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PART OF A HIGHLIGHT SECTION ON PLANT–SOIL INTERACTIONS AT LOW PH

Engineering greater aluminium resistance in wheat by over-expressing *TaALMT1*

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• *Background and Aims* Expected increases in world population will continue to make demands on agricultural productivity and food supply. These challenges will only be met by increasing the land under cultivation and by improving the yields obtained on existing farms. Genetic engineering can target key traits to improve crop yields and to increase production on marginal soils. Soil acidity is a major abiotic stress that limits plant production worldwide. The goal of this study was to enhance the acid soil tolerance of wheat by increasing its resistance to Al^{3+} toxicity.
• *Methods* Particle bombardment was used to transform wheat with *TaALMT1*, the Al^{3+} resistance gene from wheat, using the maize ubiquitin promoter to drive expression. *TaALMT1* expression, malate efflux and Al^{3+} resistance were measured in the T_1 and T_2 lines and compared with the parental line and an Al^{3+}-resistant reference genotype, ET8.
• *Key Results* Nine T_2 lines showed increased *TaALMT1* expression, malate efflux and Al^{3+} resistance when compared with untransformed controls and null segregant lines. Some T_2 lines displayed greater Al^{3+} resistance than ET8 in both hydroponic and soil experiments.
• *Conclusions* The Al^{3+} resistance of wheat was increased by enhancing *TaALMT1* expression with biotechnology. This is the first report of a major food crop being stably transformed for greater Al^{3+} resistance. Transgenic strategies provide options for increasing food supply on acid soils.

Key words: Acid soil, aluminium resistance, *TaALMT1* gene, transgenic wheat, *Triticum aestivum*, aluminum.

INTRODUCTION

The world population is predicted to increase by >2 billion by 2050. Most of this increase will occur in developing countries such as those in sub-Saharan Africa where the population is expected to double. To support this growth, cereal production for food and animal feed will need to reach 3 billion tonnes per annum, an increase of 50 % on current production levels (FAO, 2009). The majority of these gains are expected to come from improving yields and increasing cropping intensity. However, an estimated 120 million ha of extra land are also likely to come into production, mostly in Africa and Latin America. Much of the land currently not in use suffers from chemical, physical or biological constraints, making it suitable for the production of a limited range of low value crops. While conventional plant breeding and improved practices have kept up with the demands of population increases in the past, all options, including transgenic approaches, should be considered to meet the challenges of the future (Hoisington, 2002; Bhalla, 2006).

Soil acidity is one of the important abiotic stresses limiting plant production. Almost 60 % of the soil in tropical and subtropical regions is acidic (von Uexküll and Mutert, 1995), making it a major limitation to food production in many developing countries. Acid soils present many stresses to plants, but it is their high concentration of soluble aluminium cations, especially Al^{3+}, which is largely responsible for reducing root elongation, disrupting nutrient and water uptake, and increasing the susceptibility of plants to drought and heat stress. Many species have evolved mechanisms to resist Al^{3+} stress and these are generally divided into those that exclude Al^{3+} from the root and shoot tissues (resistance) and those that can safely accommodate Al^{3+} once it is taken up by the plant (tolerance). Genotypes within many species show a significant variation in their ability to cope with Al^{3+} toxicity (Taylor, 1988, 1991; Kochian *et al.*, 2004), and this variation has been used by breeders to develop more Al^{3+}-resistant lines (Garvin and Caver, 2003).

Soil acidity can be neutralized with the regular application of lime, but it can often take years to ameliorate sub-soil acidity, and these amendments are prohibitively expensive for farmers in many countries. Therefore, the combination of liming and the use of Al^{3+}-resistant germplasm is a common management strategy for cropping acid soils. Among the common cereals, rye (*Secale cereale*) and triticale (×*Triticosecale*) are generally more tolerant of acid soils than common wheat (*Triticum aestivum*), whereas durum wheat (*Triticum turgidum*) and barley (*Hordeum vulgare*) perform very poorly on acid soils. Farmers with acid soils are often left with little choice but to grow feed crops such as rye and triticale even though wheat, durum and barley are more profitable.

Although Al^{3+} resistance is a multigenic trait in wheat (Garvin and Carver, 2003; Cai *et al.*, 2008; Ryan *et al.*, 2009), most of the phenotypic variation can be attributed to a single locus on chromosome 4DL which controls the Al^{3+}-activated efflux of malate from the root apices (Delhaize *et al.*, 1993*a*, *b*; Ryan *et al.*, 1995). The malate anions protect the sensitive root apices by chelating the Al^{3+} and rendering it non-toxic. The *TaALMT1* gene controlling this response encodes an Al^{3+}-activated anion channel that is permeable to malate (Sasaki *et al.*, 2004; Raman *et al.*, 2005; Zhang *et al.*, 2008).

It is now clear that other members of the ALMT family perform similar functions in other species including arabidopsis (Hoekenga *et al.*, 2006), *Brassica napus* (Ligaba *et al.*, 2006) and rye (Fontecha *et al.*, 2007). Members of a second family of genes named the multidrug and toxic compound extrusion (*MATE*) family also contribute to the Al resistance in plants via organic anion efflux (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007; Wang *et al.*, 2007; Liu *et al.*, 2009). The evidence to date indicates that ALMT proteins facilitate malate efflux while the MATE proteins facilitate citrate efflux. In some species, such as arabidopsis and wheat, genes from both these families contribute to resistance (Liu *et al.*, 2009; Ryan *et al.*, 2009).

Enhanced Al^{3+} resistance is generally correlated with higher expression of these resistance genes, regardless of whether they are from the ALMT family or the MATE family (Raman *et al.*, 2005; Hoekenga *et al.*, 2006; Sasaki *et al.*, 2006; Magalhaes *et al.*, 2007; Fujii *et al.*, 2009). In wheat, high *TaALMT1* expression is associated with large tandem repeats of sequence in the promoter region, and it has been proposed that these repeats contain enhancer elements that increase expression of the gene (Sasaki *et al.*, 2006). The strong correlation between gene expression and Al^{3+} resistance validates transgenic strategies to increase Al^{3+} resistance in plants by increasing *TaALMT1* expression. When expressed in barley (Delhaize *et al.*, 2004) and tobacco suspension cells (Sasaki *et al.*, 2004; Zhang *et al.*, 2008) *TaALMT1* conferred an Al^{3+}-activated efflux of malate which was associated with improved resistance to Al^{3+} stress. Transgenic barley plants showed a >2-fold increase in grain production on acid soil compared with the untransformed controls (Delhaize *et al.*, 2009).

Common or bread wheat is one of world's major crops, providing one-fifth of the calories consumed worldwide (FAO, 2006). Although the variation found in Al^{3+} resistance of wheat has been successfully exploited by breeders, the level of resistance is still considerably lower than that of species such as rice and triticale. Transgenic approaches provide additional options for enhancing the acid soil tolerance of wheat, and this report describes experiments in which a wheat Al^{3+} resistance gene *TaALMT1* was over-expressed in a sensitive wheat cultivar. We show that even with a relatively small number of successful transgenic events, this strategy is a viable option for increasing the acid soil tolerance of important crop species.

MATERIALS AND METHODS

Genotypes

The wheat gene *TaALMT1* was over-expressed in the *Triticum aestivum* Bob White 26 'SH9826' line (BW26). BW26 is an Al^{3+}-sensitive wheat identified as being highly efficient for generating transgenic plants using the microparticle bombardment technique (Pellegrineschi *et al.*, 2002). The genotype ET8 was included in all experiments as a positive control line for Al^{3+} resistance (Delhaize *et al.*, 1993*a*).

Plasmid vectors

The pWUbi vector containing the *TaALMT1* coding region [allele *TaALMT1-1* (Sasaki *et al.*, 2004)] used in the transformation experiments was the same as described by Delhaize *et al.* (2004). Plasmid *pCMneoSTLS2* (Maas *et al.*, 1997), containing the neomycin phosphotransferase gene (*nptII*), that confers resistance to geneticin (G418), was used as selectable marker.

Explant preparation and microparticle bombardment

Caryopses from BW26 plants were collected 14–16 d after anthesis and seeds were sterilized (20 % sodium hypochlorite). Immature embryos, 1·0–1·5 mm in width, were excised and placed onto an osmotic medium, MSM for 2–4 h. MSM consisted of MS-based salts and vitamins (Murashige and Skoog, 1962), 0·1 g L^{-1} myo-inositol, 150 g L^{-1} maltose, pH adjusted to 5·9 and solidified with 8 g L^{-1} bactoagar.

Prior to bombardment, 5 μg of *pWUbi::TaALMT1* and 3 μg of *pCMneoSTLS2* vectors were coated on 50 μL of sub-micron (0·6 μm) gold particles (BioRad). Embryos were bombarded using the helium-driven particle delivery system, PDS-1000/He (BioRad) with 900 psi rupture discs.

Culture conditions and recovery of transformed plants

Twenty-four hours after bombardment, the immature embryos were transferred to MSE medium for callus induction. MSE is comprised of MS salts and vitamins with 30 g L^{-1} sucrose, 2·5 mg L^{-1} 2,4-D and is solidified with 3 g L^{-1} phytagel. After 14 d incubation in the dark, the calli were then transferred to regeneration medium, MSW. MSW consisted of MS salts and vitamins only, solidified with 8 g L^{-1} bactogar and supplemented with 50 mg L^{-1} geneticin (G418) as the selective agent.

Cultures were transferred to fresh MSW medium with geneticin every 2 weeks over 8–12 weeks. Geneticin-resistant shoots with root formation were then transferred to small soil pots and acclimatized in a high-humidity misting unit in the glasshouse. After 2–3 weeks, plants were transferred to pots of soil and T_1 seed were harvested from mature transgenic plants.

A total of 876 wheat embryos were isolated and bombarded over seven separate sessions. Transformation efficiency varied from 0 to 6·31 % (Supplementary Data Table S1, available online) among the sessions, with an average efficiency of 1·5 %.

DNA extraction and PCR tests for transformation

Total DNA was extracted using an SDS-based protocol (Martienssen *et al.*, 1989). After the quantification in agarose gel, 100 ng of DNA were used in PCRs with 1× HotStar Taq Master Mix (Qiagen), 10 μM of each primer and 1× Q

solution (Qiagen) (only for primers Neo5R and Neo3F). The primers FgpWUbi-F (TGCAGCATCTATTCATATGC) and TR-2 (GATGGTGCCCACCATCTCG) were used for amplification of a *TaALMT1* 289 bp fragment originated from the *pWUbi::TaALMT1* vector. Since the FgpWubi-F primer targets the maize ubiquitin promoter (intron) region of the pWUbi vector, the endogenous *TaALMT1* is not amplified. The primers Neo3F (GGCTATTCGGCTATGACTG) and Neo5R (ATCGGGAGCGGCGATACCGTA) were used for the amplification of a *nptII* 738 bp fragment originated from the *pCMneoSTLS2* vector. The amplification program used for both primer pairs was 95 °C for 15 min followed by 40 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min.

Malate efflux experiments

Seeds were grown in nutrient solution (500 μM KNO_3, 500 μM $CaCl_2$, 500 μM NH_4NO_3, 150 μM $MgSO_4$, 10 μM KH_2PO_4, 2 μM $FeCl_3$, 11 μM H_3BO_3, 2 μM $MnCl_2$, 0·35 μM $ZnCl_2$, 0·2 μM $CuCl_2$, pH 4·3) for 4 d with natural light and temperature. A total of 24–30 root apices (4 mm), collected from 6–10 different germinated seeds, were excised in Petri dishes and divided into three tubes (8–10 apices per tube). Malate efflux from the excised root apices was measured as described by Ryan *et al.* (1995) with modifications.

Analysis of TaALMT1 *expression by real-time qRT–PCR*

The *TaALMT1* expression analysis was based on the procedure previously described by Delhaize *et al.* (2004), with modifications. The total RNA from 8–10 root apices stored at −80 °C following prior malate efflux experiments was purified using the RNeasy minikit (Qiagen). The RNA purification included a DNase step to eliminate any genomic DNA contamination. The cDNA was prepared using 1 μg of total RNA, 1× RT buffer, 10 mM of each dNTP, 500 ng of oligo$(dT)_{15}$ primer, 0·2 M dithiothreitol (DTT) and 1 U of SuperScript II Reverse Transcriptase (Invitrogen). The volume of each reaction was adjusted to 20 μL and incubated at 25 °C for 5 min followed by 42 °C for 60 min. An RNaseH degradation step was performed for 30 min at 37 °C. *TaALMT1* expression relative to the endogenous gene [glyceraldehyde phosphate dehydrogenase (*GAPDH*)] was determined by real-time quantitative RT–PCR on a Rotor-Gene 3000 Real Time Cycler (Corbett Research, Australia). The samples for qPCR were prepared in a 10 μL final volume containing 5 μL of SYBR Green JumpStart Taq Ready Mix (Sigma), 0·5 μL of primer solution (mix 1 : 1 from primers Forward and Reverse at 5 μM each) and 4·5 μL of cDNA diluted 1 : 50. The primers RTFwd3 (CGTGAAAGCAGC GGAAAGCC) and RTRev3 (CCCTCGACTCACGGTACT AACAACG) were used to amplify the *TaALMT1* transcript, and the primers RiwhGAPDH-R (TCAGACTCCTCCTTGAT AGC) and RiwhGAPDH-F (GTTGAGGGTTTGATGACC AC) were used to amplify the *GAPDH* transcript, used as the endogenous reference. Thermocycling conditions were 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 40 s. At the end of the amplification steps, the reactions were incubated at 40 °C for 5 min and 55 °C for 1 min, followed by a melting curve program (increase of 1 °C from 55 ° to 99 °C holding for 5 s at each temperature).

Relative root growth (RRG) experiments in hydroponic culture

Seeds were pre-germinated in the dark for 2 d at 4 °C and 2 d at 28 °C. Once the length of the longest root was measured, the seedlings were grown over an aerated nutrient solution (as above) containing different concentrations of $AlCl_3$ for 4 d with natural light. The seedlings were then removed from the nutrient solution and the length of the longest root was measured again. The RRG was estimated as (net root growth in Al treatment/net root growth in control solution) × 100. To account for the accumulation of errors associated with deriving RRG, the errors were calculated as follows: $SE_{RRL} = RRG\,[(SE_x/x)^2 + (SE_y/y)^2]^{1/2}$ where x and y represent the mean net root length in the control treatment and the mean net root length in the Al treatment, respectively. Statistical analysis was based on the average RRG in all lines, independent of Al^{3+} concentration and experiment. A cluster analysis was employed and all the lines that were similar to BW26 were discarded. An analysis of variance (ANOVA) with 5 % significance was performed on the remaining lines using the R software.

Scoring homozygous T_2 *families*

Homozygous T_2 families were identified by a rapid screen for Al^{3+} resistance. For each T_2 family, 16–25 germinated seeds were placed in 500 mL conical flasks containing 70 mL of nutrient solution (pH 4·3) and 30 μM $AlCl_3$. The flasks were capped with aluminium foil and placed on a platform shaker (100 rpm). Solutions were replaced daily. Aluminium resistance of each seedling in each family was scored after 5 d by assessing root length and by examining the root apices for tissue damage under a dissecting microscope. Families in which all the seedlings showed relatively good root growth with undamaged apices were potentially homozygous for the transgene.

Southern analysis

Genomic DNA (10 μg) was isolated from BW26 and nine T_2 lines potentially homozygous for the transgene based on an Al^{3+} resistance assay. Following an overnight digestion with *Bam*HI, the DNA was run on a 1 % agarose gel and transferred to a PALL Biodyne B membrane using the alkaline blotting procedure. The membrane was probed with a ^{32}P-labelled 946 bp fragment targeting the maize ubiquitin intron included in the binary vector.

Soil experiments

Short-term growth experiments compared the root growth of three T_2 lines and control plants on a acidic red ferrosol obtained from the Robertson region of New South Wales in Australia (34°35′S, 150°36′E). The pH of an 0·01 M $CaCl_2$ extract was 4·33 and the exchangeable Al comprised 21 % of total cations. Half of this soil was left unamended and half was limed at a rate of 5 g $CaCO_3$ kg^{-1} dry soil to raise the pH and reduce Al^{3+} toxicity. Liming increased the pH to 5·18 and reduced the soluble Al to <1 % of the unamended soil. Field

capacity was determined to be 271 mL water kg^{-1} dry soil. Soil was wetted to 90 % field capacity and 622 g added to pots (8 cm diameter and 14·5 cm high) and arranged randomly on benches in a naturally lit glasshouse. Pre-germinated seeds were planted, two per pot at 2 cm depth, after first measuring the length of their longest root. Pots were maintained at 90 % field capacity, and after 4 d the plants were gently removed from the pots. The length of the longest root on each plant was measured a second time. The RRG of each wheat line was calculated by dividing the root lengths in the acid soil by lengths in the limed soil. The errors accounted for the cumulative errors (see above). Statistical analysis for RRG used a one-factor ANOVA where plant line was the factor.

For the long-term soil experiments three seedlings of BW26, ET8 and T2_4·4 were grown in pots (20 cm diameter and 25 cm high) containing 6 kg of the unamended acidic soil. Pots were arranged randomly in a naturally lit glasshouse and growth continued for 31 d. Roots were washed free of soil and the longest root on each plant was measured with a ruler. The roots were then scanned for total root length and root diameter using the software package WinRHIZO Pro v. 2002c (Regent Instruments Inc.). Dry weight of the roots and shoots was determined after drying at 65 °C for 48 h.

RESULTS

Generation of T_0 *plants*

Of the 18 primary transformed (T_0) plants recovered from the tissue culture, PCR determined that 13 were positive for the *pWUbi::TaALMT1* vector (T0_1, T0_2B, T0_3, T0_4, T0_5, T0_8, T0_9, T0_10, T0_12, T0_14, T0_18, T0_20A and T0_20B). The primers used spanned the ubiquitin intron in the *pWUbi* vector and the *TaALMT1* cDNA which enabled the transgene to be distinguished from the endogenous *TaALMT1* gene (Fig. 1A). A second PCR targeting the *pCMneoSTLS2* vector (providing the antibiotic resistance) revealed that T0_9 did not contain the selectable marker (Fig. 1B). These PCRs identified two additional lines, T0_2A and T0_16, that possessed the selectable marker but not the *pWUbi::TaALMT1* vector.

Analysis of the T_1 *plants*

T_1 seed were harvested from the T_0 plants, and the T_1 seedlings were analysed for *TaALMT1* expression, malate efflux and Al^{3+} resistance. All experiments included the parental cultivar BW26 and a standard Al^{3+}-resistant line ET8. Relative *TaALMT1* expression and Al^{3+}-activated malate efflux from root apices were measured over two experiments (Table 1). The relatively large errors are expected since these T_1 plants will be segregating for one or more copies of the transgene. This segregation was confirmed by scoring 16–21 seeds from each of the T_1 lines for the presence of *pWUbi::TaALMT1* with PCR (data not shown). This also identified plants that lacked the transgene (null segregants). The relationship between *TaALMT1* expression and malate efflux among the T_1 lines is shown in Supplementary Data Fig. S1A (available online).

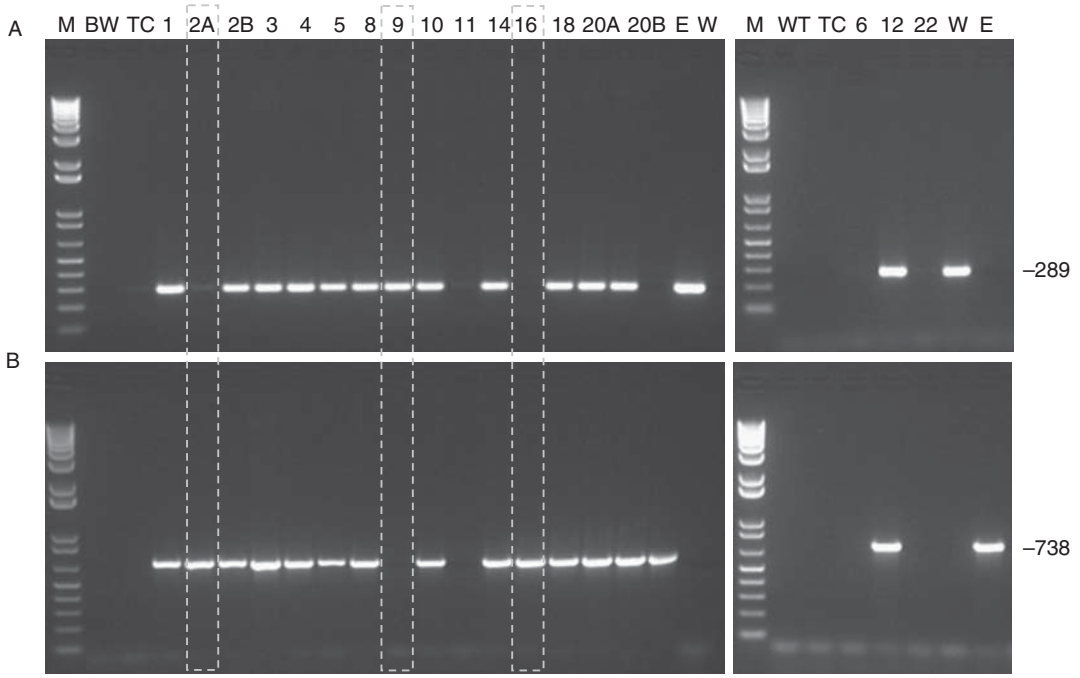

FIG. 1. Detection of transgenic lines. (A) Amplification of a 289 bp fragment specific to the inserted *TaALMT1* copy. The primer pairs do not amplify the endogenous *TaALMT1* as can be seen in the negative controls including BW26 (BW) and a tissue culture control (TC; plants regenerated in tissue culture but not transformed). (B) Amplification of the 738 bp fragment specific to the *nptII* gene from the selectable marker. M denotes the DNA size ladder while E and W indicate the vectors *pCMneoSTLS2* (selectable marker) and *pWUbi::TaALMT1*, respectively. Numbers indicate the different T_0 transformed lines recovered from the tissue culture. Dotted boxes represent lines containing only the selectable marker (T0_2A and T0_16), and a line containing the *TaALMT1* transgene but not the selectable marker (T0_9).

TABLE 1. TaALMT1 *expression and malate efflux in the* T_1 *lines*

Experiment	Wheat lines	*TaALMT1* expression (arbitrary units)	Malate efflux (nmol $apex^{-1}$ h^{-1})
1	BW26	0·033 ± 0·001	0·3 ± 0·2
	ET8	0·159 ± 0·005	1·1 ± 0·3
	T1_1	0·138 ± 0·001	1·7 ± 0·1
	T1_4	0·029 ± 0·005	0·4 ± 0·1
	T1_8	0·051 ± 0·010	0·4 ± 0·1
	T1_14	0·056 ± 0·002	1·0 ± 0·2
	T1_18	0·077 ± 0·012	1·0 ± 0·7
	T1_20B	0·134 ± 0·018	0·9 ± 0·6
2	BW26	0·016 ± 0·000	0·10 ± 0·10
	ET8	0·125 ± 0·004	0·60 ± 0·10
	T1_2B	0·083 ± 0·000	0·68 ± 0·60
	T1_3	0·056 ± 0·003	0·36 ± 0·20
	T1_5	0·035 ± 0·002	0·51 ± 0·40
	T1_9	0·022 ± 0·001	0·10 ± 0·02
	T1_10	0·019 ± 0·002	0·10 ± 0·04
	T1_20A	0·049 ± 0·004	0·50 ± 0·30

Expression in the root apices was estimated by qRT–PCR using the *GAPDH* gene as an internal reference. Data are the mean and standard error of three biological replicates. Malate efflux was measured from excised root apices in the presence of 50 μM $AlCl_3$. Data show the mean and standard error ($n = 4$).

Aluminium resistance of all the T_1 lines was estimated over three experiments by measuring net growth in nutrient solution containing 0, 5 or 20 μM $AlCl_3$ and calculating RRG (Fig. 2). Null plants were excluded from the these measurements and so all plants were either hemizygous or homozygous for the transgene. At 5 μM $AlCl_3$ most T_1 lines showed similar resistance to BW26, but, at 20 μM, $AlCl_3$ resistance in all T_1 lines except T1_9 and T1_10 was significantly greater than that of BW26 and similar to the resistant line ET8.

Analysis of T_2 *plants*

Nine T_1 transgenic lines were selected to generate families of T_2 seeds. Ten plants were grown from each of these nine lines, one of which was a null (previously determined by PCR) and the other nine plants were positive for the transgene (either homozygous or hemizygous). Likely homozygous T_2 families from each line were then identified by scoring the individual seeds in each family for Al^{3+} resistance in a rapid screen (Supplementary Data Table S2). The transgene copy number in these T_2 families was then determined by Southern blot (Supplementary Data Fig. S2). The appearance of two bands in the untransformed control plants of BW26 complicated this assessment. Nevertheless, most T_2 families had multiple inserts despite the segregation ratios for Al^{3+} resistance among T_2 families in Supplementary Data Table S2 suggesting otherwise. It is possible that the multiple transgenes segregate as a single locus if they become integrated close to another in the wheat genome. Alternatively each of the transgenes might be expressed to different levels so that one drives most of the phenotype. Transgene copy number becomes an important consideration if these transgenic lines are used in crosses to increase the Al^{3+} resistance of other elite cultivars since multiple inserts makes introgression more difficult.

Malate efflux from the root apices of the nine homozygous lines was 3- to 4-fold greater than that of the parental cultivar BW26 and most were similar to ET8 (Table 2). Quantitative RT–PCR confirmed that *TaALMT1* expression in the root apices of these T_2 plants remained greater than in those of BW26, whereas the expression level in a null-segregant line included was similar to that of BW26 (Table 2, Supplementary Data Fig. S1B).

Aluminium resistance in the T_2 homozygous lines was estimated from RRG following 4 d growth in nutrient solution containing 0, 20, 40 and 60 μM $AlCl_3$. Relative root growth of the control line BW26 was inhibited to approx. 10 % in all the $AlCl_3$ treatments (Fig. 3). All nine T_2 lines were significantly more Al^{3+} resistant than BW26 across the three Al^{3+} concentrations while eight null lines performed similarly to BW26. T2_5·4 and T2_18·1 showed statistically greater Al^{3+} resistance than ET8 at the higher Al^{3+} concentrations.

Soil experiments

Root growth of BW26, ET8 and three T_2 lines, T2_4·4, T2_5·4 and T2_20A.8, was compared in a short-term experiment using an acidic ferrosol soil with and without lime

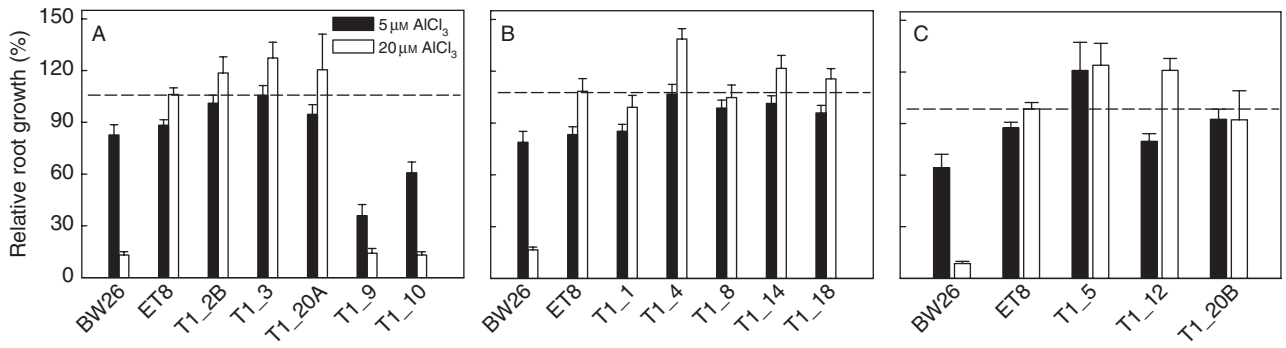

FIG. 2. Aluminium resistance of the T_1 lines. Al^{3+} resistance was estimated by measuring relative root growth in nutrient solution containing $AlCl_3$. The 13 T_1 transgenic lines were tested over three separate experiments shown in (A–C). Control lines BW26 and ET8 were included in every experiment. Net root growth in 5 and 20 μM $AlCl_3$ (pH 4·3) treatments was calculated relative to the net growth in the zero Al^{3+} control treatment. The dotted line indicates the relative root growth of the ET8 line in 20 μM $AlCl_3$ in each experiment.

TABLE 2. TaALMT1 *expression and malate efflux from* T_2 *lines*

Wheat lines	*TaALMT1* expression (relative to BW26)	Malate efflux (nmol $apex^{-1}$ h^{-1})
Controls		
BW26	1·0	0·4 ± 0·1
ET8	6·7	1·7 ± 0·2
T2_1-19 (null)	0·7	0·5 ± 0·1
T_2 lines		
T2_1·6	5·0	2·1 ± 0·2
T2_2B.1	2·3	2·2 ± 0·2
T2_3·4	1·7	1·6 ± 0·1
T2_4·4	1·8	2·0 ± 0·4
T2_5·4	3·1	1·6 ± 0·1
T2_8·5	0·8	1·2 ± 0·1
T2_12·7	4·5	1·7 ± 0·2
T2_18·1	5·1	2·1 ± 0·1
T2_20A.8	5·9	1·7 ± 0·2

Expression in the root apices was estimated by qRT–PCR using the *GAPDH* gene as an internal reference. The values shown are expressed relative to the expression in BW26 which was included in each experiment. Data are the mean of three technical replicates. Malate efflux was measured from excised root apices in the presence of 50 μM $AlCl_3$. Data show the mean and standard error ($n = 4$).

addition. Liming reduced the toxicity of the soil by raising the pH and reducing the concentration of toxic Al^{3+} cations. Root growth for the lines in each treatment and RRG (growth in acid soil relative to the limed soil) are shown in Fig. 4. All three T_2 lines performed better than BW26, and line T2_20A.8 showed significantly greater resistance than ET8.

A longer term soil experiment compared the root growth of BW26, ET8 and a single homozygous line (T2_4·4) after 31 d in the unamended and unfertilized acid soil. T2_4·4 and ET8 plants had significantly larger root systems than BW26 based on both total root length and length of the longest root, and T2_4·4 had greater root dry weight than either of the other two lines (Table 3). Representative root systems from each genotype are shown in Fig. 5. Roots of BW26 also appeared to be generally thicker with shorter laterals than the other lines. This observation was confirmed when the distribution of root diameters was analysed on each plant (Fig. 6). As a percentage of the total root system, BW26 had significantly fewer roots with diameters ≤0·28 mm compared with T2_4·4 and ET8. This change in root morphology helps explain why, although the root system of BW26 appears smaller than that of ET8 in Fig. 5, their dry weights are similar. In contrast to the root data, shoot dry weight for T2_4·4 and BW26 plants was significantly greater than for ET8 (Table 3), which is consistent with previous reports suggesting that shoot growth can be relatively unaffected by the early stages of Al^{3+} toxicity.

DISCUSSION

Over-expression of *TaALMT1* in wheat conferred greater Al^{3+}-activated malate efflux from the roots and improved Al^{3+} resistance which was maintained in the T_1 and T_2 generations. This is the first report of stably increasing the Al^{3+} resistance of a major food crop by genetic engineering. A previous report decribed the transformation of wheat with *SbMATE*, the Al^{3+} resistance gene from sorghum which encodes a citrate transporter (Magalhaes *et al.*, 2007). However, the increased Al^{3+} resistance measured in T_1 plants did not appear to be as robust as described for *TaALMT1* (Magalhaes *et al.*, 2007) and was not maintained in subsequent generations (L. V. Kochian, USDA-ARS, USA, pers. comm.). Other studies have increased the Al^{3+} resistance of plants by mutagenesis (Rounds and Larsen, 2008), by over-expressing genes involved in membrane physiology and stress responses (Ezaki *et al.*, 2000; Basu *et al.*, 2001; Stival da Silva *et al.*, 2006; Ryan *et al.*, 2007) or by targeting genes involved in organic anion synthesis and metabolism (de la Fuente *et al.*, 1997; Koyama *et al.*, 1999; Tesfaye *et al.*, 2001; Anoop *et al.*, 2003; Barone *et al.*, 2008; Trejo-Téllez *et al.*, 2010). Although a number of these studies showed enhanced Al^{3+} resistance in hydroponics, few have demonstrated enhanced tolerance to acid soils. Furthermore, transgenic plants modified for the expression of enzymes involved in malate and citrate synthesis have not consistently increased organic anion concentration in tissues or Al^{3+} resistance (Delhaize *et al.*, 2001, 2003).

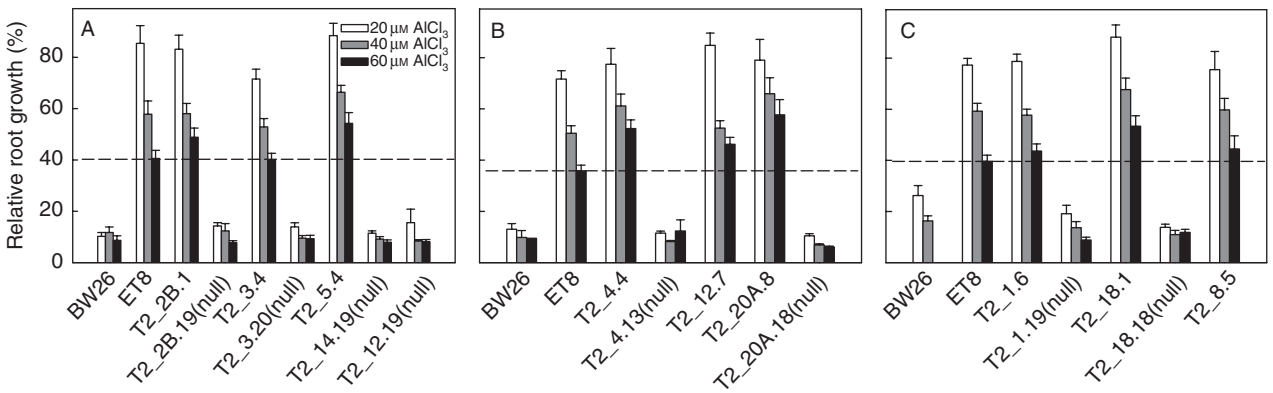

FIG. 3. Aluminium resistance in the T_2 transgenic lines. Al^{3+} resistance was estimated in T_2 lines by measuring relative root growth after 4 d in nutrient solution containing 20, 40 and 60 μM $AlCl_3$ (pH 4·3) compared with zero $AlCl_3$ controls. Eight null-segregant lines were also included. The dotted line indicates the relative root growth of the ET8 line in 60 μM Al^{3+} in each experiment.

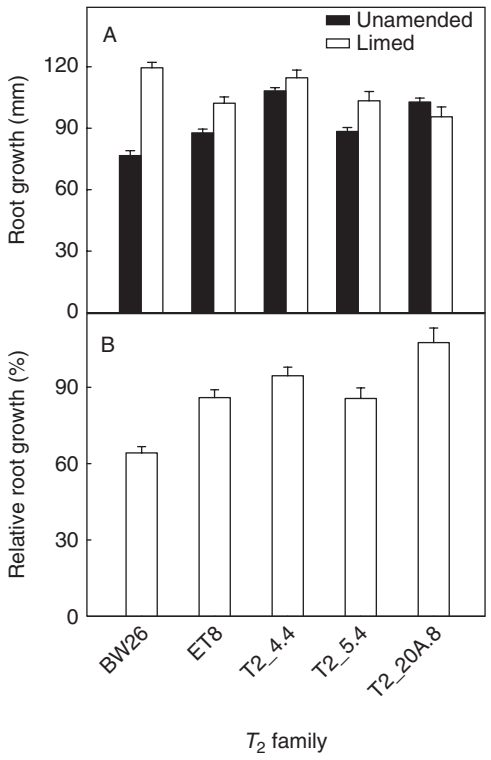

FIG. 4. Aluminium resistance of T_2 families in a short-term soil experiment. Seedlings of three T_2 families homozygous for the transgene as well as BW26 and ET8 were grown on an acid soil with and without lime addition. Seeds were pre-germinated on wetted filter paper and planted in pots. After 4 d the plants were removed and the length of the longest root on each plant measured. (A) Net root growth on unamended acid soil and limed soil, as indicated. (B) Relative root growth of each wheat line was calculated as a percentage of net root growth on acid soil relative to the net root growth in limed soil. Data show the mean and standard error ($n = 5$) taking into account the cumulative errors.

In the present study, nine of the 13 primary trangenic lines generated T_2 lines with greater Al^{3+} resistance than the parental cultivar and some even exceeded the resistance of ET8 in nutrient solution and acid soil (Figs 3 and 4). It is possible that screening of a larger number of transgenic events will identify plants that exceed the Al^{3+} resistance of even the most resistant wheat cultivars currently available.

We used a constitutive promoter to drive *TaALMT1* expression, but future studies could use promoters that restrict expression to the roots. In particular, the use of promoters and coding sequences derived from the species being transformed may enhance public acceptance of genetically modified crops. Furthermore, public acceptance of transgenic crops might also be improved if the selectable marker is absent from the cultivars that are finally commercialized. Including the transgene and selectable marker on different plasmids provides a means of removing the antibiotic resistance in subsequent generations because the two plasmids do not always insert into the same region of the genome.

When grown in acidic soil the T2_4·4 plants generated root systems that were considerably larger than those of the parental line BW26 and more similar to those of the Al^{3+}-resistant genotype ET8 (Fig. 5). The transgenic line had a greater total root length, longer individual roots and a greater percentage of fine roots than BW26, all of which would benefit the plants by improving water and nutrient uptake on acid soil (Table 3).

The finding that the Al^{3+} resistance of several T_2 lines exceeded that of ET8 highlights the potential of genetic engineering to increase the Al^{3+} resistance of wheat above what is naturally available and opens up the possibility of combining traits. What remains unclear is whether increasing the expression of *TaALMT1* in cultivars that are already Al^{3+} resistant provides an even stronger phenotype or whether other factors become limiting. Interestingly, while the transgenic lines displayed a wide range of *TaALMT1* expression levels, none exceeded those measured in ET8 (Table 2). Although the number of transgenic lines is small, this may indicate that there is an upper limit for *TaALMT1* expression in wheat. It is unclear what the physiological basis for such a limit would be, but ongoing crosses between the T_2 lines and other resistant genotypes will establish whether two loci of the *TaALMT1* gene (one transgenic and the other endogenous) generate greater expression than a single locus. Furthermore, the constitutive ubiquitin promoter may extend *TaALMT1* expression to additional cells in the root apex or other tissues not normally targeted by the native promoter. Efflux from these new cells might provide relatively greater Al^{3+} resistance to the plants, perhaps because they are nearer the root surface or because they protect new cells important for growth.

Additional benefits may also be gained by introgressing other Al^{3+} resistance loci into these transgenic lines. For instance, another mechanism for Al^{3+} resistance in plants relies on the efflux of citrate (Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007; Wang *et al.*, 2007; Ryan *et al.*, 2009). In wheat, this mechanism confers weaker resistance than the malate efflux controlled by *TaALMT1* and appears to be restricted to relatively few genotypes derived from Brazil (Ryan *et al.*, 2009). Nevertheless over-expression of the candidate gene controlling this trait, *TaMATE1*, could offer other advantages because the stability of the Al^{3+}–citrate complex is substantially stronger than that of the the

TABLE 3. *Root and shoot analyses from the longer term soil experiment*

Wheat line	Total root length (mm)	Longest root (mm)	% Roots ≤0·28 mm diameter	Root d. wt (mg)	Shoot d. wt (mg)
BW26	300 ± 57^a	258 ± 23^a	10 ± 3^a	56 ± 4^a	95 ± 22^a
T2_4·4	598 ± 21^b	451 ± 57^b	24 ± 1^b	77 ± 5^b	110 ± 9^a
ET8	756 ± 128^b	360 ± 37^b	41 ± 13^b	58 ± 3^a	51 ± 3^b

Plants were grown in the acidic ferrosol soil for 31 d. Provided for each wheat line are the total root length, length of the longest root, the proportion of the total root system with diameters ≤0·28 mm and the total root and shoot dry weights. Data show the mean and standard error ($n = 3$). Means with the same letter are not significantly different from one another ($P > 0·05$).

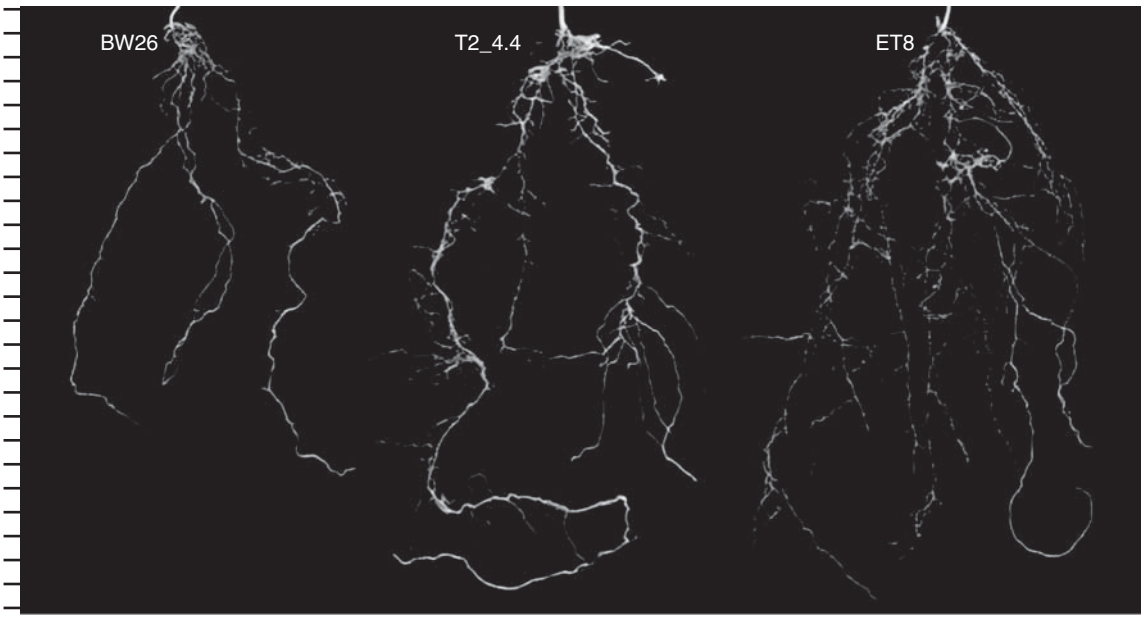

FIG. 5. Root systems collected from the long-term soil experiment. Plants were grown for 31 d in an acidic ferrosol soil and the soil gently washed from the roots. Photographs were taken of a representative root system from the replicates of each genotype BW26, ET8 and the transgenic T_2 line T2_4·4. The intervals on the left-hand scale denote centimetres.

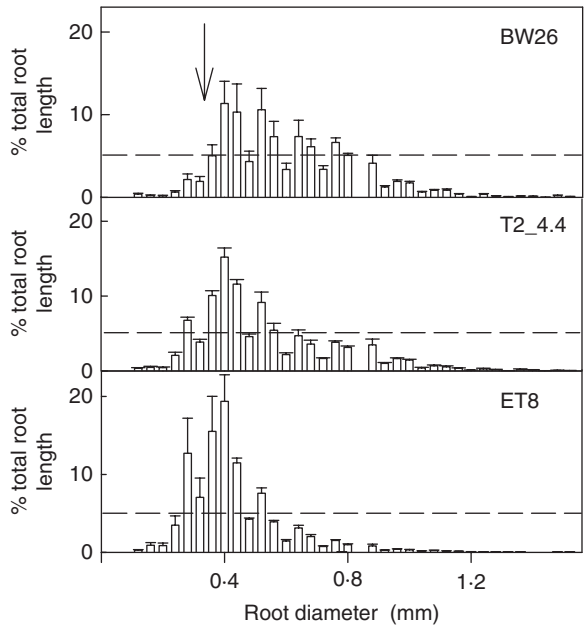

FIG. 6. Distribution of root diameters in a T_2 family and control plants grown in a longer term soil experiment. Plants of BW26, ET8 and the T2_4·4 line were grown for 31 d in the unamended acid soil. Roots were washed from the soil and analysed using the WinRHIZO scanner. The *x*-axis shows the classes of root diameter increasing by 0·04 mm. Data are the mean frequencies of roots in each diameter class ($n = 3$). The horizontal line represents the 5 % value and the arrow delineates the 0·28 mm diameter size class below which the data in Table 3 were calculated.

Al^{3+}–malate complex (Hue *et al.*, 1986). In addition, it is possible to use related genes from barley and sorghum to generate transgenic wheat, although difficulties with this approach are noted above for *SbMATE*. An additive effect on Al^{3+} resistance might be achieved by over-expressing two or more genes at the same time, particularly if they control the efflux of different organic anions. If the supply of organic anions becomes limiting for efflux, genes involved in enhancing organic anion biosynthesis could be co-transformed with genes involved in organic anion transport, which might generate transgenics with even greater levels of resistance.

Chief among the challenges facing plant scientists and agronomists are practical options for increasing food production on land presently under cultivation as well as raising the productivity of marginal land which will need to be appropriated for food production in the future. Careful land management is essential for sustainability. Yet the expense of even basic practices such as the application of lime can be prohibitive to many farmers. These expenses are due not only to the costs of the commodities but also to their transportation and distribution. Transgenic strategies, such as the one described here, provide opportunities for sustaining and even increasing grain production on acid soils. We have demonstrated that over-expression of the *TaALMT1* gene can increase the Al^{3+} resistance of wheat, a major food crop. This provides options for improving wheat yields on acid soils as well as helping to expand food production in the future to more marginal lands.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following tables and figures. Table S1. Efficiency of transformation of wheat with *TaALMT1*. Table S2. Scoring T_2 families for Al^{3+} resistance. Fig. S1. Relationship between *TaALMT1* expression and malate efflux in the T_1 and T_2 lines. Fig. S2. Southern analysis of the T_2 transgenic plants.

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