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The multiple origins of aluminium resistance in hexaploid wheat include *Aegilops tauschii* and more recent *cis* mutations to *TaALMT1*

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SUMMARY

Acid soils limit plant production worldwide because their high concentrations of soluble aluminium cations (Al^{3+}) inhibit root growth. Major food crops such as wheat (*Triticum aestivum* L.) have evolved mechanisms to resist Al^{3+} toxicity, thus enabling wider distribution. The origins of Al^{3+} resistance in wheat are perplexing because all progenitors of this hexaploid species are reportedly sensitive to Al^{3+} stress. The large genotypic variation for Al^{3+} resistance in wheat is largely controlled by expression of an anion channel, TaALMT1, which releases malate anions from the root apices. A current hypothesis proposes that the malate anions protect this sensitive growth zone by binding to Al^{3+} in the apoplasm. We investigated the evolution of this trait in wheat, and demonstrated that it has multiple independent origins that enhance Al^{3+} resistance by increasing *TaALMT1* expression. One origin is likely to be *Aegilops tauschii* while other origins occurred more recently from a series of *cis* mutations that have generated tandemly repeated elements in the *TaALMT1* promoter. We generated transgenic plants to directly compare these promoter alleles and demonstrate that the tandemly repeated elements act to enhance gene expression. This study provides an example from higher eukaryotes in which perfect tandem repeats are linked with transcriptional regulation and phenotypic change in the context of evolutionary adaptation to a major abiotic stress.

Keywords: evolution, aluminium, resistance, tolerance, *Triticum aestivum, Aegilops tauschii,* acid soil.

INTRODUCTION

Acid soils inhibit root growth and limit plant production worldwide. Forty per cent of total arable land is affected by acidity, and this is especially common in tropical and sub-tropical soils due to the higher rainfall (von Uexküll and Mutert, 1995). Consequently, acid soils threaten sustainable food production in many developing countries in Asia, Africa and Latin America. Plants growing on acid soils are exposed to many stresses, but inhibition of root elongation is primarily caused by the prevalence of soluble aluminium ions (Al^{3+}), which damage the sensitive root apices (Taylor, 1988; Ryan *et al.*, 1993; Horst, 1995; Sivaguru and Horst, 1998; Matsumoto, 2000; Kochian *et al.*, 2004). Shorter roots with impaired function take up less water and nutrients, retarding plant growth.

Many species have evolved mechanisms to resist Al^{3+} stress, either by excluding it from the root and shoot tissues or by safely accommodating the cations taken up by the plant (Taylor, 1988, Taylor, 1991; Kochian *et al.*, 2004; Hiradate *et al.*, 2007). Major food crops such as wheat (*Triticum aestivum*), rice (*Oryza sativa*) and maize (*Zea mays*) display some resistance to Al^{3+} stress, suggesting that this trait is important for their wider cultivation (Aniol and Gustafson, 1984; Nguyen *et al.*, 2003; Ninamango-Carenas *et al.*, 2003). The significant variation between genotypes within species has been exploited by plant breeders to develop more Al^{3+}-resistant crops (Garvin and Carver, 2003).

In many species, Al^{3+} resistance is a multigenic trait. This is apparent based on several lines of evidence, including

segregation analysis and genetic mapping (Hoekenga *et al.*, 2003; Ninamango-Carenas *et al.*, 2003; Matos *et al.*, 2005; Zhou *et al.*, 2007; Hu *et al.*, 2008; Raman *et al.*, 2010), transcriptional analysis (Maron *et al.*, 2008), screening of mutagenized seed (Larsen *et al.*, 1996) and chromosomal deletions or substitutions (Aniol and Gustafson, 1984; Papernik *et al.*, 2001). However, a single locus can dominate the resistance phenotype even in species where multiple resistance loci have been confirmed. One or two loci often explain most of the genotypic variation in populations of wheat (Luo and Dvorak, 1996; Riede and Anderson, 1996; Ma *et al.*, 2005; Raman *et al.*, 2005; Ryan *et al.*, 2009), barley (*Hordeum vulgare*) (Tang *et al.*, 2000; Furukawa *et al.*, 2007; Wang *et al.*, 2007), *Arabidopsis thaliana* (Hoekenga *et al.*, 2003), rye (*Secale cereale*) (Collins *et al.*, 2008) and sorghum (*Sorghum bicolor*) (Magalhaes *et al.*, 2004). Some of the genes controlling resistance in these species, as well as their transcriptional regulators, have been isolated and characterized. For example, a large proportion of the natural genotypic variation for Al^{3+} resistance in several *Poaceae* species, as well as Arabidopsis, is controlled by members of the *ALMT* (aluminium-activated malate transporter) and *MATE* (multidrug and toxic compound extrusion) gene families (Sasaki *et al.*, 2004; Hoekenga *et al.*, 2006; Delhaize *et al.*, 2007; Furukawa *et al.*, 2007; Magalhaes *et al.*, 2007; Wang *et al.*, 2007; Collins *et al.*, 2008; Ryan *et al.*, 2009; Maron *et al.*, 2010). These genes encode transport proteins that reside on the plasma membrane of root cells and release organic anions (malate or citrate) into the apoplasm. The evolution of this resistance mechanism and its surprising prevalence in so many different species have been discussed recently (Magalhaes, 2006; Ryan and Delhaize, 2010). Other resistance genes in rice and Arabidopsis encode transporters from the ATP binding cassette (ABC) family, but their function is less certain. For example, the STAR1 and STAR2 (sensitive to Al rizotoxicity) proteins in rice (Huang *et al.*, 2009) appear to protect the root cells by releasing sugars into the apoplasm, while ALS1 and ALS3 (Al sensitive) proteins in Arabidopsis (Larsen *et al.*, 2005, 2007) may export Al^{3+} to the apoplasm or vacuoles to reduce concentrations in the cytosol. More recently, the C2H2 zinc finger-type transcription factors *STOP1* (sensitive to pH) and *ART1* (aluminium resistance transcription factor 1) from Arabidopsis and rice, respectively, have been shown to regulate the expression not only of *ALMT*, *MATE* and *ABC* genes mentioned above, but also of other genes conferring tolerance to low pH (Iuchi *et al.*, 2007; Yamaji *et al.*, 2009).

Common wheat (*T. aestivum* spp. *aestivum* L.; genome BBAADD) is a hexaploid species that emerged approximately 10 000 years ago from a spontaneous hybridization between tetraploid emmer wheat (*T. turgidum* ssp. *dicoccum*; genome BBAA) and the diploid goatgrass (*Aegilops tauschii*; genome DD). Domestication and distribution of this new species was assisted by mutations that resulted in free-threshing grains and a non-fragile rachis (McFadden and Sears, 1946; Salamini *et al.*, 2002). Although the genotypic variation in Al^{3+} resistance among genotypes of *T. aestivum* is substantial, no resistance has been reported in any of its tetraploid or diploid progenitor species (Slootmaker, 1974; Berzonsky and Kimber, 1986; Cosic *et al.*, 1994). Indeed, *T. turgidum* (emmer and durum wheat) is noted for its sensitivity to Al^{3+} stress (Cosic *et al.*, 1994), a characteristic that is likely to have restricted its wider distribution in agriculture. As a consequence, Al^{3+} resistance is thought to have evolved in hexaploid wheat after hybridization of the tetraploid and diploid progenitors (Garvin and Carver, 2003).

Al^{3+} resistance is a multigenic trait in *T. aestivum* with at least three resistance loci identified on chromosomes 4DL, 4BL and 3BL (Riede and Anderson, 1996; Papernik *et al.*, 2001; Ma *et al.*, 2005; Raman *et al.*, 2005, 2010; Zhou *et al.*, 2007; Cai *et al.*, 2008; Ryan *et al.*, 2009). Genetic analysis of wheat genotypes collected from various regions indicates that Al^{3+} resistance may have originated independently in the USA, Brazil and China (Hu *et al.*, 2008; Raman *et al.*, 2008). Nevertheless, the variation among a wide range of genotypes is dominated by a major genetic locus on chromosome 4DL, where the *TaALMT1* gene is located. The product of the *TaALMT1* gene controls the Al^{3+}-activated efflux of malate (Delhaize *et al.*, 1993; Ryan *et al.*, 1995a; Sasaki *et al.*, 2004; Ma *et al.*, 2005; Raman *et al.*, 2005; 2010). *TaALMT1* encodes an anion channel (Zhang *et al.*, 2008), and constitutive expression of *TaALMT1* in *Xenopus* oocytes, tobacco suspension cells, barley and wheat plants results in Al^{3+}-activated efflux of malate and enhanced Al^{3+} resistance (Delhaize *et al.*, 2004; Sasaki *et al.*, 2004; Pineros *et al.*, 2008; Pereira *et al.*, 2010). Malate is thought to protect the sensitive root apices by binding Al^{3+} in the extracellular spaces to form non-toxic complexes (Delhaize *et al.*, 1993; Ryan *et al.*, 1995b). The variation of malate efflux between genotypes of wheat is unrelated to the coding regions of *TaALMT1* but is significantly correlated with the level of *TaALMT1* expression (Sasaki *et al.*, 2004; Zhang *et al.*, 2008). High levels of expression in Al^{3+}-resistant genotypes is almost always associated with tandemly repeated elements 33–803 bp long located immediately upstream of the *TaALMT1* coding region. These repeats define several alleles of the *TaALMT1* promoter, previously designated as types II–VII (Sasaki *et al.*, 2006; Raman *et al.*, 2008). Al^{3+}-sensitive genotypes, by contrast, have low *TaALMT1* expression, low malate efflux and type I and I^a promoter alleles that lack tandem repeats. The strong association between the tandem repeats and *TaALMT1* expression suggests that they enhance gene expression (Sasaki *et al.*, 2006). However, direct experimental evidence demonstrating any influence of these repeats on *TaALMT1* expression is lacking.

We investigated the evolution of the major Al^{3+} resistance mechanism in *T. aestivum* by examining its progenitor species and by directly testing the effect of the various

promoter alleles on *TaALMT1* expression. We conclude that the resistance mechanism involving malate efflux has multiple independent origins, many of which involve *cis* mutations that increase *TaALMT1* expression.

RESULTS

We performed an extensive screen of Al^{3+} resistance in *Triticum* species to date, focusing on the likely progenitors of hexaploid wheat (Table 1). The germplasm included accessions from the Watkin's collection, which includes a diverse range of accessions of *T. turgidum*, *T monococcum*, *T. urartu*, *T. dicoccoides*, *T. timopheevii* and *T. zhukovskyi* (Miller *et al.*, 2000), as well as accessions of *Ae. tauschii* collected throughout its distribution range (Lagudah *et al.*, 1991). Of the 760 accessions screened, none possessing only the A and/or B genomes displayed significant Al^{3+} resistance (Table 1). However, five of the *Ae. tauschii* (DD) accessions screened did display moderate levels of resistance compared to the resistant hexaploid genotype ET8 (Figure 1a and Table S1). In this paper, we refer to the orthologue of *TaALMT1* in *Ae. tauschii* as *AetALMT1*. Al^{3+} resistance in the five accessions of *Ae. tauschii* was positively correlated with malate efflux from roots and with the level of *AetALMT1* expression (Figure 1), reflecting the major resistance mechanism in hexaploid wheat encoded by *TaALMT1*. To test whether this trait is expressed in a hexaploid plant, we examined the progeny from a hybridization between one of the Al^{3+}-resistant *Ae. tauschii* accessions, AUS18913, and the tetraploid species *T. turgidum* spp. *durum* (cv. Langdon). The resulting synthetic hexaploid plants were found to be more resistant to Al^{3+} and showed a greater Al^{3+}-activated efflux of malate than the tetraploid parent (Figure 2). These results demonstrate that a moderate level of Al^{3+} resistance occurs in *Ae. tauschii*, and this probably predates the appearance of hexaploid wheat.

We investigated the allelic diversity of *AetALMT1* in 55 accessions of *Ae. tauschii*, including the five Al^{3+}-resistant ones, by sequencing three polymorphic regions of the gene (regions 1, 2 and 3), and scoring them with a cleaved amplified polymorphic sequence (CAPS) marker that differentiates the two alleles of the *TaALMT1* coding region (Figure 3a) (Sasaki *et al.*, 2004). In phylogenetic trees generated by analysing the sequence from regions 2 and 3, the five Al^{3+}-resistant accessions clustered together, indicating a common origin (Figure S1a,b). However the resistant accessions were not differentiated from the others in a phylogenetic tree generated from the promoter sequences of region 1 (Figure S1c). All 55 *Ae. tauschii* accessions, including the Al^{3+}-resistant ones, lacked the tandemly repeated elements commonly detected in the *TaALMT1* promoters of Al^{3+}-resistant hexaploid wheat. We then tested an additional 305 accessions of *Ae. tauschii* (Table S2) by PCR, and none possessed tandem repeats upstream of *AetALMT1*. The absence of these repeats in a total of 360 accessions collected throughout the distribution range of this species (Table S2) suggests that they do not occur in *Ae. tauschii*. Instead, most of the *AetALMT1* promoter sequences (region 1) were identical to the type I and I^a alleles of hexaploid

Table 1 The number of diploid and polyploid accessions of *Triticum* species screened for Al^{3+} resistance

Species	Genome	Number of accessions screened	Number of accessions showing resistance
Triticum monococcum	A^mA^m	49	0
Triticum dicoccoides (wild emmer)	BBAA	19	0
Triticum zhukovskyi	AAAABB	4	0
Triticum timopheevii	A^tA^tGG	8	0
Triticum uratu	A^uA^u	20	0
Triticum turgidum (durum)	BBAA	631	0
Aegilops tauschii	DD	29	5

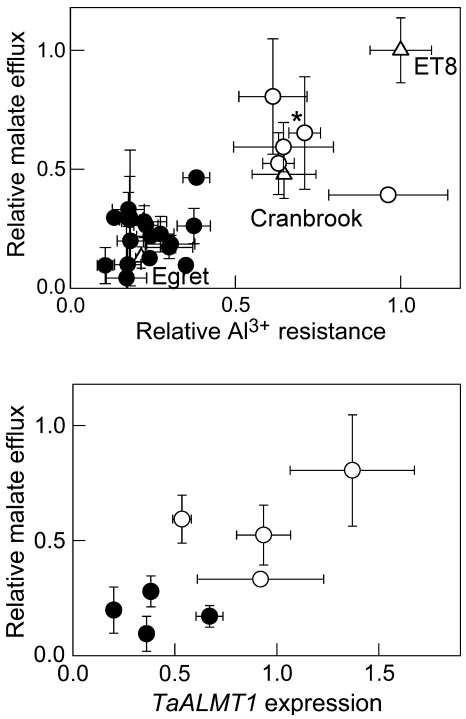

Figure 1. Al^{3+} resistance in *Aegilops tauschii*.
(a) Plot of Al^{3+}resistance versus malate efflux for a range of *Ae. tauschii* accessions. The hexaploid genotypes (Δ) that are also included are ET8 (Al^{3+}-resistant), Cranbrook (moderately Al^{3+}-resistant) and Egret (Al^{3+}-sensitive). Al^{3+} resistance and malate efflux were calculated relative to ET8. The asterisk indicates the accession AUS18913 that was used to generate a synthetic hexaploid in Figure 2.
(b) Plot of malate efflux versus *AetALMT1* expression among Al^{3+}-resistant (open circle) and -sensitive (closed circle) *Ae. tauschii* accessions (r = 0.71). Endogenous transcript levels were measured by real-time quantitative PCR using *PT1* as a reference gene. Data are means ± standard error for three replicates.

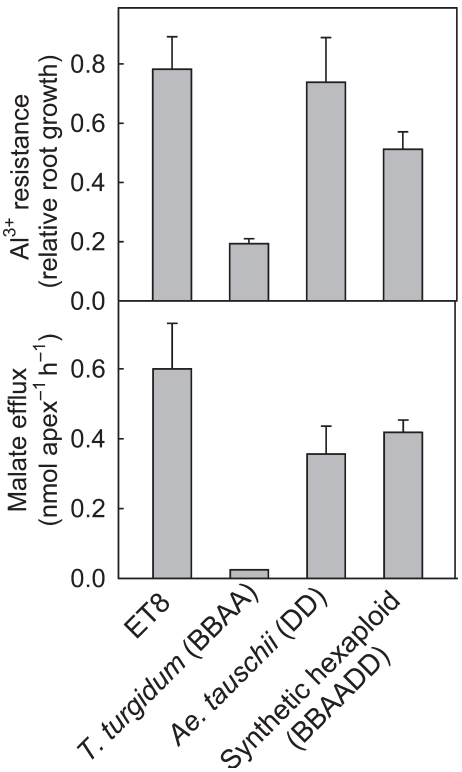

Figure 2. Al^{3+} resistance from an *Ae. tauschii* donor functions in synthetic hexaploid wheat.
Al^{3+} resistance (a) and malate efflux (b) were measured in the tetraploid species *T. turgidum* (durum, cv. Langdon), an Al^{3+}-resistant accession of *Ae. tauschii* (AUS18913) and a synthetic hexaploid generated by crossing these parental lines. The standard Al^{3+}-resistant hexaploid genotype ET8 is also included. Data are means and SE using three replicates for malate efflux and six to eight replicates for resistance.

wheat which, with few exceptions, are associated with Al^{3+} sensitivity (Sasaki *et al.*, 2006). Additional single nucleotide polymorphisms (SNPs) and indels were also identified, and these define additional promoter alleles now designated as I^b, I^c, I^d, I^e, I^f and I^g (Tables S1 and S3).

We tested whether the tandem repeats upstream of *TaALMT1* contribute to Al^{3+} resistance in hexaploid wheat by enhancing *TaALMT1* expression. Two promoter alleles containing mutliple repeats (types V and VI) and two without repeats (types I and I^a) were used to drive GFP expression in transgenic rice (Figure 3b). The type I promoter allele is common in Al^{3+}-sensitive hexaploid wheat, including ES8 (Sasaki *et al.*, 2006). Type I^a differs from type I by eleven SNPs or indels, and is present in Al^{3+}-resistant and Al^{3+}-sensitive accessions of *Ae. tauschii* (Table S1). The type V and VI promoter alleles each contain three perfect tandem repeats in different patterns, and have only been detected in Al^{3+}-resistant hexaploid wheat (Sasaki *et al.*, 2006; Raman *et al.*, 2008). The relative strengths of these promoters were compared by quantifying fluorescence intensity and transcript level in transgenic calli and in the root apices of regenerated plants. Rice transformation is considerably more efficient than wheat transformation, and promoter efficiency was therefore analysed in this heterologous system. In a time-course experiment, the type V promoter generated greater GFP fluorescence in transgenic calli than the type I promoter after 6 days on regeneration medium (Figure 4). When all four promoters were compared directly in the calli under similar conditions, the two promoters with tandem repeats generated significantly more fluorescence and accumulated more transcript than the promoters without repeats

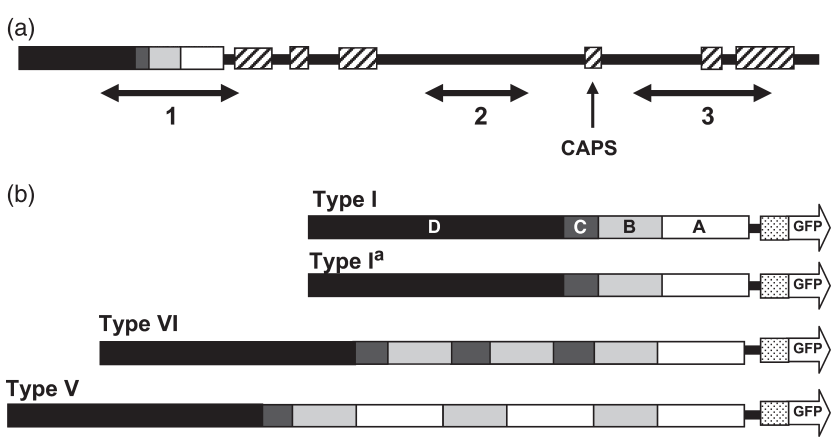

Figure 3. Structure of the coding and promoter regions of *TaALMT1*.
(a) Structure of *TaALMT1* showing exons (hatched boxes) and introns (thin black lines). The black, grey and white boxes upstream of the first exon indicate a type I promoter allele. Numbered horizontal arrows illustrate the three sequenced regions used to assess allelic diversity in 55 accessions of *Ae. tauschii*. The vertical arrow at exon 4 marks the position of the CAPS marker.
(b) The four promoter constructs used to drive GFP (arrows) expression in transgenic rice. The type I promoter allele lacks any repeats of the A, B or C blocks of sequence (172, 108 and 97 bp, respectively), the type I^a promoter allele differs from type I by 11 SNPs or indels, and types V and VI possess three perfect tandem repeats of these blocks in different patterns. D represents the sequence common to all genotypes that is upstream of the region containing the repeats.

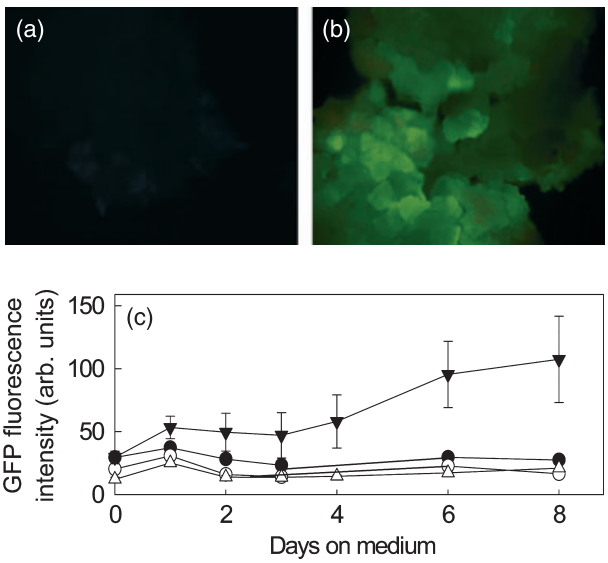

Figure 4. Tandem repeats from the *TaALMT1* promoter enhance gene expression in callus.
GFP expression in transgenic rice callus was driven by two *TaALMT1* promoter alleles shown in Figure 3(b).
(a,b) Fluorescence from rice callus expressing GFP with the type I (a) and type V (b) promoters after 8 days on regeneration medium.
(c) GFP fluorescence driven by the type I (circles) and type V (triangles) promoters through time on callus induction medium (open symbols) and regeneration medium (closed symbols). Data are means ± standard error (n = 7). Each replicate represents an independent transformation event.

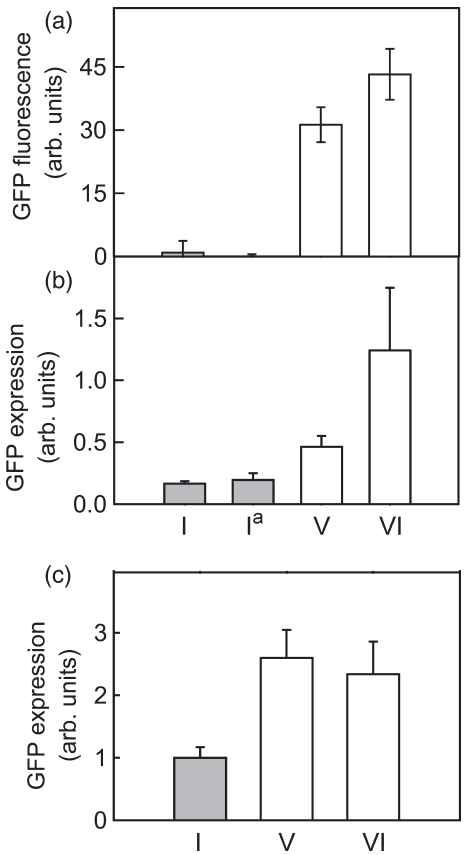

Figure 5. Comparison of promoter alleles driving GFP expression in calli and root apices.
GFP expression driven by the promoter alleles was quantified in rice callus and regenerated plants.
(a) GFP fluorescence in callus (arbitrary units). Data are means ± standard error (n = 12).
(b) GFP transcript levels in callus measured using real-time quantitative PCR and expressed relative to the hygromycin resistance gene *Hpt*. Data are means and standard errors (n = 9–12).
(c) GFP transcript levels in root apices measured using real-time quantitative PCR and expressed relative to the hygromycin resistance gene *Hpt*. Data are means and standard errors (n = 20, 20 and 9 for types I, V and VI respectively). Each replicate represents independent transformation events. For the calli, one-way ANOVA showed that the transcript levels for the type I and I^a promoters (grey bars) are significantly different from those for type V and VI promoters (white bars). For root apices, one-way ANOVA showed that the transcript level for the type I promoter (grey bar) is significantly different from those from the type V and VI promoters (white bars).

(Figure 5a,b). Measurements on plants regenerated from the transgenic callus yielded similar results, although the differences were not as large. Seedlings transformed with the type V and VI promoters accumulated significantly more GFP transcript in the root apices than those transformed with the type I promoter that lacks repeats (Figure 5c). Data from experiments with the type I^a promoter are not included here because insufficient plants were regenerated from the callus. Further experiments expressing these constructs in Al^{3+}-sensitive and -resistant wheat would help to establish whether additional factors affect the level of *TaALMT1* expression between genotypes.

DISCUSSION

This study extends our understanding of the origins and evolution of Al^{3+} resistance in hexaploid wheat in two ways. First, we establish that Al^{3+} resistance occurs in *Ae. tauschii*, the D-genome donor of hexaploid wheat. This provides experimental evidence that a moderate level of Al^{3+} resistance pre-dates the appearance of *T. aestivum*. The mechanism involves malate efflux and enhanced *AetALMT1* expression, but tandemly repeated elements are not present in the promoter of *AetALMT1*. Second, we demonstrate that the tandemly repeated elements upstream of *TaALMT1* contribute directly to the Al^{3+} resistance of hexaploid wheat. We speculate that the repeats contain regulatory elements that enhance *TaALMT1* expression when present in multiple copies.

Allelic variation among accessions of *Ae. tauschii* is generally greater than in the corresponding regions on the D-genome of hexaploid wheat, reflecting the genetic bottleneck that results from polyploidization (Caldwell *et al.*, 2004). However, the tandem repeats upstream of *TaALMT1* represent allelic variation in *T. aestivum* that is absent from *Ae. tauschii.* The simplest explanation for the absence of

these promoter alleles among the 360 *Ae. tauschii* accessions tested here is that they appeared in hexaploid wheat during the last approximately 10 000 years. How these tandem repeats arose is unclear, but the rolling-circle DNA replication machinery used by transposable elements such as *Helitrons* might be involved, as suggested previously for the appearance of repeated regions within the barley *Mlo* gene (Piffanelli *et al.*, 2004), especially as mobile elements can be activated by allopolyploidy in cereals (Feldman and Levy, 2005). This burst of allelic variation in hexaploid wheat is consistent with the genome plasticity that arises from polyploidy, and exemplifies how allopolyploidy can accelerate evolution (Feldman and Levy, 2005; Dubcovsky and Dvorak, 2007). The alternative explanation is that the seven promoter alleles detected in hexaploid wheat are present in *Ae. tauschii* but not in the accessions that we examined. This explanation is less likely as we tested several hundred accessions collected from throughout its distribution range. Furthermore, hexaploid wheat would need to have been generated by at least five independent hybridization events between the tetraploid and diploid progenitors to explain the allelic diversity present in modern genotypes (Sasaki *et al.*, 2006; Raman *et al.*, 2008). Current evidence supports a model in which modern hexaploid wheat is largely derived from two independent hybridization events (Caldwell *et al.*, 2004; Dubcovsky and Dvorak, 2007).

Seven promoter alleles have been identified in *TaALMT1* to date, based on the various patterns of tandemly repeated elements (Sasaki *et al.*, 2006; Raman *et al.*, 2008). Some of these alleles have the same pattern of repeats and differ only in the number of repeats (e.g. types IV and V, and types VI and VII; Sasaki *et al.*, 2006). It is plausible that these are derived from one another as a result of unequal cross-overs during recombination (Delhaize *et al.*, 2007; Raman *et al.*, 2008). However, alleles with distinct patterns of repeats, such as types V and VI examined here, are likely to have arisen independently. As none of the *Ae. tauschii* accessions had *AetALMT1* promoters that contained repeats, the enhanced *AetALMT1* expression measured in the five Al^{3+}-resistant accessions must be controlled by sequences beyond the promoter region analysed here. Most transcriptional enhancers lie within the first kilobase upstream of the transcription start site, but regulatory elements can be more distant or occur elsewhere in the genome (Blackwood and Kadonaga, 1998). 'Cranbrook' is one of very few Al^{3+}-resistant cultivars of hexaploid wheat that lacks tandem repeats upstream of *TaALMT1*. Interestingly, Cranbrook is a moderately resistant cultivar (Figure 1a) that shares identical coding and promoter sequences with the resistant accessions of *Ae. tauschii* and aligns near them in cladograms generated from regions 2 and 3 (Figure S1) (Raman *et al.*, 2008). One explanation for this finding is that Cranbrook inherited its Al^{3+} resistance trait unchanged from the original D-genome donor.

Large-scale comparisons of genomes from related species indicate that coding and non-coding sequences are subject to similar selection pressure (Shabalina *et al.*, 2001). Nevertheless, until recently, the contribution of non-coding DNA to species diversification and evolution has received less attention than changes in protein function. Indeed, the hypothesis that mutations in *cis*-regulatory elements represent a major force for evolutionary change via transcriptional activity was initially controversial (Wray *et al.*, 2003; Pennisi, 2008). An increasing number of studies now support a key role for *cis* mutations in controlling phenotype and speciation (Wray *et al.*, 2003; Wittkopp, 2006; Hanikenne *et al.*, 2008; Chan *et al.*, 2010). For instance, a recent study in baboon populations demonstrated that allelic variation in a *cis*-regulatory region of the *FY* gene influenced gene expression and the susceptibility of individuals to the *Hepatocystis* pathogen (Tung *et al.*, 2009). Fewer examples of *cis* mutations involving tandemly repeated elements have been reported. One study in yeast established a link between transcriptional activity and tandem repeats involving small A/T-rich repeats. Because these repeats were unstable, they allowed rapid changes in gene expression to occur, which could then be subject to selection (Vinces *et al.*, 2009). Larger repeats have also been implicated in gene regulation. In certain strains of *Penicllium digitatum*, five tandemly arranged copies of a 126 bp transcriptional enhancer increase expression of cytochrome P450 sterol 14α-demethylase and provide resistance to fungicides containing demethylation inhibitors (Hamamoto *et al.*, 2000). The promoter of the human prodynorphin gene contains an allelic variation involving tandem repeats of a 68 bp region. This region contains a putative binding site for an AP-1 transcription factor, and the multiple repeats enhance gene transcription (Zimprich *et al.*, 2000; Rockman *et al.*, 2005). A recent example from plants involves a series of 23 bp repeats that increase the expression of a gene (*MYB10*) encoding an anthocyanin-regulating transcription factor in apples (*Malus* x *domestica*) (Espley *et al.*, 2009). The present study is an example from higher plants that links large tandemly repeated elements (200–300 bp) with phenotypic changes that facilitate adaptation to a major abiotic stress.

Aegilops tauschii is divided into the subspecies (ssp.) *tauschii* and *strangulata*. The original D-genome progenitors of hexaploid wheat most likely come from the *strangulata* gene pool (Dvorak *et al.*, 1998; Ogbonnaya *et al.*, 2005). *Ae. tauschii* has several varieties based on morphology and early classification systems included three of these in ssp. *tauschii* (*anthera*, *meyeri* and *typica*) and one in ssp. *strangulata* (*strangulata*). More recent genetic analyses indicate that, in some geographical regions, ssp. *strangulata* also encompasses the *meyeri* and *typica* varieties (Dvorak *et al.*, 1998). Two of the five

resistant accessions identified in the present study show *strangulata* traits (AUS110802 and AUS21711), two show *meyeri* traits (AUS18913 and AUS110812) and one is intermediate (AUS110668) (Table S1). All of the resistant accessions were collected from Rasht and Gorgan in northern Iran and from regions in the southern Caucasus, regions that overlap with the likely origin of hexaploid wheat (Dvorak *et al.*, 1998). Interestingly, the frequency of the CAPS marker scores in the *Ae. tauschii* accessions varied with geographical locations. This marker was initially developed to distinguish the major coding alleles in hexaploid wheat (Sasaki *et al.*, 2004; Raman *et al.*, 2005, 2008). Both CAPS alleles are common in accessions collected from the southern and western shores of the Caspian Sea, the likely origin of hexaploid wheat, but the *TaALMT1-2* (*AetALMT1-2*) allele occurs less frequently at locations further from this area (e.g. Armenia), and is absent in accessions collected from Pakistan, Afghanistan and Turkey. These findings are consistent with previous analyses of allelic distributions in *Ae. tauschii* that show greater diversity along the southern reaches of the Caspian sea (Dvorak *et al.*, 1998; Lelley *et al.*, 2000).

As Al^{3+}-sensitive genotypes of *Ae. taushchii* and *T. aestivum* express the same functional protein as resistant genotypes, but at lower levels, it is interesting to consider how the gene has persisted in Al^{3+}-sensitive genotypes. One explanation is that even low expression of this gene conferred some benefit under the selection pressure of mildly acidic soils. The distribution of acid soils in the regions in which *Ae. tauschii* evolved over the past 2–4 million years is not known, but some soils adjacent to the Black Sea are known to be acidic today (Adiloglu and Adiloglu, 2003). Therefore, it is plausible that soils in North-Eastern Turkey, perhaps extending up to Georgia, provided sufficient selection pressure to fix the Al^{3+} resistance trait in specific accessions of *Ae. tauschii*. Further selection pressure will have been exerted as the distribution of hexaploid wheat was extended by humans into regions in which acid soils are more prevalent. An alternative explanation is that *AetALMT1* provided other benefits that helped it persist in the absence of direct selection pressure from acid soils (Ryan and Delhaize, 2010).

We provide evidence that a major mechanism for Al^{3+} resistance in hexaploid wheat has multiple independent origins that function by enhancing the expression of *TaALMT1*. One origin is *Ae. tauschii* while others probably arose since the appearance of hexaploid wheat and rely on *cis* mutations in the *TaALMT1* promoter. These mutations were able to increase the Al^{3+} resistance above the levels present in *Ae. tauschii*. These promoter alleles, together with other minor resistance loci (Cai *et al.*, 2008; Ryan *et al.*, 2009), help to explain the variation of Al^{3+} resistance evident among modern wheat cultivars.

EXPERIMENTAL PROCEDURES

Plant material

Seed was obtained from CSIRO Plant Industry (Canberra, Australia), the Industry and Investment NSW (Wagga Wagga, Australia) and the Australian Winter Cereal Collection of the Industry and Investment NSW (Tamworth, Australia). The 360 *Ae. tauschii* accessions studied here were collected throughout their distribution range in eastern Europe, the Middle East and Western Asia. Geographic origins for each are provided in Tables S1 and S2. Although the *TaALMT1* genes of hexaploid wheat are derived from *Ae. tauschii*, we use the terminology *AetALMT1* to distinguish the *Ae. tauschii* genes from those of hexaploid wheat and from the *AtALMT1* gene of *Arabidopsis thaliana* (Hoekenga *et al.*, 2006).

Growth conditions and measurement of Al^{3+} resistance

Seeds were planted in 20 L of aerated nutrient solution (Ryan *et al.*, 1995b). Al^{3+} resistance was estimated on the basis of relative root length (RRL) and haematoxylin staining. RRL was estimated by measuring the length of the longest root on each seedling before and after 4 days of growth in control solution or 10 μM $AlCl_3$. RRL was calculated as (net root growth under Al treatment/net root growth in control solution) × 100. Al^{3+} resistance for the *Ae. tauschii* accessions is presented relative to ET8, a standard Al^{3+}-resistant hexaploid that was included in each trial to account for variation between experiments.

Malate efflux

Malate efflux was measured from excised root apices in 50 μM $AlCl_3$ as described previously (Ryan *et al.*, 1995b). Malate efflux from each accession of *Ae. tauschii* is presented relative to that for a standard hexaploid genotype (ET8) that was included in each trial to account for variation between experiments.

Gene expression

Relative expression of the *ALMT1* genes was analysed by real-time quantitative PCR on a Rotor-Gene (Corbett Research, http://www.corbettlifescience.com) using comparative quantification with Rotor-Gene software version 6.1. cDNA prepared from 5–10 root apices (4 mm) using a Qiagen RNeasy kit (http://www.qiagen.com/) was amplified using the forward and reverse primers 5′-CGTGAAAGCAGCGGAAAGCC-3′ and 5′-CCCT CGACTCACGGTACTAACAACG-3′, respectively. The two internal reference genes used were the phosphate transporter gene *PT1* (Genbank accession number AF110180), which was amplified using forward and reverse primers 5′-GAAGGACATCTTCACGGCGATC-3′ and 5′-CACGGCCATGAAGAAGAAGC-3′, respectively, and the glyceraldehyde-3-phosphate dehydrogenase gene (Genbank accession number EF592180) using the forward and reverse primers 5′-TGTTGAGGGTTTGATGACCAC-3′ and 5′-TCAGACTCCTCCTTGATAGC-3′, respectively. The reference genes yielded similar results, and data are presented for *PT1* only.

Allelic diversity in *Ae. tauschii*

Allelic diversity of *AetALMT1* was first examined in 55 accessions of *Ae. tauschii* (Tables S1 and S3) by sequencing three regions of the gene (regions 1, 2 and 3) and scoring the accession for a cleaved amplified polymorphic sequence (CAPS) marker (Figure 3a). Region 1 spans approximately 610 bp of the promoter, encompassing the tandem repeats present in Al^{3+}-resistant hexaploid genotypes. Amplification was achieved using the forward and reverse primers 5′-GCTCCTACCACTATGGTTGCG-3′ and 5′-CAGGCCGACTTTGA-

GCGAG-3′. Region 2 spans 519 bp in intron 3 and was amplified using the forward and reverse primers 5′-GACCAGGACCA GTTGCGCAC-3′ and 5′-GCCAGCGGACCTAAGGTTGC-3′. Region 3, a 634 bp fragment straddling intron 4 to exon 6, was amplified using the forward and reverse primers 5′-GGATACAGAGGGTGCGGGT-TAC-3′ and 5′-AATGCAAGCTCATTTCGCCAC-3′. The CAPS marker was used as described previously (Sasaki *et al.*, 2004). This marker targets a SNP in exon 4 of *TaALMT1* that distinguishes the two major alleles in coding the coding region called *TaALMT1-1* and *TaALMT1-2*. The presence or absence of tandem repeats in the promoter of 305 additional accessions (Table S2) was assessed on the basis of the lengths of PCR products amplified using primers 5′-GCTCCTACCACTATGGTTGCG-3′ (forward) and 5′-CTTGAGCTTG-CATTGCATCTG-3′ (reverse). The promoter allele previously designated type I′ by Sasaki *et al.* (2006) is named type I^a in this study to allow for the additional allele types I^b–I^g detected in *Ae. tauschii* (Table S1).

Promoter analyses

Four promoter alleles of *TaALMT1* and *AetALMT1* representing types I, I^a, V and VI (Genbank accession numbers AB243162.1, AB243170.1, AB243166.1 and AB243167.1) were amplified using primers 5′-GATGAGGCGCGCCGGCAGATGCAATGCAAGCTC-3′ and 5′-CACTAGAGCGGCCGCTTAATTAATGAGCTTCCATGTCTATTG-3′ (restriction sites for *Pac*I and *Asc*I are underlined). Digested PCR products were ligated into the HvPht1 binary expression cassette to drive GFP expression (Schunmann *et al.*, 2004). Rice was transformed with *Agrobacterium* using the procedure described by Toki *et al.* (2006). Expression was determined by quantifying GFP fluorescence and by real-time quantitative PCR. Each replicate represents an independently transformed callus or primary transgenic (T_0) plant regenerated from the callus. GFP fluorescence in calli was quantified using a fluorescence microscope (Leica MZFLIII, http://www.leica.com/) and AnalySIS Five software (Olympus Soft Imaging Systems, http://www.olympus-global.com/). The green wavelengths in each image were first separated from the others. They were then converted to a grey scale so their intensity could be measured and compared with the intensities in other figures. Regions to be analysed were selected using a freehand polygon tool, and the mean grey scale value of the selection was then determined. A 'blank' value obtained from tissue transformed with a vector lacking the GFP gene was subtracted from the raw data. The transcript level in the calli and root apices (5 mm) was analysed by real-time quantitative PCR as described by Ryan *et al.* (2009). For GFP, the forward and reverse primers were 5′-GGTCACGAACTCCAGCAGGA-3′ and 5′-AGAACGGCATCAAGGTGAAC-3′, respectively. The reference for these experiments was *Hpt*, the antibiotic selection gene included in the binary vector that confers resistance to hygromycin. Use of *Hpt* as the reference gene enabled us to account for the likelihood that not all cells in the callus were transformed and for variations in gene expression as a consequence of where the transgenes were inserted into the genome. Forward and reverse primers for the reference gene were 5′-TCGGTTTCCACTATCGGCGAGTACTTC-3′ and 5′-ATCTTCTTCTGGAGGCCGTGGTTG-3′, respectively. Statistical analysis of GFP expression in transgenic calli and regenerated plants was performed using one-way analysis of variance.

Phylogenetic analysis

Phylogenetic analyses were performed using Phylip-3.68 (Felsenstein, 1989). Base changes along the tree were analysed using the assumptions outlined previously (Fitch, 1971) and the sites were unweighted. Additional details are presented in the online legend to Figure S1.

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SUPPORTING INFORMATION

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

Figure S1. Phylogenetic analyses of *AetALMT1* sequences from various *Ae tauschii* accessions.

Table S1. *Aegilops tauschii* accessions assessed for allelic variation in *AetALMT1*.

Table S2. Additional *Aegilops tauschii* accessions assessed for promoter alleles.

Table S3. Genbank accession numbers for the three sequenced regions of *AetALMT1*.

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