand cDNA was obtained by reverse transcription using an Invitrogen kit with an oligo(dT) primer. The forward and reverse primers targeting *HvAACT1* were 5'-AGCAGCCAAGACCTTGAGAA-3' and 5'-GCCGAAAAGATCAGGAACAC-3', respectively. The reference gene used for expression analysis in barley was actin, and the primers were 5'-GACTCTGGTGATGGTGTCAGC-3' (forward) and 5'-GGCTGGAAGAGGACCTCAGG-3' (reverse). The reference gene used for expression analysis in wheat was glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and the primers were 5'-TCAGACTCCTCCTTGATAGC-3' (forward) and 5'-GTTGAGGGTTTGATGACCAC-3' (reverse). The parental barley line 'Golden Promise' is an Al^{3+} -sensitive cultivar and therefore shows low expression of *HvAACT1*, and wild-type wheat does not express *HvAACT1*.

Assaying citrate efflux and malate efflux from root apices

Seedlings were grown for 4 d in the nutrient solution described below (without added $AlCl_3$). Root apices (5 mm) were excised

from seedlings and washed for 20 min on a platform shaker (60 rpm) in sealed glass vials with 1 mL of control solution (0.2 mM CaCl₂, pH 4.3). The control solution was then discarded and 1 mL of treatment solution (with or without AlCl₃) was added. The vials containing the root tips were shaken (60 rpm) for 2 h. The enzyme assay used to determine citrate concentration is described by Wang *et al.* (2007). Briefly, the samples were dried on a rotary vacuum drier and resuspended in 114 µL of assay solution consisting of 100 mM Tris, pH 8.0, 0.25 µL of malate dehydrogenase (Sigma-Aldrich Cat. no. M-7383), 0.25 µL of lactate dehydrogenase (Roche Cat. no. 107042) and 14 µL of NADH (Sigma-Aldrich Cat. no. N8129) which was prepared by dissolving 16 mg of NADH and 15 mg of NaHCO₃ in 2 mL of water. NADH consumption, read as the decrease in A₃₄₀, was monitored after the reaction was initiated with 1 µL of citrate lyase (Sigma-Aldrich Cat. no. C0897) prepared by dissolving 5 mg of citrate lyase in 100 µL of water. The initial citrate content in each sample was calculated from a standard curve. Malate concentration was measured with an enzyme assay as described previously (Ryan *et al.*, 1995).

Measurements of Al³⁺ tolerance in hydroponic culture

Seed were sterilized with 20 % bleach for 15 min, rinsed thoroughly and germinated in the dark for 2 d at 4 °C and then 2 d at 28 °C. Root length of the seedlings was measured and they were placed in an aerated nutrient solution containing 500 µM KNO₃, 500 µM CaCl₂, 500 µM NH₄NO₃, 150 µM MgSO₄, 10 µM KH₂PO₄, 2 µM Fe:EDTA, 11 µM H₃BO₃, 2 µM MnCl₂, 0.35 µM ZnCl₂ and 0.2 µM CuCl₂. For barley, Al³⁺ tolerance was estimated by measuring net root length after 4 d in 0, 1, 2 and 4 µM AlCl₃. For wheat, the concentrations were 0, 4 and 8 µM AlCl₃ for the 'BW26'-derived lines and 0, 4 and 30 µM AlCl₃ for the 'Fielder'-derived line. Relative root length (RRL) was estimated as: (net root growth in Al³⁺ treatment/net root growth in control solution) × 100 %.

Measurements of Al³⁺ tolerance in soil

An acidic red ferrosol soil was obtained from the Robertson region of New South Wales, Australia (34°35'S, 150°36'E). The pH of a 0.01 M CaCl₂ extract was 4.33 and the exchangeable Al³⁺ was 21 % of the total cations in solution. A proportion of the soil was treated by adding 5 g of lime kg⁻¹ dry soil, which raised the pH to 5.18 and reduced the exchangeable Al³⁺ to <1 % of the total cations in solution. The soil moisture was maintained at 90 % field capacity (333 mL kg⁻¹ dry soil). Seeds were surface-sterilized and germinated as described above. For each line, seedlings with similar root lengths were planted into pots (two seedling per pot) that contained 1.3 kg of either acid or limed soil. After 6 d, the seedlings were harvested and the fresh shoot weight, fresh root weight and length of the two longest roots were measured. The lengths of the two longest roots were combined and the RRL was calculated as the value in acid soil relative to that in limed soil. The root system from each plant was scanned and analysed with WinRHIZOTM system to determine total root length. Errors associated with RRL (SE_{RRL}) were calculated as described below except *x* and *y* represent the mean net root length in the limed soil and the mean net root length in the acid soil. Relative root and shoot

weight for each plant was calculated as the total dry weight of the roots or shoots in the acid soil compared with the limed soil.

Statistical analyses

Experiments performed in this study often compared a parameter, such as root length, in a treatment (e.g. Al³⁺ concentration or acid soil), with a control condition (e.g. no Al³⁺ or limed soil). This was done to account for the inherent differences in root growth that occur in control conditions. For instance, root growth of 'Dayton' plants was consistently shorter than that of 'Bob White 26' and the other lines when grown in the absence of Al³⁺. Since each measurement of root length has a mean and standard error associated with it, the ratio of the means to estimate the relative change has a new accumulated standard error. For instance, RRL is calculated as the ratio of root length in acid soil to that in limed soil. Therefore, RRL = *x*/*y* where *x* and *y* represent the mean net root length in the treatment and control conditions, respectively. The standard error for this ratio (SE_{RRL}) was estimated by eqn (1):

$$SE_{RRL} = RRL[(SE_x/x)^2 + (SE_y/y)^2]^{1/2} \quad (1)$$

where SE_{*x*} is the standard error of the Al³⁺ treatment and SE_{*y*} is the standard error of the control.

Determining whether two RRL values are different from one another is problematic if there is no *a priori* reason to pair the original plants in the treatment and control conditions. To compare relative data (see Figs 2–4), we adapted an approach based on overlapping confidence limits (CLs) described by Payton *et al.* (2003). In brief, Payton *et al.* (2003) showed that the 84 % CL of means sampled from a normally distributed population will overlap 95 % of the time (if the standard errors are similar sizes). As the two standard errors become less homogenous (ratio ≥ 2), the size of the CL increases to maintain the same probability of overlap. The present study used this principle to compare T₂ transgenic and null lines. Initially, the values of SE_{RRL} were calculated for the quotients [eqn (1)] and the 84 % CLs were calculated from the Z distribution by multiplying the standard error by 1.33. If the CLs did not overlap, the means were considered significantly different from one another at *P* < 0.05.

RESULTS

Characterization of transgenic barley lines

Barley ('Golden Promise') was transformed with the HvAACT1 gene with an efficiency of approx. 30 %. Four transgenic T₂ lines (Hv:T2_17A, Hv:T2_33A, Hv:T2_51 and Hv:T2_52B) and three null lines (Hv:T2_17A_null, Hv:T2_33A_null and Hv:T2_51_null) were selected for further detailed analysis. Transgenic and null lines with the same number are sister lines (e.g. Hv:T2_17A_null is a null segregant line of transgenic line Hv:T2_17A). The segregation ratio of the HvAACT1 transgene was assessed in the T₁ lines by PCR. Plants of Hv_T1_51 segregated in a 3:1 ratio (transgenic to non-transgenic) and therefore this line was likely to contain a single transgenic insertion (χ² tested at *P* < 0.05). The other lines segregated with ratios that differed significantly from 3:1, indicating that it was likely that

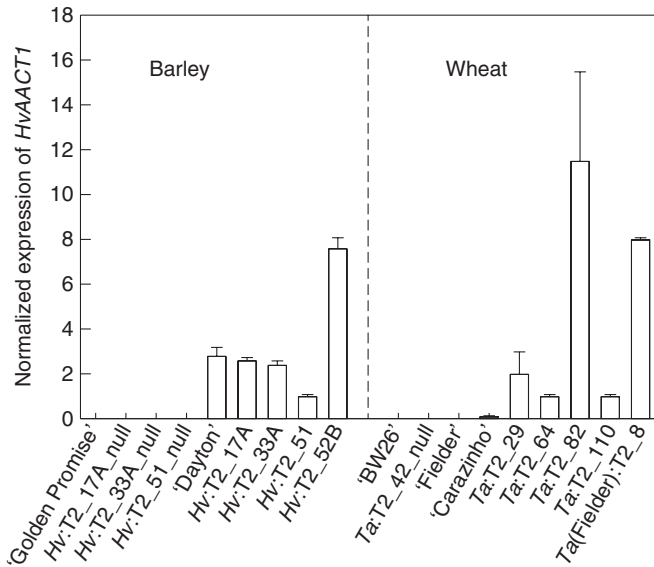


FIG. 1. *HvAACT1* expression in transgenic T_2 barley and wheat lines. Data show the mean of the expression level normalized to *actin* (barley) and *GAPDH* (wheat), and the standard error from two biological replicates each with two technical replicates. Left: ‘Golden Promise’ (GP) is the Al^{3+} -sensitive parental barley cultivar for the transgenic barley lines and ‘Dayton’ is an Al^{3+} -tolerant barley cultivar. Right: ‘Bob White 26’ (‘BW26’) is the Al^{3+} -sensitive parental wheat cultivar of most transgenic lines and ‘Fielder’ is the Al^{3+} -tolerant parent wheat cultivar for one transgenic line. ‘Carazinho’ is an Al^{3+} -tolerant wheat cultivar.

more than one insertion of the transgene had occurred at unlinked loci.

Expression of *HvAACT1* in these lines was compared with the wild-type parental line and with ‘Dayton’, an Al^{3+} -tolerant barley, which expresses the endogenous *HvAACT1* gene. Line *Hv:T2_52B* showed the highest expression among the transgenic lines, with nearly 3-fold greater expression than ‘Dayton’ (Fig. 1). Lines *Hv:T2_17A* and *Hv:T2_33A* had similar *HvAACT1* expression levels to ‘Dayton’, whereas expression in *Hv:T1_51* was about 30 % of that in ‘Dayton’. As expected, no expression was detected in the null lines or in ‘Golden Promise’.

Organic anion efflux from excised root apices was measured in the presence of $50 \mu M Al^{3+}$ because *HvAACT1* is activated by Al^{3+} (Fujii *et al.*, 2012). Citrate efflux from each of the T_2 transgenic lines was 3- to 4-fold greater than that in the null control lines and about half the efflux measured from ‘Dayton’. The Al^{3+} -activated malate efflux was low and similar for all transgenic lines, as well as ‘Golden Promise’ (Table 1).

The Al^{3+} tolerance was first evaluated by measuring root growth in hydroponic solutions containing 0, 1, 2 and $4 \mu M Al^{3+}$. In the absence of Al^{3+} , the length of the longest root was similar for all lines (55–65 mm; data not shown). The RRL in all four transgenic lines at $1 \mu M Al^{3+}$ was approx. 110 %, which was similar to that of ‘Dayton’ (Fig. 2A). In contrast, RRL in ‘Golden Promise’ and the three null lines was < 60 %. Similar differences were detected at the higher Al^{3+} concentrations, with the transgenic lines consistently showing a 2-fold greater RRL than

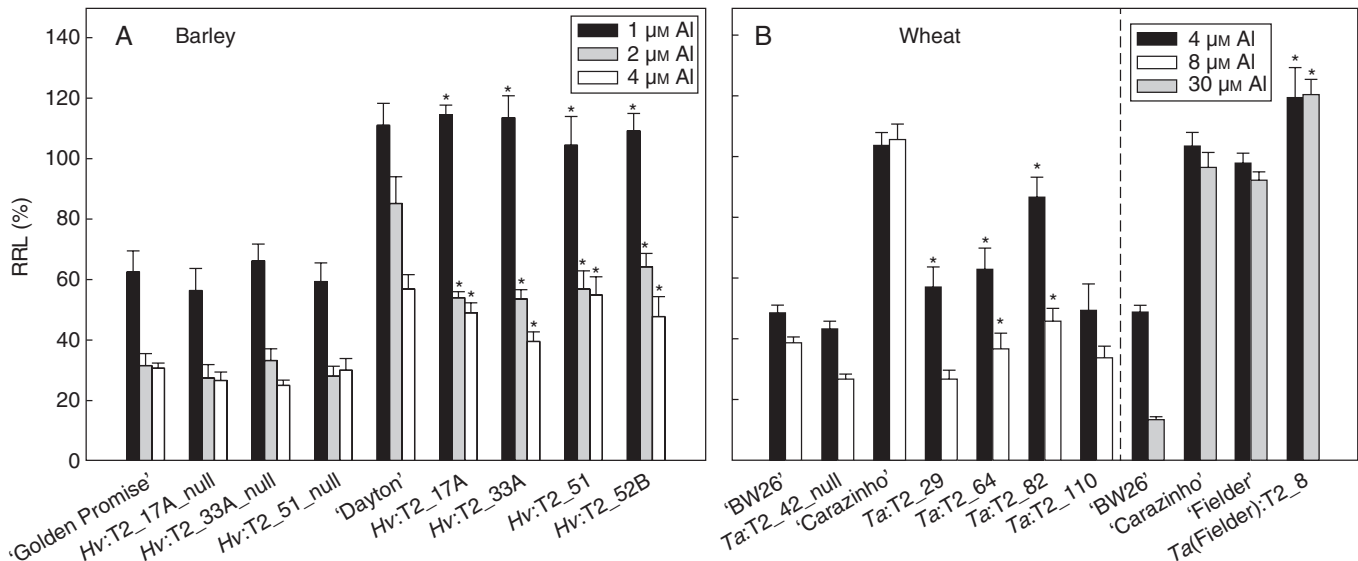


FIG. 2. Al^{3+} tolerance of transgenic T_2 barley and wheat lines in hydroponic culture. Net root growth was measured on seedlings after 4 d in nutrient solution containing different concentrations of $AlCl_3$. (A) Relative root growth of transgenic barley lines, null lines, ‘Golden Promise’ and ‘Dayton’. Data show the means and standard error calculated from eqn (1) ($n = 7$). Asterisks above transgenic lines *Hv:T2_17A*, *Hv:T2_33A* and *Hv:T2_51* indicate significant differences from their respective null lines at each Al^{3+} concentration ($P < 0.05$). Asterisks above line *Hv:T2_52B* indicate significant differences from the null lines. (B) Relative root growth of transgenic and control wheat lines including ‘Bob White 26’ (‘BW26’), the Al^{3+} -sensitive parental cultivar of most transgenic lines, ‘Fielder’, the Al^{3+} -tolerant parent wheat cultivar for one transgenic line, and ‘Carazinho’, an Al^{3+} -tolerant cultivar. The ‘BW26’-derived lines (left) were tested at 4 and $8 \mu M AlCl_3$, while the ‘Fielder’-derived line (right) was tested at 4 and $30 \mu M AlCl_3$. Data show the mean and standard error calculated from eqn (1) ($n = 7$). Asterisks above the ‘BW26’-derived transgenic lines *Ta:T2_29*, *Ta:T2_64* and *Ta:T2_82* indicate significant differences from the null line at each Al^{3+} concentration ($P_{0.05}$). Asterisks above the ‘Fielder’-derived transgenic line *Ta(Fielder):T2_8* indicate significant differences from the ‘Fielder’ parental line at each Al^{3+} concentration ($P < 0.05$).

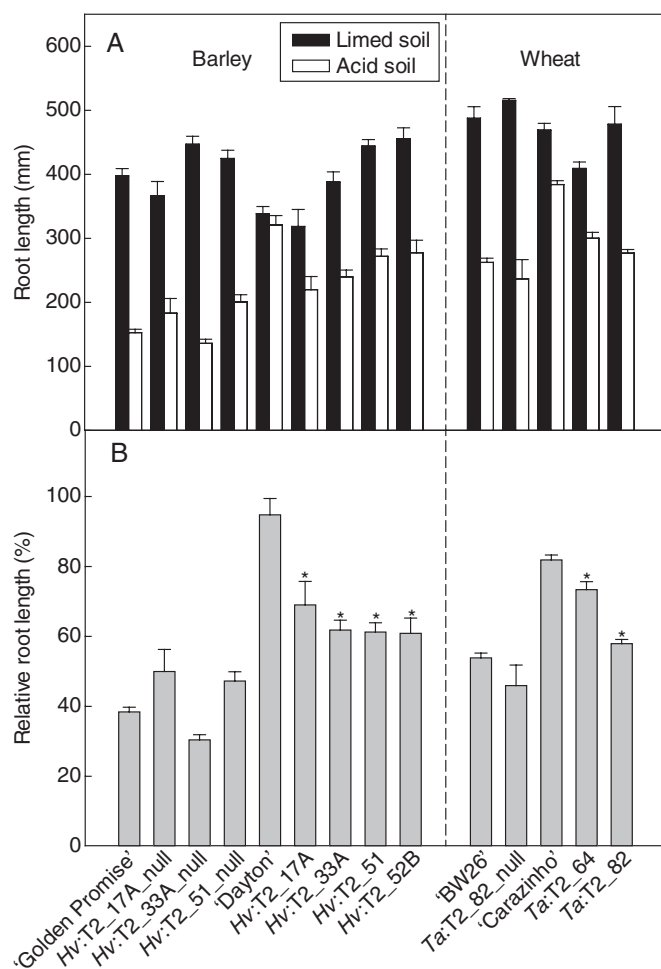


FIG. 3. Relative root length of transgenic T_2 barley and wheat lines grown in soil. (A) Combined length of the two longest roots from barley and wheat plants grown in acid and limed soils for 6 d. Data show the mean and standard error ($n = 6$). (B) Relative root length (RRL) of barley and wheat lines. RRL is calculated as the length of the two longest roots in acid soil expressed as a percentage of the two longest roots in limed soil. Transgenic lines, null lines and other control lines are indicated as for Fig. 2. Data show the mean of RRL and the accumulated standard error ($n = 6$). For the barley results, asterisks above transgenic lines *Hv:T2_17A*, *Hv:T2_33A* and *Hv:T2_51* indicate significant differences from their respective null lines at each Al^{3+} concentration ($P < 0.05$). Asterisks above line *Hv:T2_52B* indicate significant differences from all the null lines. For the wheat results, asterisks above the transgenic lines *Ta:T2_64* and *Ta:T2_82* indicate significant differences from the null line *Ta:T2_82_null* ($P < 0.05$).

the null lines. A statistical analysis based on overlapping CLs indicated that the transgenic lines *Hv:T2_17A*, *Hv:T2_33A* and *Hv:T2_51* were significantly different from their respective nulls at all Al^{3+} concentrations. Transgenic line *Hv:T2_52B* was also significantly different from these nulls.

The Al^{3+} tolerance of the transgenic and control lines was also compared by measuring root length after 6 d growth in an acid soil with and without added lime (Fig. 3A). Representative photographs of these plants at the end of the experiment are shown in Fig. 4. The RRL was calculated as the combined length of the two longest roots in the acid soil compared with that in the limed soil. In the four transgenic lines, RRL was approx. 60%, which was significantly greater ($P < 0.05$) than

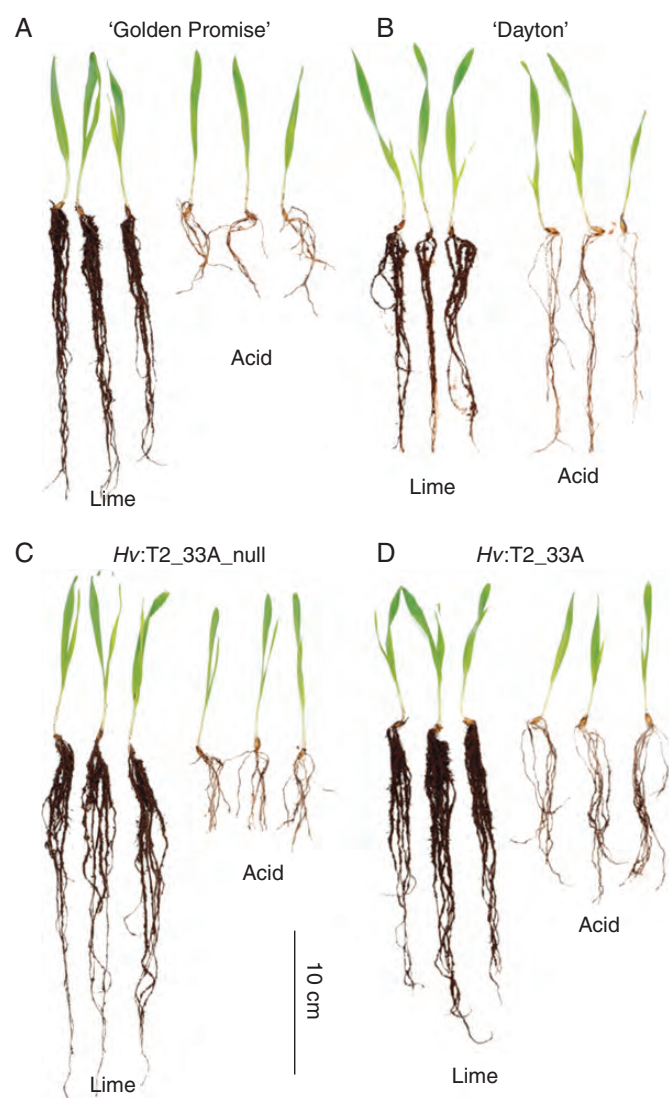


FIG. 4. Representative photographs of transgenic and control barley plants harvested from soil experiments. Plants were grown for 6 d in an acid soil and the same soil amended with lime as indicated. (A) 'Golden Promise', the Al^{3+} -sensitive parental cultivar; (B) 'Dayton', an Al^{3+} -tolerant cultivar; (C) *Hv:T2_33A_null* is a null (non-transgenic) segregant line; (D) *Hv:T2_33A* is a T_2 transgenic line over-expressing *HvAACT1*. Note that *Hv:T2_33A_null* and *Hv:T2_33A* are sister lines. The scale bar represents 10 cm.

that of their respective null sister lines and 'Golden Promise', all of which showed an RRL of between 30 and 50% (Fig. 3B). 'Dayton' was the most tolerant genotype in this experiment because RRL was 100%. However, the size of the rhizosphere (soil loosely clinging to the root) of all lines was visibly smaller in the acid soil compared with the limed soil (Fig. 4), indicating that root hair growth was inhibited in the acid soil (Delhaize *et al.*, 2012b). This indicates that over-expression of *HvAACT1* increased the Al^{3+} tolerance of the roots more than the root hairs, even though a constitutive promoter was used. This could be related to the fact that root hairs are tip-growing cells and the *HvAACT1* proteins may not be able to function well at the tip of these growing cells.

TABLE 1. Organic anion efflux from wild-type and transgenic barley and wheat lines expressing *HvAACT1* *

Lines	Citrate efflux (pmol per apex h^{-1})	Malate efflux (nmol per apex h^{-1})
Barley		
'Golden Promise'	3.8 ± 0.2	0.3 ± 0.0
'Dayton'	33.2 ± 6.4	0.3 ± 0.1
<i>Hv:T2_17A</i> _null	1.4 ± 0.6	0.2 ± 0.1
<i>Hv:T2_33A</i> _null	3.8 ± 1.1	0.2 ± 0.0
<i>Hv:T2_51</i> _null	1.7 ± 0.1	0.3 ± 0.1
<i>Hv:T2_17A</i>	14.9 ± 1.4	0.3 ± 0.0
<i>Hv:T2_33A</i>	9.7 ± 0.4	0.3 ± 0.1
<i>Hv:T2_51</i>	14.7 ± 1.6	0.3 ± 0.1
<i>Hv:52B</i>	21.3 ± 4.4	0.3 ± 0.0
Wheat		
'BW26'	6.8 ± 1.2	0.1 ± 0.0
<i>Ta:T2_82</i> _null	6.8 ± 1.4	0.2 ± 0.0
'Carazinho'	95.5 ± 4.9	1.0 ± 0.2
<i>Ta:T2_29</i>	8.6 ± 2.5	0.1 ± 0.0
<i>Ta:T2_64</i>	14.4 ± 2.8	0.2 ± 0.1
<i>Ta:T2_82</i>	26.6 ± 3.9	0.1 ± 0.1
<i>Ta:T2_110</i>	7.9 ± 0.5	0.2 ± 0.0
'Fielder'	11.7 ± 2.7	1.8 ± 0.2
<i>Ta(Fielder):T2_8</i>	53.6 ± 3.5	1.5 ± 0.6

* Citrate and malate efflux from excised root apices of transgenic T_2 lines, null lines and wild-type controls. Efflux was measured in the presence of 50 μM $AlCl_3$. Data show the mean and standard error ($n = 4$).

'Golden Promise' is the Al^{3+} -sensitive parental barley cultivar of the transgenic lines and 'Dayton' is an Al^{3+} -tolerant barley cultivar. 'Bob White 26' ('BW26') is the Al^{3+} -sensitive parental wheat cultivar of most transgenic lines and 'Fielder' is the Al^{3+} -tolerant parent wheat cultivar for one transgenic line as indicated. 'Carazinho' is an Al^{3+} -tolerant wheat used as another control.

Total root length of 'Dayton' grown in acid soil was similar to total root length of plants grown in the limed soil (Fig. 5A). All other lines had shorter roots in the acid soil but differences in relative total root length (RTRL) were detected among some of the lines (Fig. 5B). For instance, RTRL in transgenic lines *Hv:T2_17A* and *Hv:T2_33A* was greater than in their respective null sister lines ($P_{0.05}$), whereas *Hv:T2_51* did not differ from its null sister line (Fig. 5B). Line *Hv:T2_52B* also did not differ from the other null lines. Root weight of these lines was also compared in the acid and the limed soil. Relative root weight (acid/limed) in the transgenic lines was 1.5- to 2-fold greater than the null lines (Table 2). Line *Hv:T2_17A* had the greatest relative root weight of the transgenic lines and was similar to 'Dayton' (Table 2). In contrast, no consistent differences were detected in shoot weight or relative shoot weight between transgenic and null lines (Table 2).

Characterization of transgenic wheat lines

The barley *HvAACT1* gene was expressed in wheat cultivars 'Bob White 26' ('BW26', Al^{3+} sensitive) and 'Fielder' (Al^{3+} tolerant). Experiments were performed on four T_2 transgenic lines (*Ta:T2_29*, *Ta:T2_64*, *Ta:T2_82* and *Ta:T2_110*) and two null lines (*Ta:T2_42*_null and *Ta:T2_82*_null) generated from 'BW26'. Only a single transgenic T_2 line named *Ta(Fielder):T2_8* was generated in the 'Fielder' cultivar, but

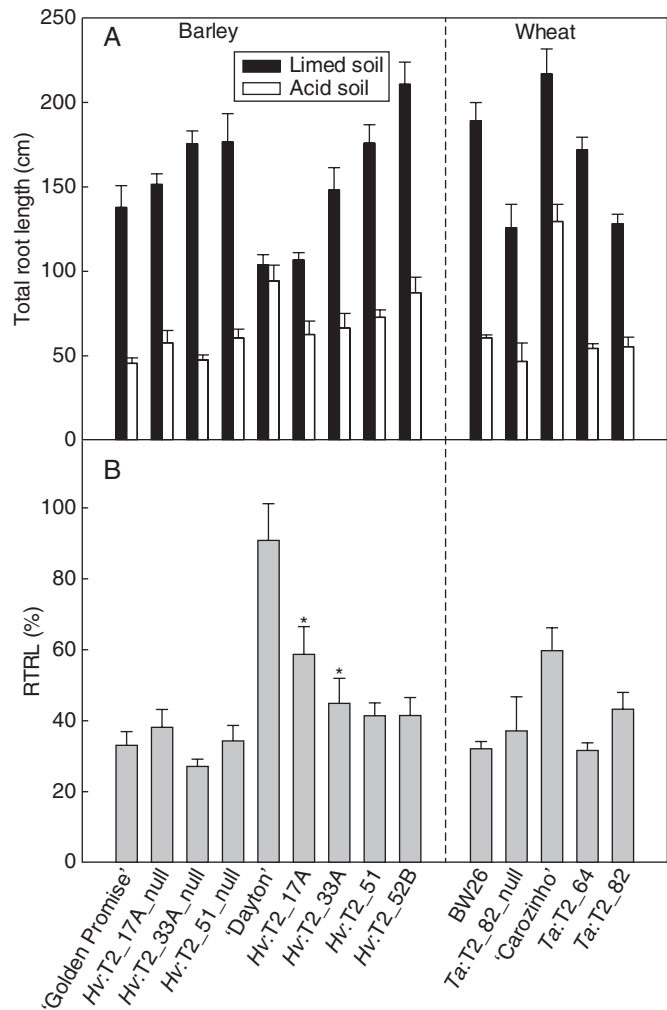


FIG. 5. Total root length of transgenic T_2 barley and wheat lines grown in soil. (A) Total root length after 6 d growth in acid and limed soils. (B) Relative total root length (RTRL) is expressed as a percentage of the total root length in acid soil relative to limed soil. The data show the mean of RTRL and the standard error ($n = 6$). Lines are described in Fig. 3. For the barley results, asterisks above transgenic lines *Hv:T2_17A* and *Hv:T2_33A* indicate significant differences from their respective null lines ($P < 0.05$).

no null sister line could be isolated from this family, indicating that it contains multiple inserts.

HvAACT1 expression was detected in the root apices of the T_2 transgenic lines, with *Ta:T2_82* and *Ta(Fielder):T2_8* showing the highest levels (Fig. 1). No expression was detected in one null line tested, or in the wild-type wheat cultivars 'BW26', 'Fielder' or 'Carazinho'.

Citrate and malate efflux was measured from excised root apices of the transgenic lines and controls in the presence of 50 μM Al^{3+} . 'Carazinho' was included since it has citrate efflux which is controlled by a different gene, the endogenous *TaMATE1B* gene in wheat (Ryan *et al.*, 2009; Tovkach *et al.*, 2013). Citrate efflux from three transgenic lines [*Ta(Fielder):T2_8*, *Ta:T2_64* and *Ta:T2_82*] was significantly greater than from the null and their respective wild-type cultivars, 'Fielder' and 'BW26' (Table 1). The two remaining T_2 lines (*Ta:T2_29* and *Ta:T2_110*) did not differ from the controls in these experiments. No malate efflux was

TABLE 2. Shoot and root fresh weights of transgenic barley and wheat lines compared with control lines grown in acid and limed soils*

Lines	Shoot weight limed soil (g)	Shoot weight acid soil (g)	Relative shoot weight (%)	Root weight limed soil (g)	Root weight acid soil (g)	Relative root weight (%)
Barley						
'Golden Promise'	0.217 ± 0.017	0.170 ± 0.005	78.4 ± 6.6	0.186 ± 0.018	0.111 ± 0.010	59.4 ± 7.9
'Dayton'	0.188 ± 0.005	0.159 ± 0.017	84.4 ± 9.4	0.148 ± 0.005	0.187 ± 0.019	126.6 ± 13.7
<i>Hv:T2_17A_null</i>	0.222 ± 0.012	0.146 ± 0.011	65.9 ± 6.0	0.176 ± 0.012	0.139 ± 0.014	78.7 ± 9.7
<i>Hv:T2_33A_null</i>	0.242 ± 0.009	0.171 ± 0.007	70.8 ± 4.0	0.206 ± 0.01	0.118 ± 0.007	57.5 ± 4.4
<i>Hv:T2_51_null</i>	0.247 ± 0.009	0.173 ± 0.006	70.3 ± 3.5	0.203 ± 0.014	0.148 ± 0.011	72.8 ± 7.6
<i>Hv:T2_17A</i>	0.167 ± 0.014	0.134 ± 0.015	80.2 ± 11.2	0.115 ± 0.01	0.153 ± 0.020	132.9 ± 20.7
<i>Hv:T2_33A</i>	0.204 ± 0.015	0.120 ± 0.011	58.9 ± 6.8	0.169 ± 0.018	0.160 ± 0.021	95.1 ± 15.9
<i>Hv:T2_51</i>	0.252 ± 0.014	0.152 ± 0.013	60.4 ± 6.1	0.157 ± 0.012	0.177 ± 0.01	112.4 ± 10.4
<i>Hv:T2_52B</i>	0.257 ± 0.011	0.173 ± 0.013	67.2 ± 6.0	0.224 ± 0.018	0.215 ± 0.021	96.3 ± 12.3
Wheat						
'BW26'	0.236 ± 0.010	0.181 ± 0.004	77 ± 3.6	0.254 ± 0.018	0.177 ± 0.005	70 ± 5.4
'Carazinho'	0.219 ± 0.010	0.167 ± 0.009	76 ± 5.3	0.245 ± 0.015	0.201 ± 0.012	82 ± 7.0
<i>Ta:T2_42_null</i>	0.170 ± 0.013	0.112 ± 0.010	66 ± 7.5	0.196 ± 0.018	0.113 ± 0.011	57 ± 7.9
<i>Ta:T2_29</i>	0.186 ± 0.004	0.130 ± 0.013	70 ± 7.1	0.215 ± 0.011	0.142 ± 0.013	66 ± 6.8
<i>Ta:T2_64</i>	0.141 ± 0.012	0.098 ± 0.014	69 ± 11.5	0.148 ± 0.013	0.109 ± 0.022	74 ± 16.3
<i>Ta:T2_82</i>	0.158 ± 0.02	0.111 ± 0.005	70 ± 9.3	0.186 ± 0.021	0.147 ± 0.006	79 ± 9.5

* Shoot and root fresh weight of plants grown in acid soil and limed soil for 6 d. Data show the mean of shoot and root fresh weight (g) and standard error ($n = 6$), and the mean of relative shoot and root weight and standard error ($n = 6$).

Plant lines are the same as described for Table 1.

detected from 'BW26' or any of the 'BW26'-derived transgenic lines. Malate efflux was detected from the Al^{3+} -tolerant cultivars 'Carazinho' and 'Fielder' and the transgenic line generated from 'Fielder', *Ta*(Fielder):T2_8. This is consistent with the known mechanism of Al^{3+} tolerance in wheat encoded by *TaALMT1*.

The Al^{3+} tolerance of the transgenic wheat lines was first evaluated by measuring RRL in hydroponic solutions containing increasing concentrations of Al^{3+} (Fig. 2B). For the 'BW26'-derived lines, the concentrations were 0, 4 and 8 μM Al^{3+} and for the 'Fielder'-derived line the concentrations were 0, 4 and 30 μM Al^{3+} . A higher range of concentrations was used for 'Fielder' because it possesses an Al^{3+} -tolerant allele of the *TaALMT1* gene whereas 'BW26' possesses an Al^{3+} -sensitive allele (see below). Two of the 'BW26'-derived lines, *Ta*:T2_64 and *Ta*:T2_82, showed greater RRL than the null line and 'BW26' at both 2 and 4 μM Al^{3+} (Fig. 2B). Of the remaining two lines, *Ta*:T2_29 showed a greater RRL than the null at 4 μM only, and *Ta*:T2_110 was not significantly different from the null line at either concentration (Fig. 2B). These results are generally consistent with the measured citrate efflux from these lines. For the single 'Fielder'-derived line, RRL was approx. 120 % in both 8 and 30 μM Al^{3+} , which was significantly greater than in the wild-type 'Fielder' and 'Carazinho' in these short-term experiments. The inclusion of 'BW26' in this experiment demonstrates its greater sensitivity to Al^{3+} compared with 'Fielder' and 'Carazinho'.

The Al^{3+} tolerance of a selection of these wheat lines was examined in an acid soil and in the same soil amended with lime. This experiment included the two 'BW26'-derived transgenic lines (*Ta*:T2_64 and *Ta*:T2_82), a null line (*Ta*:T2_82_null), and wild-type cultivars 'BW26' and 'Carazinho'. The 'Fielder'-derived transgenic line was not included because there was insufficient seed. Relative shoot and root weights of the transgenic lines were not consistently different from those of the null lines and 'BW26' (Table 2). However the transgenic lines had longer roots in acid soil (Fig. 3), with RRL being significantly larger in the two transgenic lines than in the null line (Fig. 3B).

'Carazinho' had the greatest RRL at >80 % (Fig. 3B). 'Carazinho' showed a significantly greater RRL than the other lines (Fig. 5B), but no differences were detected between the transgenic and null lines.

DISCUSSION

We have demonstrated that expression of *HvAACT1* in barley and wheat with a constitutive promoter enhances Al^{3+} -activated citrate efflux from roots and confers increased Al^{3+} tolerance. In these short-term experiments, effects on shoot growth were not apparent and would probably appear over a longer period as changes in root growth and function affect water and nutrient uptake (Zhao *et al.*, 2003). The results were generally similar in the hydroponic experiments and soil trials, which reinforces the value of short-term hydroponic screens for assessing lines. In the hydroponic experiments, root growth of transgenic barley lines and 'Dayton' was stimulated in 1 μM Al^{3+} . This response has been observed previously in other species and is interpreted as low concentrations of Al^{3+} alleviating pH toxicity by reducing the concentration of the more toxic H^+ on, or adjacent to, the charged membrane surface of the root cells (Kinraide *et al.*, 1992; Kinraide, 1993). Although the transgenic barley lines were significantly more Al^{3+} tolerant than null lines, they did not reach the level of tolerance displayed by the tolerant wild-type cultivar 'Dayton'. Furthermore, *HvAACT1* does not appear to be as effective for increasing Al^{3+} tolerance in transgenic barley as the wheat tolerance gene *TaALMT1* (Delhaize *et al.*, 2004). Barley plants expressing *TaALMT1* are tolerant of 20 μM Al^{3+} (RRL >80 %) (Delhaize *et al.*, 2004) whereas in the present study, plants expressing *HvAACT1* are inhibited by 4 μM Al^{3+} in a hydroponic solution of similar composition. This indicates that Al^{3+} tolerance is determined not only by the effectiveness of organic acid in chelating Al^{3+} , but also by the amount of organic acid released from root apices. In transgenic barley lines expressing *TaALMT1* or *HvAACT1*, malate efflux was about 50 times greater than citrate efflux. For instance, in

transgenic barley plants over-expressing *TaALMT1*, malate efflux was up to 1000 pmol per apex per hour (Delhaize *et al.*, 2004), while in barley plants over-expressing *HvAACT1* citrate efflux was about 20 pmol per apex per hour. Nevertheless, *HvAACT1* expression does increase tolerance and provides opportunities for further enhancing the Al³⁺ tolerance of barley and wheat, as discussed later.

Furukawa *et al.* (2007) reported a positive relationship between *HvAACT1* expression level and citrate efflux among wild-type genotypes of barley. In the current study, *HvAACT1* expression in one of the transgenic barley lines (*Hv*:T2_52B) was almost 3-fold greater than in 'Dayton' despite showing less citrate efflux and a lower relative tolerance. Several technical and biological factors could explain the poor correlation between expression and citrate efflux when comparing wild-type plants with transgenic plants. First, gene expression is not necessarily directly correlated with the protein content so expression levels of the gene might not reflect expression of the phenotype. More importantly, the constitutive promoter in the transgenic plants might obscure the correlation detected among the wild-type plants because the pattern of expression in roots of the transgenic plants may differ from the pattern occurring naturally. For instance, *HvAACT1* expression in wild-type barley is driven by its native promoter which confers relatively high expression in the epidermal cells compared with the other cells of the apex (Fujii *et al.*, 2012). A correlation between relative *HvAACT1* expression in these specific cells and citrate efflux from roots could be obscured in transgenic plants expressing *HvAACT1* with a constitutive promoter where expression occurs in all cells. Not all root cells may contribute to the measured citrate efflux due to other anatomical, regulatory or metabolic restraints. It also remains possible that other factors such as the rate of citrate synthesis within the cells or the genetic background of barley influences the capacity to release citrate from roots.

Fujii *et al.* (2012) also expressed *HvAACT1* in 'Golden Promise', but the aims of that study were quite different from those of the present work. Fujii *et al.* (2012) primarily investigated the allelic variation in *HvAACT1* promoters and used native promoters derived from different genotypes to drive *HvAACT1* expression. They analysed T₁ lines, not T₂ lines, and compared citrate efflux and Al³⁺ tolerance with wild-type 'Golden Promise' but not with null segregant lines or Al³⁺-tolerant wild-type cultivars. Furthermore, they measured citrate efflux from the whole root system of young seedlings instead of the root apex, which is more relevant for Al³⁺ tolerance. This could explain why they reported smaller increases in citrate efflux from their T₁ lines compared with controls (2- to 4-fold) than we measured from T₂ lines in the present study (4- to 10-fold). Nevertheless, Fujii *et al.* (2012) showed that *HvAACT1* expression in transgenic barley lines using the native promoter from the Al³⁺-tolerant Murasakimochi cultivar could increase Al³⁺ tolerance above that measured in the parental cultivar 'Golden Promise'.

Two of the transgenic wheat lines generated in this study with the highest expression levels, *Hv*(Fielder):T2_8 and *Hv*:T2_82, also exhibited the greatest citrate efflux and Al³⁺ tolerance in both hydroponic culture and acid soil. The 'Fielder'-derived line showed citrate efflux in addition to malate efflux (controlled by the endogenous *TaALMT1* gene) and was more tolerant of Al³⁺ than wild-type 'Fielder' in hydroponic culture. This suggests

that citrate efflux and malate efflux may confer additive levels of Al³⁺ tolerance. However, since only a single transgenic line was characterized, this conclusion needs to be confirmed with additional lines and soil experiments. None of the transgenic barley lines or 'BW26'-derived wheat lines showed Al³⁺-activated malate efflux, which is consistent with *HvAACT1* specifically facilitating citrate efflux from these roots.

The efflux of organic anions from root cells involves two main processes: synthesis of organic anions and their transport across the plasma membrane. Ryan *et al.* (2001) argued that the transport step was more likely to be rate limiting since organic anions such as malate and citrate are common cellular components with high turnover rates. None of the transgenic barley lines expressing *HvAACT1* showed greater Al³⁺ tolerance than the tolerant wild-type cultivar 'Dayton'. It is possible that as the capacity to transport citrate out of the cell is increased, other processes begin to limit efflux. Consistent with this idea are the findings from other studies which show that enhanced expression of genes involved in organic anion synthesis can also increase Al³⁺ tolerance (see Ryan *et al.*, 2011). For instance, expressing genes encoding organic acid synthesis in tobacco (*Nicotiana tabacum*) (de la Fuente *et al.*, 1997; Han *et al.*, 2009), alfalfa (*Medicago sativa*) (Barone *et al.*, 2008) and arabidopsis (Koyama *et al.*, 1999; Koyama *et al.*, 2000) is reported to increase citrate efflux and Al³⁺ tolerance. Although other studies have not observed these same responses (Delhaize *et al.*, 2001, 2003), it remains plausible that transgenic strategies which combine enhanced organic anion synthesis and transport will provide an additional benefit if organic anion supply begins to limit efflux. Alternative transgenic strategies which have successfully increased Al³⁺ tolerance to some degree have targeted genes involved in membrane lipid composition, stress responses and general metabolism (Ezaki *et al.*, 2000; Basu *et al.*, 2001; da Silva *et al.*, 2006; Ryan *et al.*, 2007; Trejo-Tellez *et al.*, 2010). Future studies will explore whether combining natural tolerance traits in barley and wheat with transgenic strategies is effective for increasing Al³⁺ tolerance.

In conclusion, this study has demonstrated that genetically modifying barley and wheat to express the barley gene *HvAACT1* gene can increase their Al³⁺ tolerance in hydroponic experiments and short-term soil trials. This transgene could be introgressed into naturally Al³⁺-tolerant genotypes of wheat and barley to assess whether the effects of the endogenous genes and transgenes are additive. To date, *TaALMT1* confers the most effective level of Al³⁺ tolerance in transgenic barley (Delhaize *et al.*, 2004) and wheat (Pereira *et al.*, 2010), and combining it with *HvAACT1* may further enhance the tolerance of these species. Further studies need to test the effectiveness of these transgenes in increasing grain yield as established for barley expressing *TaALMT1* (Delhaize *et al.*, 2009). Ultimately, field trials are needed to establish whether these strategies can increase production on acid soils above what is currently possible with natural germplasm.

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