

# Introgression of genes from bread wheat enhances the aluminium tolerance of durum wheat

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## Abstract

**Key message** The aluminium tolerance of durum wheat was markedly enhanced by introgression of *TaALMT1* and *TaMATE1B* from bread wheat. In contrast to bread wheat, *TaMATE1B* conferred greater aluminium tolerance than *TaALMT1*.

**Abstract** Durum wheat (tetraploid AABB, *Triticum turgidum*) is a species that grows poorly on acid soils due to its sensitivity of Al<sup>3+</sup>. By contrast, bread wheat (hexaploid AABBDD, *T. aestivum*) shows a large variation in Al<sup>3+</sup> tolerance which can be attributed to a major gene (*TaALMT1*) located on chromosome 4D as well as to other genes of minor effect such as *TaMATE1B*. Genotypic variation for Al<sup>3+</sup> tolerance in durum germplasm is small and the introgression of genes from bread wheat is one option for enhancing the ability of durum wheat to grow on acid soils. Introgression of a large fragment of the 4D chromosome previously increased the Al<sup>3+</sup> tolerance of durum wheat

demonstrating the viability of transferring the *TaALMT1* gene to durum wheat to increase its Al<sup>3+</sup> tolerance. Here, we used a *ph1* (pairing homoeologous) mutant of durum wheat to introgress a small fragment of the 4D chromosome harboring the *TaALMT1* gene. The size of the 4D chromosomal fragment introgressed into durum wheat was estimated by markers, fluorescence in situ hybridisation and real-time quantitative PCR. In a parallel strategy, we introgressed *TaMATE1B* from bread wheat into durum wheat using conventional crosses. Both genes separately increased the Al<sup>3+</sup> tolerance of durum wheat in both hydroponics and soil cultures. In contrast to bread wheat, the *TaMATE1B* gene was more effective than *TaALMT1* in increasing the Al<sup>3+</sup> tolerance of durum wheat grown on acid soil.

## Introduction

Durum wheat (*Triticum turgidum* var durum) has a genetic makeup comprising the A and B genomes ( $2n = 4x = 28$ ; AABB), but lacks the D-genome of bread wheat (*T. aestivum*) where major genes for aluminium and salt tolerances are located. Durum wheat is grown in many regions of the

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world for the production of pasta and bread (Sissons 2008). Drought and temperature extremes have been identified as limiting factors for durum production in many regions of the world (Habash et al. 2010). Extensive root systems are clearly attributes that enable plants to take up water from depth, improving a plant's ability to tolerate drought. Durum as a species is particularly sensitive of acid soils which can be attributed to its sensitivity of  $Al^{3+}$ . Acid soils typically liberate poorly soluble forms of Al into toxic  $Al^{3+}$  that inhibits growth by causing roots to become stunted and thickened (Delhaize and Ryan 1995). Acid soils are widespread globally (von Uexküll and Mutert 1995) and can reduce yields or limit the area that an  $Al^{3+}$ -sensitive species such as durum wheat is grown. Shortened roots reduce the ability of plants to take up water and nutrients resulting in reduced grain yields. Screens of durum wheat genotypes showed that this species is  $Al^{3+}$ -sensitive (Cosic et al. 1994; Moustakas et al. 1992) and an extensive screen of over 600 genotypes failed to identify any useful levels of tolerance (Ryan et al. 2010). Reports of  $Al^{3+}$ -tolerant durum genotypes have either proven to be due to contaminating  $Al^{3+}$ -tolerant varieties of hexaploid wheat (Han et al. 2014) or are yet to be confirmed.

In contrast to durum wheat, hexaploid wheat ( $2n = 6x = 42$ ; AABBDD, bread wheat) shows a large variation in  $Al^{3+}$  tolerance largely due to allelic variation in the major  $Al^{3+}$  tolerance gene *TaALMT1* located on chromosome 4D (Delhaize et al. 2012). *TaALMT1* encodes a transport protein located on the plasma membrane that has been characterized as a malate-permeable anion channel that is activated by  $Al^{3+}$ . The malate secreted by  $Al^{3+}$  tolerant wheat is thought to protect roots by chelating the  $Al^{3+}$  in the apoplast and rendering it non-toxic.

When limited genetic variation for a trait exists within durum germplasm, the use of related species to donate genes is an option for crop improvement. For instance, the introgression of genes encoding gluten proteins from the D-genome of hexaploid wheat is a strategy for improving durum for bread making, while maintaining pasta quality (Sissons et al. 2014). Similarly, the  $Al^{3+}$  and  $Na^{+}$  tolerances of durum wheat have been improved by introgression of fragments of chromosome 4D (Dubcovsky et al. 1996; Dvorak and Gorham 1992; Dvorak et al. 1994; Han et al. 2014; Luo et al. 1996). The starting germplasm for the introgression of 4D-chromosomal fragments was a substitution line of durum where the 4D chromosome of hexaploid wheat replaced the 4B chromosome of durum wheat. Subsequently, the use of the *ph1c* mutant enabled the transfer of chromosomal fragments with the desired gene into the durum genetic background (Dvorak and Gorham 1992; Luo et al. 1996). In the absence of the *ph1* locus, a spontaneous translocation of a fragment from chromosome 4D to chromosome 4B was identified and used to develop durum

lines that were both  $Al^{3+}$ - and  $Na^{+}$ -tolerant while maintaining the semi-dwarf habit of an elite durum cultivar (Han et al. 2014). The translocation allowed both *TaALMT1* and *KNa1* on chromosome arm 4DL to be transferred to durum wheat while maintaining the semi-dwarf allele of *Rht-B1* on chromosome arm 4BS of durum.

A mutant *ph1* locus has enabled efficient transfer of relatively small chromosomal regions from alien chromosomes to homeologous chromosomes of wheat. The *ph1* mutants have been used by breeders to not only allow the transfer of desirable genes into wheat germplasm but also to avoid detrimental phenotypes due to linkage drag of undesirable genes (Able and Atienza 2014; Ayala-Navarrete et al. 2013; Marais et al. 2010; Niu et al. 2014; Qi et al. 2007). The introgression of a 4D chromosomal fragment into durum wheat did not show any obvious detrimental phenotypes apart from reduced grain size, which was presumably caused by genes other than *TaALMT1* located on the introgressed fragment (Han et al. 2014). To avoid other subtle effects on grain quality or yield, the *ph1c* mutation of durum wheat could be used to reduce the size of 4D chromosomal fragment introgressed into durum while maintaining  $Al^{3+}$  tolerance. Alternatively, if suitable genes are located on either the A or B genomes of hexaploid wheat or related diploid species, they can be introgressed into durum wheat without the need of the *ph1c* mutation. For example, the *Nax* loci from *T. monococcum* ( $2n = 2x = 14$ ; AA) were introgressed into durum germplasm to enhance its ability to exclude and tolerate  $Na^{+}$  (James et al. 2012). Bread wheat and durum wheat can be crossed to generate pentaploid progeny and these progeny backcrossed to durum wheat to quickly eliminate the D-genome and to introgress the desired genes into elite cultivars (Ceoloni et al. 1996).

While *TaALMT1* is the major gene for  $Al^{3+}$  tolerance in hexaploid wheat, other genes of minor effect have been identified on the A and B genomes (Cai et al. 2008; Ma et al. 2006; Navakode et al. 2014; Navakode et al. 2009; Raman et al. 2010; Ryan et al. 2009; Zhou et al. 2007). The only  $Al^{3+}$  tolerance gene other than *TaALMT1* that has been isolated from hexaploid wheat and its function characterized is *TaMATE1B* located on chromosome 4B (Ryan et al. 2009; Tovkach et al. 2013). *TaMATE1B* also encodes a transport protein but the protein belongs to a family unrelated to *TaALMT1*. In contrast to the  $Al^{3+}$ -activated efflux of malate conferred by *TaALMT1*, *TaMATE1B* confers constitutive citrate efflux from root apices and like malate, citrate is thought to bind  $Al^{3+}$  to form a non-toxic complex. Although *TaALMT1* generates a stronger  $Al^{3+}$  tolerance phenotype than *TaMATE1B* in bread wheat, the location of *TaMATE1B* on the B-genome indicates that it should be relatively simple to introgress this gene into durum wheat since the B-genome is shared by both species. Here, we

describe the introgression and characterization of a small fragment of chromosome 4D containing *TaALMT1* into a durum genetic background as well as the development of durum lines where *TaMATE1B* has been introgressed from bread wheat. We describe crosses that avoid hybrid necrosis when durum wheat was crossed to bread wheat and compare the effectiveness of the two genes to confer  $Al^{3+}$  tolerance in a durum genetic background in both hydroponic and soil cultures.

## Materials and methods

### Germplasm

A durum line with the Langdon genetic background where the 4B chromosome is substituted with the 4D chromosome of hexaploid wheat (Joppa and Williams 1988) was crossed with a homozygous *ph1c* mutant (cv Cappelli) as the female parent. The 4D(4B) substitution line possesses an  $Al^{3+}$ -tolerant allele of *TaALMT1* derived from Chinese Spring (Sasaki et al. 2004). The *TaALMT1* allele of Chinese Spring has a large duplication in its promoter region associated with  $Al^{3+}$  tolerance. The progeny were assessed for  $Al^{3+}$  tolerance by hydroponics using previously described methods (Han et al. 2014) to confirm that the crosses were successful. The  $F_1$  plants as pollen donors were backcrossed to the homozygous *ph1c* mutant to generate 40 grains which were germinated and screened for  $Al^{3+}$  tolerance. Two plants were  $Al^{3+}$  tolerant with the remainder found to be  $Al^{3+}$  sensitive. The  $Al^{3+}$ -tolerant plants were screened for the *ph1c* mutation by PCR (Wang et al. 2002). We established that one  $Al^{3+}$ -tolerant plant was homozygous for the *ph1c* mutation. Since the plant was  $Al^{3+}$  tolerant and homozygous for *ph1c*, it would have been heterozygous for the 4D chromosome and progeny suitable to screen for recombinations between chromosomes 4D and 4B. To generate sufficient grains to screen by PCR for identifying recombinant chromosomes,  $Al^{3+}$ -tolerant progeny of the single plant was grown to maturity and the grain collected. This not only generated additional grain for screening but also enhanced the likelihood that further recombinations could occur. Collecting progeny from heterozygous  $Al^{3+}$ -tolerant plants that were homozygous for *ph1c* was repeated a number of times. At each generation, progeny seedlings were screened for  $Al^{3+}$  tolerance and only those progeny batches that were segregating were analyzed by PCR. If a batch of progeny was segregating for  $Al^{3+}$  tolerance, then the parental plant must have been heterozygous for the 4D-chromosomal fragment. Therefore, recombinations between the 4D and 4B chromosomes could have occurred. If a batch of progeny was all  $Al^{3+}$  tolerant, then the parental plant would have been homozygous for the 4D

chromosomal fragment and would not have enabled recombination with the 4B chromosome so these populations were discarded. After screening the batches of progeny, DNA from  $Al^{3+}$ -tolerant seedlings was extracted only from populations that were segregating for  $Al^{3+}$  tolerance. DNA samples were analyzed by PCR and seedlings at each generation that possessed the smallest recombination based on markers proximal and distal to *TaALMT1* were identified. These seedlings were allowed to self-fertilize to generate further potential recombinations in subsequent populations. After four rounds of selfing, the seedling with *TaALMT1* and with the smallest amount of recombined 4D chromosome as identified with markers was crossed to the elite Australian durum cv Jandaroi. The progeny were backcrossed twice more to cv Jandaroi to generate lines that possessed the small 4D introgression (SF *TaALMT1* line) and sister lines that lacked the introgression.

An allele of *TaMATE1B* conferring  $Al^{3+}$  tolerance to bread wheat was introgressed into durum wheat. The *TaMATE1B* gene is located on chromosome 4B and in theory could already be present in some durum genotypes. However, to-date no  $Al^{3+}$ -tolerant allele of *TaMATE1B* has been identified in durum germplasm. We crossed durum wheat with hexaploid wheat lines including a line with the cv Westonia background that possessed the  $Al^{3+}$ -tolerant allele of *TaMATE1B* donated by cv Carazinho. When either Carazinho or Westonia was crossed to several Australian durum cultivars (Jandaroi, Tamaroi and Bellaroi), all  $F_1$  progeny showed symptoms of hybrid necrosis and subsequently died before setting seed. Hybrid necrosis is a syndrome that is commonly encountered in crosses between bread and tetraploid wheats. It is controlled by interaction of complementary genes *Ne1* and *Ne2* (Tsunewaki 1992). To develop successful crosses between cv Carazinho or cv Westonia and durum germplasm, we crossed both hexaploid genotypes to each of 10 durum cultivars selected at random and originating from diverse countries to establish if any could act as a “bridge” between hexaploid wheat and elite Australian durum cultivars. The durum cultivars used in the crosses to hexaploid wheat included Bouffarick, Forex, Durex, Icaro, Greece 14, Langdon, Kalka, Arrivato, Leeds Dwarf and Castelporziano. The progeny were grown and of the crosses, the cultivars Greece 14 and Leeds Dwarf were the only durum parents to produce  $F_1$  progeny that did not develop hybrid necrosis. The  $F_1$  progeny of the cross between Leeds Dwarf and the cv Westonia line that possessed *TaMATE1B* were crossed to cv Jandaroi. The resulting progeny was crossed again to cv Jandaroi to develop homozygous *TaMATE1B* lines that had been effectively crossed three times to durum wheat including a backcross to cv Jandaroi. The availability of a co-dominant molecular marker for *TaMATE1B* (Tovkach et al. 2013) facilitated the screening of progeny and allowed the rapid development

of lines homozygous for the  $Al^{3+}$ -tolerant allele of *TaMATE1B* in a durum background. The availability of a dominant marker for *TaALMT1* (Sasaki et al. 2004) allowed us to verify that the lines only possessed the *TaMATE1B* gene and that *TaALMT1* had not been introgressed by a spontaneous translocation.

Methods previously described for short-term hydroponic and soil cultures were used to assess the  $Al^{3+}$  tolerance of the durum germplasm (Han et al. 2014). For soil experiments, we used soil that had been amended with various amounts of lime to generate a range of  $Al^{3+}$  toxicities with 4 g of lime per kg soil totally detoxifying the  $Al^{3+}$  toxicity. Pots (1.3 kg) were prepared and plants grown as described previously (Han et al. 2014). Plants were harvested after six (*SF TaALMT1* lines) or seven (*TaMATE1B* lines) days, roots washed and lengths measured by scanning and WinRhizo Pro V software (2002). For hydroponic experiments, seedlings were grown in separate 11 L containers for each  $Al^{3+}$  treatment. Within a container, individual seedlings from each line were randomized with 7–10 replicate seedlings planted for each line. For experiments that used soil, pots were set up in a randomized block arrangement with 5 or 6 replicate blocks used in each experiment. Data were analyzed by one-way ANOVA, two-way ANOVA or Student's *t* test as specified in figure legends using SigmaPlot v 12.3. Where required to ensure normal distributions, data were  $\log_{10}$  transformed prior to statistical analysis.

## Markers

Various markers located on chromosome 4D of hexaploid wheat were sourced from the Graingenes database (<http://wheat.pw.usda.gov/cgi-bin/graingenes>). Markers for *TaALMT1* and *TaMATE1B* were implemented as described previously (Sasaki et al. 2004; Tovkach et al. 2013). A deletion series of chromosome 4D (Mickelson-Young et al. 1995) was used to verify the location of markers.

## Fluorescence in situ hybridisations (FISH)

FISH analyses were undertaken using previously described methods (Zhang et al. 2004).

Bacterial artificial chromosomes 676D4 and 9M13 contain dispersed repetitive sequences specifically derived from the A- and D-genomes, respectively (Zhang et al. 2004). One microgram each of 676D4 and 9M13 DNA was labeled with tetramethyl-rhodamine-5-dUTP (Roche Diagnostics Australia P/L, NSW) using nick-translation and biotin-14-dATP (BioNick Labeling System, Invitrogen Australia P/L, Vic.), respectively. The hybridisation and post-hybridisation washes were conducted as described in Zhang et al. (2004). The biotin-labeled probe was detected with fluorescein-avidin DN (Vector Laboratories,

Burlingame, CA, USA). Chromosome preparations were analyzed with an epifluorescence Zeiss Axio Imager microscope (Carl Zeiss Microimaging GmbH, Göttingen, Germany). Images were captured with a Retiga EXi CCD (charge-coupled device) camera (QImaging, Surrey, BC, Canada) operated with Image-Pro Plus 7.0 software (Media Cybernetics Inc., Bethesda, MD, USA) and processed with Photoshop version 8.0 software (Adobe Systems, San Jose, CA, USA).

## Real-time PCR

To rapidly estimate the amount of D-genome recombined into durum lines, we used real-time quantified PCR (RT-qPCR). The Dgas44 sequence is a member of a family of repeated elements (Dgas) specific to the D-genome (McNeil et al. 1994) and primers were generated as described previously (Han et al. 2014). Primers (CTGATCTTCTGTGAA-GGGT forward primer; TGATAGA AACTCGTAATGGGC reverse primer) that amplified 28S ribosomal RNA genes were used as reference genes for RT-qPCR undertaken as described previously (Delhaize et al. 2004). Ribosomal RNA genes are repeated within genomes and are sequences common to all the A, B and D-genomes. Expressing the amount of Dgas DNA as a ratio of the genes encoding 28S ribosomal RNA would provide a measure for the relative abundance of Dgas between different lines and hence could be used to estimate the relative amount of the D-genome introgressed into the durum background. To test the method, we used DNA (approximately 10 ng) from lines that possessed differing amounts of D-genome ranging from hexaploid wheat (cv Chinese Spring; full complement of the D-genome), a 4D(4B) durum substitution line (possesses only the 4D chromosome), a durum line where a large fragment of chromosome 4D (LF line) had been introgressed (Han et al. 2014) and a durum line that lacked the D-genome altogether.

## Results

### Introgression of *TaALMT1* into durum wheat

We verified the published locations of markers on chromosome 4D (<http://wheat.pw.usda.gov/cgi-bin/graingenes>) using a 4D-chromosomal deletion series and mapped a homolog of *TaMATE1B* to a particular bin on the long arm of chromosome 4D (Fig. 1a). The *TaALMT1* gene was previously mapped to a bin delineated by break-points 4DL-12 and 4DL-14 in hexaploid wheat with marker *wmc331* identified as closely linked. Use of the *ph1c* mutation resulted in progressively smaller introgressions of chromosome 4D that possessed *TaALMT1* and a seedling was identified

where the only marker from chromosome 4D that remained linked to *TaALMT1* was *wmc331* (Fig. 1b). Marker *wmc331* is located in the same bin as *TaALMT1* and is in a position distal to *TaALMT1* on chromosome 4D. The closest marker proximal to *TaALMT1* had been recombined. Chromosome 4B of this line that had the smallest fragment of D-genome introgressed (SF line) is shown in Fig. 1b.

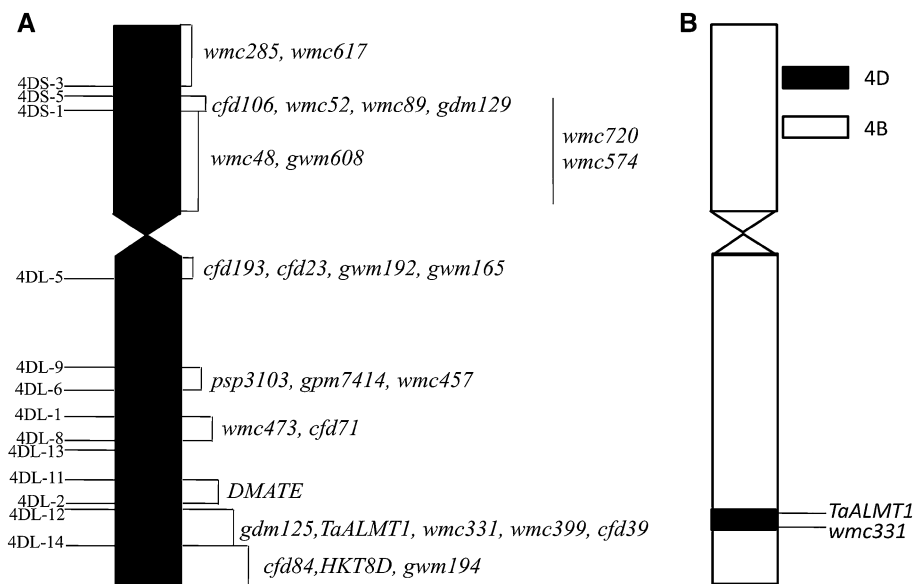
To assess the relative size of 4D chromosomal introgressions into a durum genetic background, we used a method based on RT-qPCR. This allowed rapid estimation of the amount of D-genome introgressed and was tested using DNA derived from wheat lines with varying amounts of D-genome. The cultivar Chinese Spring showed the largest relative amount of D-genome consistent with it having a full complement of the D-genome chromosomes (Fig. 2). The line identified with markers (Fig. 1) as having the smallest amount of 4D chromosome introgressed into a durum background (SF line) had the smallest amount of DNA estimated by RT-qPCR and could not be distinguished from the durum cv Jandaroi (Fig. 2).

The amount of D chromosome was assessed visually by FISH using genome-specific probes. Figure 3a shows a pair of complete chromosomes in substitution line 4D(4B) as being derived from the D-genome, while a

chromosome arm of the LF line hybridized with the probe for the D-genome (Fig. 3b). Only a relatively small signal of D-genome was present in the arm of a chromosome for the SF line (Fig. 3c) and this line had the smallest fragment of chromosome 4D introgressed as identified by markers (Fig. 1) and RT-qPCR (Fig. 2).

### Introgression of *TaMATE1B* into durum wheat

*TaMATE1B* is located on chromosome 4B and durum wheat possesses the  $Al^{3+}$  sensitive allele (lacks citrate efflux). A co-dominant marker was used to track the introgression of *TaMATE1B* into durum. Because the hexaploid donor also possessed a tolerant allele of *TaALMT1*, we needed to verify that any  $Al^{3+}$  tolerance was caused solely by *TaMATE1B* and not due to a spontaneous translocation of *TaALMT1* from chromosome 4D to either the A- or B-genomes of durum wheat. The marker for *TaALMT1* indicated that this gene had not been introgressed into the durum lines and the RT-qPCR assay also showed little or no D-genome had been introgressed into the durum line (Supplementary Fig. S1). Based on these findings, we were confident that *TaMATE1B* was responsible for the observed increases in  $Al^{3+}$  tolerance.

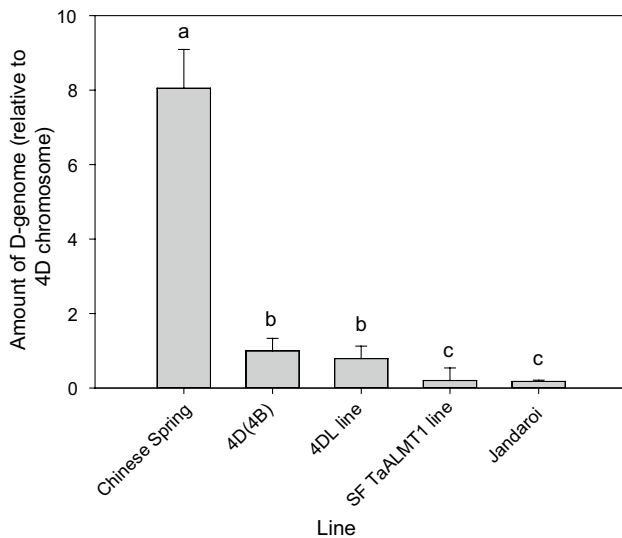


**Fig. 1** Introgression of a fragment of chromosome 4D containing *TaALMT1* into chromosome 4B of durum wheat. **a** Markers on chromosome 4D used to analyze DNA samples by PCR. The markers were used on deletion lines of chromosome 4D to verify to which "deletion bin" they belonged. The designations on the left of chromosome 4D denote the chromosomal break points and the name of deletion lines as described previously (Mickelson-Young et al. 1995). The designations on the right of chromosome 4D denote markers most of

which were dominant for the 4D chromosome although a few were co-dominant since a fragment amplified from chromosome 4B could be distinguished from the fragment(s) amplified from chromosome 4D. A homolog of *TaMATE1B* (on chromosome 4B) was mapped to the interval between break points 4DL-11 and 4DL-2 (*DMATE*). **b** The size of the 4D chromosomal fragment introgressed into chromosome 4B as estimated from marker analysis. The D-genome is represented in black and the B-genome in white

### Al<sup>3+</sup> tolerance of durum introgression lines

The Al<sup>3+</sup> tolerance of SF *TaALMT1* and *TaMATE1B* durum lines was assessed by hydroponics with varying Al<sup>3+</sup>



**Fig. 2** Quantification of D-genome introgressed into a durum background using RT-q PCR. The amount of Dgas sequence is expressed as a ratio of the rRNA sequence with the data normalized to show the 4D(4B) substitution line having a value of 1. Lines Chinese Spring with the full complement of D chromosomes, 4D(4B) is a durum line with chromosome 4B substituted with chromosome 4D, 4DL is a durum line that has a large fragment of chromosome 4D recombined with chromosome 4B (Han et al. 2014), SF TaALMT1 is the durum line which incorporates a small fragment of the 4D chromosome described in this paper and Jandaroi is a durum cultivar with no D-genome chromosome introgressed. Error bars show the SE (standard error) for two to four independent DNA extractions of each line. Data were log<sub>10</sub> transformed before a one-way ANOVA. Bars with different letters signifying statistically significant differences at  $P < 0.05$

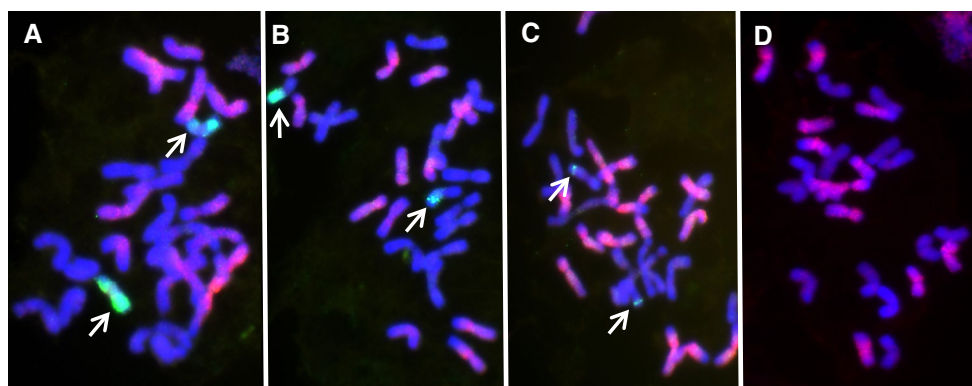
concentrations (Fig. 4). Both *TaALMT1* and *TaMATE1B* enhanced the Al<sup>3+</sup> tolerance of durum with *TaALMT1* conferring the stronger phenotype. The level of Al<sup>3+</sup> tolerance conferred by the SF line in hydroponics was comparable to that conferred by the full 4D chromosome (Fig. 4a).

When assessed in an acid soil, *TaALMT1* enhanced root growth of cv Jandaroi, but this was only apparent in the length of thick roots in the most acidic soils (Fig. 5a). By contrast, the growth of fine roots was not enhanced on any of the soils (Fig. 5b). When total roots were measured, the *TaALMT1* line had improved root growth but this could be attributed entirely to enhanced growth of thick roots (Fig. 5). The 4D(4B) line had better growth of both thick and fine roots in soil than the SF *TaALMT1* line (Fig. 5a).

The *TaMATE1B* line of durum had a markedly improved growth of both thick and fine roots on acid soils (Fig. 6). Consequently, the enhanced growth of total root length was a result of increased growth of both classes of roots. Both thick and fine roots of cv Jandaroi behaved similarly in the two soil experiments (SF *TaALMT1* and *TaMATE1B* lines), indicating that a similar range of Al<sup>3+</sup> toxicity was encompassed in both experiments. In contrast to hydroponic culture, the *TaMATE1B* line showed greater Al<sup>3+</sup> tolerance than the SF *TaALMT1* line when assessed on this acid soil (Figs. 5, 6).

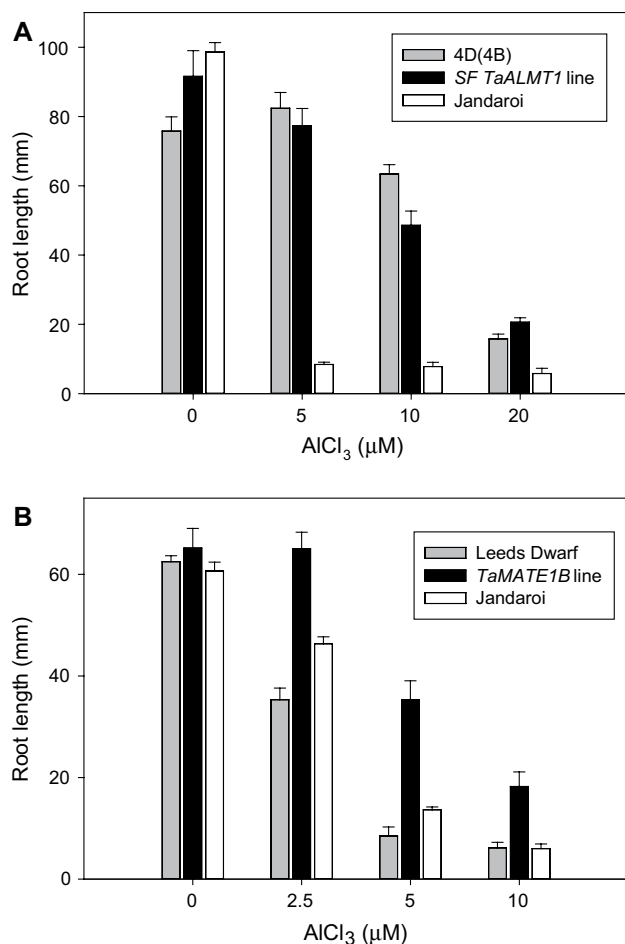
### Al<sup>3+</sup> tolerance of near-isogenic hexaploid wheat lines possessing different *TaMATE1B* alleles

The greater Al<sup>3+</sup> tolerance on soil conferred by *TaMATE1B* than *TaALMT1* in a durum genetic background was intriguing and prompted us to determine whether hexaploid wheat behaved in a similar fashion. Previously, it was shown that hexaploid wheat lines that possessed the Al<sup>3+</sup>-tolerant allele of *TaMATE1B* had improved Al<sup>3+</sup> tolerance



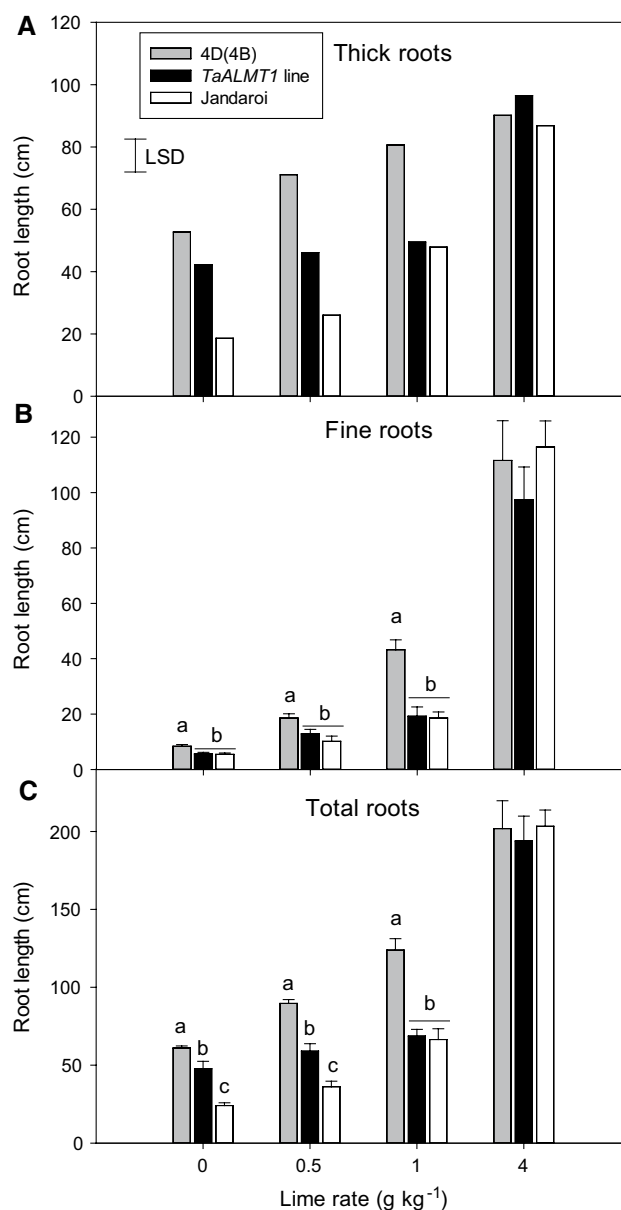
**Fig. 3** FISH analysis identifies an introgression of a small fragment of D-genome into durum wheat. The green signals denote the presence of D-genome, whereas pink and blue denote the A- and B-genomes. a The 4D(4B) substitution line where the whole 4D chromosome has replaced the 4B chromosome of durum wheat. b A spontaneous translocation of a relatively large fragment from chromosome

4D into durum wheat (Han et al. 2014) consistent with a whole-arm translocation. c The small 4D fragment recombined into chromosome 4B using the *ph1c* mutant as described in this paper. d The durum cv Jandaroi that lacks any D-genome. The white arrows indicate signals for D-genome in each panel



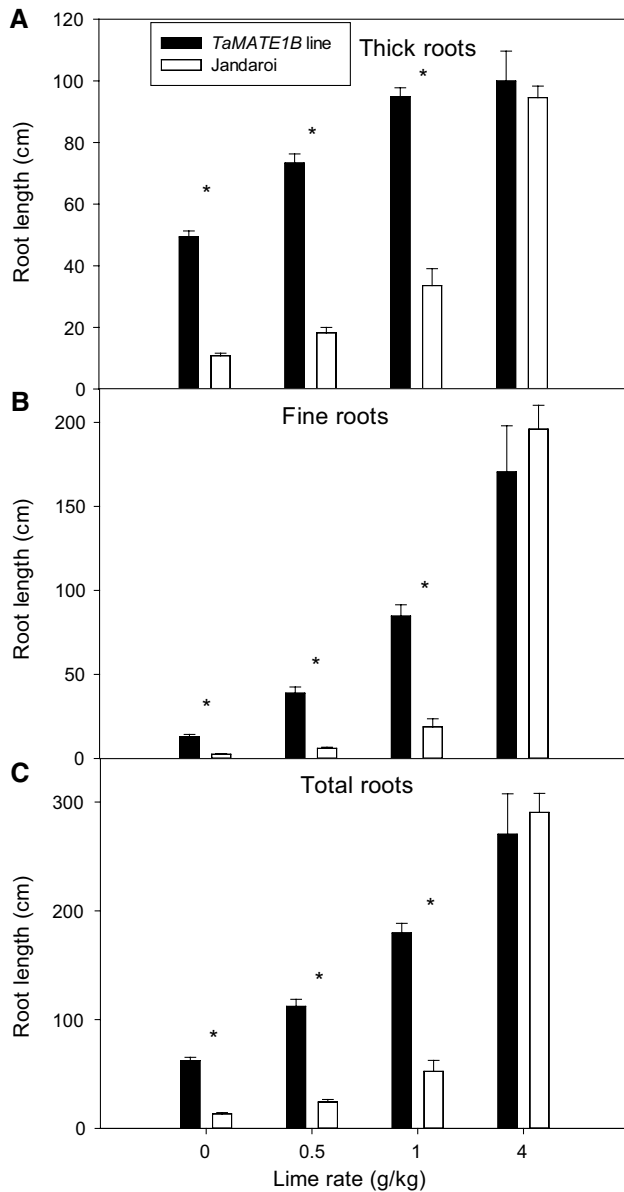
**Fig. 4** Introgression of *TaALMT1* or *TaMATE1B* into durum wheat enhances  $Al^{3+}$  tolerance as assessed by hydroponics. Effects of **a** SF *TaALMT1* and **b** *TaMATE1B* on  $Al^{3+}$  tolerance of durum wheat. Seed was pre-germinated prior to planting and seedlings were grown in solutions that contained various concentrations of  $AlCl_3$ . The data show root length after 5 days of the longest seminal root with the black bar denoting the introgression line and the white bar the cultivar Jandaroi ( $n = 7$ ; error bars show the SE). For **a**, the gray bar is a line where chromosome 4B is substituted with chromosome 4D and for **b** the gray bar is cv Leeds Dwarf which was the line used as a “bridge” to transfer *TaMATE1B* from bread wheat to durum wheat before the resulting line was crossed to cv Jandaroi. Note that the maximum  $AlCl_3$  concentration used was 10  $\mu M$  for the *TaMATE1B* line and 20  $\mu M$  for the SF *TaALMT1* line

in hydroponic culture, but this tolerance was considerably less than observed for lines that possessed *TaALMT1* on its own (Ryan et al. 2009). The lines used in that study were  $F_3$  families derived from single crosses between cvs Carazinho and Egret so were not necessarily genetically similar to one another. Here, we used backcrossed germplasm where *TaMATE1B* was introgressed into cultivars that were either homozygous for a tolerant (cv EGA-Burke) or sensitive (cv Egret) *TaALMT1* allele. In the presence of the sensitive *TaALMT1* allele, *TaMATE1B* conferred improved  $Al^{3+}$



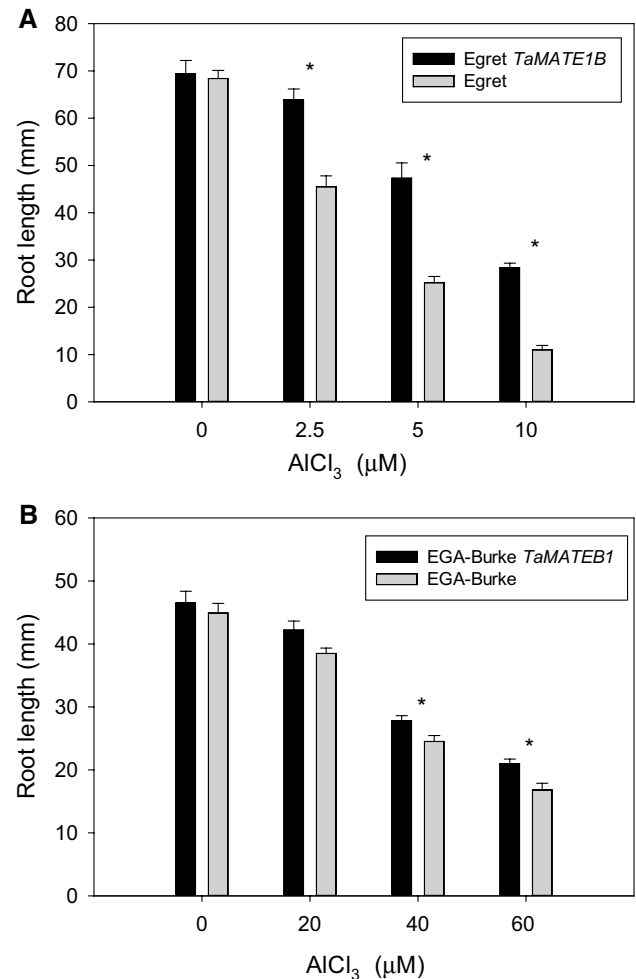
**Fig. 5** The SF *TaALMT1* fragment improves growth of thick (seminal) but not fine (lateral) roots of durum grown in acid soil. Lengths of **a** thick roots, **b** fine roots and **c** total roots. Pre-germinated seed were planted into 1.3 kg pots of soil and seedlings grown for 6 days before roots were washed out and scanned. After scanning, data were analyzed in groups as thick roots composed mainly of seminal roots, fine roots composed mainly of lateral roots and the total composed of combined data for thick and fine roots. Data show the mean of five replicates and error bars denote the SE. For **a** there was a significant interaction identified from a two-way ANOVA between genotype and treatment and the LSD for  $P < 0.05$  is shown. For **b** and **c** data needed to be  $\log_{10}$  transformed before two-way ANOVA. For each lime rate, the different letters indicate significant differences between genotypes at  $P < 0.05$

tolerance in hydroponic culture (Fig. 7a). When *TaMATE1B* was present in combination with a tolerant *TaALMT1* allele, it conferred a small increase in  $Al^{3+}$  tolerance (Fig. 7b). The



**Fig. 6** *TaMATE1B* improves growth of both thick (seminal) and fine (lateral) roots in acid soil. Lengths of **a** thick roots, **b** fine roots and **c** total roots. Pre-germinated seeds were planted into 1.3 kg pots of soil and seedlings grown for 7 days before roots were washed out and scanned. Data show the mean of six replicates and *error bars* denote the SE. The *asterisks* denote significant differences ( $P < 0.001$ ) between genotypes at each lime rate as determined by a *t* test

effectiveness of *TaALMT1* could be seen in the considerably higher  $\text{AlCl}_3$  concentration used to screen EGA-Burke (60  $\mu\text{M}$ ) compared to Egret (10  $\mu\text{M}$ ). In contrast to durum wheat, the effectiveness of the lines on a range of amended acid soils showed a similar pattern to the hydroponics experiments with *TaMATE1B* improving  $\text{Al}^{3+}$  tolerance in the Egret background but not improving  $\text{Al}^{3+}$  tolerance in the EGA-Burke background above the high background of  $\text{Al}^{3+}$  tolerance conferred by *TaALMT1* (Fig. 8).

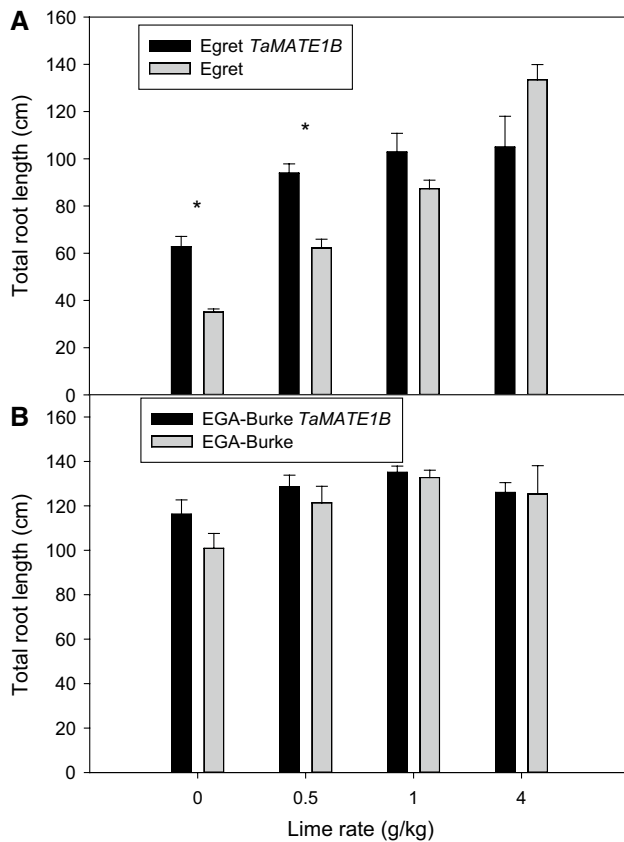


**Fig. 7** *TaMATE1B* improves the  $\text{Al}^{3+}$  tolerance of hexaploid wheat cultivars grown in hydroponic culture. **a** Cultivar Carazinho was backcrossed to cv Egret six times using Egret as the recurrent parent to generate near isogenic lines (NILs) that differ in *TaMATE1B* alleles. The *line* that has the *TaMATE1B* allele that confers citrate efflux is named Egret *TaMATE1B*. Egret has an  $\text{Al}^{3+}$ -sensitive allele of *TaALMT1* and lacks  $\text{Al}^{3+}$ -activated malate efflux. **b** Cultivar Carazinho was backcrossed to cv EGA-Burke nine times using EGA-Burke as the recurrent parent to generate NILs that differ in *TaMATE1B* alleles. The line that has the *TaMATE1B* allele that confers citrate efflux is named EGA-Burke *TaMATE1B*. EGA-Burke has an  $\text{Al}^{3+}$ -tolerant allele of *TaALMT1* that confers  $\text{Al}^{3+}$ -activated malate efflux. Data show the means of 10 replicates and *error bars* denote the SE. For **a** data were normally distributed but for **b** data were  $\log_{10}$  transformed prior to a two-way ANOVA. For **a** there was a significant interaction between genotype and treatment ( $P < 0.001$ ). For **b** there were significant genotype and treatment effects ( $P < 0.001$ ), but they did not interact. The *asterisks* denote significant differences between genotypes at each  $\text{Al}$  treatment

## Discussion

When *TaALMT1* and *TaMATE1B* were introgressed separately into a durum wheat background, they each enhanced the  $\text{Al}^{3+}$  tolerance of roots in hydroponic culture and





**Fig. 8** *TaMATE1B* improves the tolerance of an  $\text{Al}^{3+}$ -sensitive wheat cultivar grown in acid soil. **a** The cv Carazinho was backcrossed to cv Egret six times using Egret as the recurrent parent to generate NILs that differ in *TaMATE1B* alleles. The line that has the *TaMATE1B* allele that confers citrate efflux is named Egret *TaMATE1B*. Egret has an  $\text{Al}^{3+}$ -sensitive allele of *TaALMT1* that lacks  $\text{Al}^{3+}$ -activated malate efflux. **b** The cv Carazinho was backcrossed to cv EGA-Burke nine times using EGA-Burke as the recurrent parent to generate NILs that differ in *TaMATE1B* alleles. The line that has the *TaMATE1B* allele that confers citrate efflux is named EGA-Burke *TaMATE1B*. EGA-Burke has an  $\text{Al}^{3+}$ -tolerant allele of *TaALMT1* that possesses  $\text{Al}^{3+}$ -activated malate efflux. Data show the mean of six replicates and error bars denote the SE. The asterisks denote significant differences ( $P < 0.05$ ) between genotypes at each lime rate as determined by a *t* test

improved root growth on acid soil. Previously, when a large fragment of chromosome 4D was introgressed into durum wheat, the  $\text{Al}^{3+}$  tolerance of roots was increased in hydroponic culture and only growth of thick (seminal) roots was improved in acid soil without improving the growth of fine (lateral) roots. Here, we show that a similar phenotype was obtained when a much smaller fragment of chromosome 4D that possessed the *TaALMT1* gene was introgressed into durum. By contrast, the *TaMATE1B* gene enhanced the  $\text{Al}^{3+}$  tolerance of both thick and fine roots of durum when grown in acid soil. However, when assessed in hydroponic culture *TaMATE1B* was not as effective as *TaALMT1* in conferring  $\text{Al}^{3+}$  tolerance (Fig. 4). Although both genes

enhanced  $\text{Al}^{3+}$  tolerance of hexaploid wheat, *TaALMT1* conferred the stronger phenotype in both hydroponic and soil cultures (Figs. 7, 8).

It is not clear why *TaMATE1B* conferred a stronger phenotype than *TaALMT1* in durum wheat, but not bread wheat and specifically in soil. This illustrates that while hydroponic culture has proven to be useful to screen plants for  $\text{Al}^{3+}$  tolerance, the findings are not always reflected in experiments using acid soils. Similar to our findings with durum wheat, the  $\text{Al}^{3+}$  tolerance rankings of barley in hydroponic culture did not necessarily reflect rankings when the same lines were assessed on acid soil (Moroni et al. 2010). It is probable that the root systems of bread and durum wheat grown in acid soil differ sufficiently from one another such that the efflux of citrate was more effective for durum wheat roots whereas malate efflux was more effective for hexaploid wheat roots.

A relatively small fragment of chromosome 4D was introgressed into durum wheat and this was verified using three methods. Molecular markers located on chromosome 4D provided a genetic estimate of the size of fragments introgressed (Fig. 1), whereas FISH provided a direct image of the size of the fragment (Fig. 3). The use of a PCR-based method to quantify the relative amount of D-genome in the lines was in general agreement with both the marker and FISH analyses (Fig. 2). It provided a rapid method to estimate the relative sizes of fragments and another tool for selecting the recombinants with the smallest introgressions. A background signal was found in the absence of D-genome (cv Jandaroi; Fig. 2; Supplementary Fig. S1) which can be attributed to the primers annealing to related sequences in the A- and B-genomes albeit at lower efficiency relative to the D-genome. Here, we also show that the LF *TaALMT1* durum line generated previously (Han et al. 2014) by a spontaneous translocation had transferred the long arm of chromosome 4D to the short arm of chromosome 4B (Fig. 3). While the RT-qPCR method is useful to estimate the amount of D-genome introgressed, in some cases the method could provide distorted estimates if the distributions of the two classes of genes vary throughout the genome. Nevertheless, the technique proved useful in establishing that the D-genome in the *TaMATE1B* lines had been quickly eliminated by backcrossing the bread wheat/durum wheat hybrids to durum wheat (Supplementary Fig. S1).

The *TaALMT1* and *TaMATE1B* genes each independently improved the  $\text{Al}^{3+}$  tolerance of durum wheat. It should be possible to combine the genes to determine if their effects are additive and to further increase the acid soil tolerance of durum wheat. Figure 7b shows that *TaMATE1B* increased the  $\text{Al}^{3+}$  tolerance in hydroponics of a bread wheat cultivar that possesses a tolerant *TaALMT1* allele although the effect was small in the hexaploid wheat background. Combining the 4D chromosomal fragment containing *TaALMT1* with

*TaMATE1B* into a durum wheat background will require that the durum lines with each gene introgressed be crossed and a suitable recombination event identified. If *TaMATE1B* in durum is located in a homeologous region to where *TaMATE1B* is located in bread wheat, then it will be closely linked to the fragment containing *TaALMT1* (Fig. 1a). This could make identifying a suitable recombination event difficult. However, once generated it would simplify the introgression into other cultivars as the closely linked genes would generally be transferred together in crosses.

The development of a durum line that possesses a small fragment from the D-genome showed a similar  $Al^{3+}$  tolerance phenotype to lines that possessed the whole 4DL arm. The introgression of the small fragment containing *TaALMT1* appears to have avoided the smaller grain phenotype that was observed when the full 4DL arm was introgressed (Han et al. 2014) but the *Kna1* salt tolerance locus was absent from this fragment. To ensure that lines are also salt tolerant, a small fragment possessing the *Kna1* in a durum wheat background (Luo et al. 1996) could be recombined with the  $Al^{3+}$  tolerance genes on chromosome 4B. Alternatively, the *Nax* genes that confer salt tolerance to durum wheat could be other sources of genes that are located on chromosomes 2A (*Nax1*) and 5A (*Nax2*) (Byrt et al. 2007; Lindsay et al. 2004). Nevertheless, the small fragment harboring *TaALMT1* should enable the *TaMATE1B* gene to be combined with *TaALMT1* as discussed above, whereas the full 4DL chromosome arm would not allow straightforward recombination of both genes.

**Author contribution statement** ED directed the work, undertook some of the experimental work and wrote the manuscript. CH undertook the bulk of the experimental work to identify recombinants and cross genotypes to develop germplasm. CH also revised the manuscript. PZ undertook the FISH experimental work and revised the manuscript, PRR revised the manuscript, TR undertook real-time PCR aspects of the work and revised the manuscript, Z-H Y revised the manuscript.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no conflict of interest.

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