Plant Biotechnology Journal

Plant Biotechnology Journal (2016) **14**, pp. 313–322 doi: 10.1111/pbi.12385

The *TaDREB3* transgene transferred by conventional crossings to different genetic backgrounds of bread wheat improves drought tolerance

Yuri Shavrukov*,†, Manahil Baho, Sergiy Lopato and Peter Langridge

Australian Centre for Plant Functional Genomics, University of Adelaide, Urrbrae, SA 5064, Australia

Received 13 February 2015;
revised 20 March 2015;
accepted 20 March 2015.
**Correspondence* (Tel +61 8 7220 5758;
fax +61 8 8313 7402;
email yuri.shavrukov@adelaide.edu.au)
†Present address: School of Agriculture, Food and Wine, University of Adelaide, Urrbrae, SA 5064, Australia.

Keywords: backcrossing, DREB transcription factor, drought, frost, hybrids, transgenic wheat.

Summary

Drought tolerance of the wheat cultivar Bobwhite was previously enhanced by transformation with a construct containing the wheat *DREB3* gene driven by the stress-inducible maize *Rab17* promoter. Progeny of a single T_2 transgenic line were used as pollinators in crosses with four elite bread wheat cultivars from Western Australia: Bonnie Rock, IGW-2971, Magenta and Wyalkatchem, with the aim of evaluating transgene performance in different genetic backgrounds. The selected pollinator line, BW8-9-10-3, contained multiple transgene copies, had significantly improved drought tolerance compared with wild-type plants and showed no growth and development penalties or abnormalities. A single hybrid plant was selected from each cross-combination for three rounds of backcrossing with the corresponding maternal wheat cultivar. The transgene was detected in all four F_1BC_3 combinations, but stress-inducible transgene expression was found in only three of the four combinations. Under well-watered conditions, the phenotypes and grain yield components of the F_2BC_3 transgene-expressing lines were similar to those of corresponding recurrent parents and null-segregants. Under severe drought conditions, the backcross lines demonstrated 12–18% higher survival rates than the corresponding control plants. Two from four F_3BC_3 transgenic lines showed significantly higher yield (18.9% and 21.5%) than control plants under limited water conditions. There was no induction of transgene expression under cold stress, and therefore, no improvement of frost tolerance observed in the progenies of drought-tolerant F_3BC_3 lines.

Introduction

Drought is a major abiotic stress affecting global crop production. Many genes are involved in plant responses to drought, and some of these can be used for the improvement of drought tolerance by genetic engineering (reviewed in Shanker *et al.*, 2014). Regulatory genes that activate or deactivate suites of drought-responsive genes are of particular interest to biotechnologists (Huang *et al.*, 2008; Ni *et al.*, 2009; Singh *et al.*, 2013). Genes encoding dehydration-responsive element-binding (DREB) transcription factors (TFs) comprise one of the major groups of genes involved in drought response regulation (Yamaguchi-Shinozaki and Shinozaki, 2006).

Strong constitutive over-expression of *DREB* genes in transgenic plants often provides improvement of plant survival under strong or prolonged drought by regulation of large numbers of stress-responsive genes (reviewed in Agarwal *et al.*, 2006; Lata and Prasad, 2011; Yamaguchi-Shinozaki and Shinozaki, 2009). However, unnecessarily high levels of TFs delivered by strong constitutive promoters often lead to the development of pleiotropic phenotypes, including dwarfism, delayed transition to flowering and decreased grain yield (Ito *et al.*, 2006; Li *et al.*, 2012; Morran *et al.*, 2011). It has been reported that phenotypes of transgenic plants can be improved by the use of stress-inducible promoters, which restrict transgene overexpression to the stress period, when plant growth is significantly affected by the stress. Low levels of transgene expression in non-stressed conditions either decrease or eliminate any negative influences of the transgene on plant phenotypes (Cui *et al.*, 2011; Fu *et al.*, 2007; James *et al.*, 2008; Kovalchuk *et al.*, 2013; Morran *et al.*, 2011; Pellegrineschi *et al.*, 2004; Xue *et al.*, 2011). Over-expression of *TaDREB3* in transgenic wheat and barley using stress-inducible promoters improved tolerance to dehydration and considerably preserved the original phenotypes of transformed wheat and barley cultivars (Kovalchuk *et al.*, 2013; Morran *et al.*, 2011). It has been demonstrated that in transgenic wheat, the *TaDREB3* gene driven by the maize *Rab17* promoter (Vilardell *et al.*, 1991, 1994) had no basal expression and was strongly activated by dehydration but showed a minimal response to cold (+4 °C) treatment (Morran *et al.*, 2011). By contrast, use of the same construct in barley resulted in moderate basal levels of *TaDREB3* expression, with a mild negative influence of the transgene on plant development and significant enhancement of the frost tolerance of transgenic lines (Morran *et al.*, 2011).

Biolistic bombardment is a more efficient method of transformation of wheat compared with the *Agrobacterium*-mediated transformation (Ismagul *et al.*, 2014; Kovalchuk *et al.*, 2009). Unfortunately, biolistic bombardment often delivers multiple copies of the transgene into the bread wheat genome (reviewed in Mitchell *et al.*, 2004; Sahrawat *et al.*, 2003; Terzi *et al.*, 2005). These copies may be either spread across the genome of transgenic plants, or clustered as closely linked loci and/or tandem repeats. In the first case, the transgenes may segregate

independently, and plants with a single transgene copy and stable inheritance may be obtained following a number of generations of characterization and selection (Rooke *et al.*, 2003; Tsai *et al.*, 2012; Yao *et al.*, 2006). In the second case, multiple copy numbers and tightly linked inheritance of the transgene cluster have been reported (Mitchell *et al.*, 2004; Sahrawat *et al.*, 2003).

However, abnormal transgene inheritance can sometimes be observed in transgenic wheat even with a single transgene insertion. An example of this is a report for transgenic bread wheat cv. Riband expressing the *Rubisco* gene (*Ubi-rbcS*). Almost 50% of T_1 lines containing a single transgene copy demonstrated segregation ratios inconsistent with Mendelian inheritance. It was speculated that such result can be a consequence of reduced production of fertile gametes in these lines (Mitchell *et al.*, 2004).

It has also been reported that transgenes in bread wheat can show unexpected expression profiles. For example, some T_3 transgenic wheat plants transformed with *1Dx5* and *1Dy10* genes, which encode high molecular weight glutenin subunits, showed transgene expression with stable inheritance up to the T_5 generation. However, in the same study, another transgene, *1Bx8* from the same gene family, caused a silencing of the endogenous gene in two of 60 transgenic lines (Zhang *et al.*, 2003). For wheat lines showing inheritance of transgenes as a single Mendelian locus, both stable (Yao *et al.*, 2006) and unstable (Rooke *et al.*, 2003) transgene expression patterns have been reported.

Generation-dependent decreases in transgene expression levels have been observed in some transgenic wheat lines. For instance, luciferase gene expression in transgenic wheat was decreased by around three-fold in twenty T_2 transgenic lines of cv. Bobwhite compared with the expression levels in T_1 plants (Bourdon *et al.*, 2002). In the T_3 generation, luciferase expression was reduced by further 60%. The authors suggested that one or several transgene copies provided low levels of expression in subsequent generations due to possible genome rearrangements during meiosis (Bourdon *et al.*, 2002). Similarly, a significant reduction in transgene expression levels has been reported for the *Rubisco* gene in all five investigated independent transgenic lines of bread wheat cv. Riband, which were carried through T_1 and T_2 generations (Mitchell *et al.*, 2004).

In some cases, unstable functional performance of the transgene has been observed. For example, transgenic T_4 lines of bread wheat cv. Hi-Line transformed with the barley *HVA1* gene, encoding a late embryogenesis abundance (LEA) protein, and driven by the constitutive *Ubiquitin* (*Ubi*) promoter, were tested under drought conditions in the field (Bahieldin *et al.*, 2005). Transgenic wheat plants demonstrated an increase of total plant biomass and improved grain yield per plot compared with wild-type (WT). However, the phenotypic effect of the transgene in some transgenic lines was dependent on environmental conditions (Bahieldin *et al.*, 2005).

Very little is known about the effects of introgression of transgenes into different genetic background by conventional backcrossing. The *RTBV* transgene encoding resistance to rice *Tungro bacilliform* virus showed close to classical transgene segregation ratios (1 : 1) in two consequent backcrossings after two sets of crosses with non-transgenic rice (Roy *et al.*, 2012). However, in one set of crosses, the selectable marker *Hygromycin phosphotransferase* (*hpt*), incorporated into the construct, was missing. Nevertheless, the marker-free segregants in F_1BC_2 still contained a functional transgene. Loss of the selectable marker incorporation was proposed to have occurred as a result of recombination between the *RTBV* transgene and *hpt* marker gene within the same genetic construct (Roy *et al.*, 2012).

In this study, conventional hybridization and backcrossing were used for transgene transfer from transgenic plants generated by biolistic bombardment of wheat cv. Bobwhite, a cultivar with high transformation and regeneration efficiency, to four low transformation-efficient elite Australian wheat cultivars. The study includes the following: (i) investigation of the inheritance of constitutive and inducible levels of transgene expression in hybrid plants and progeny during three consecutive backcrosses, (ii) evaluation of the transgene influence on development of the recipient cultivars in the absence of stress, and (iii) confirmation of the transgene product functionality in backcross-derived lines by comparison of (i) drought and frost tolerance and (ii) grain yields of control and transgene-containing plants under well-watered conditions and under moderate drought at flowering.

Results

Transgene transfer to elite Australian wheat cultivars

The scheme of crosses and performed genetic analyses is demonstrated in Figure 1, using the cultivar IGW-2971 as an example. It shows the initial cross and three subsequent backcrosses, with semi-nested PCR used to detect the presence or absence of the transgene. Although small numbers of siblings were obtained and analysed for each backcross event (F_1BC_1, F_1BC_2 and F_1BC_3 generations), segregation rates for the transgene followed the expected ratio of 1 : 1 predicted by classical Mendelian inheritance (Figure 1).

Analysis of transgene expression in F_1BC_3 progenies of four hybrids

The expression of the transgene driven by the *ZmRab17* promoter was analysed in F_1BC_3 progenies of four backcross lines. Northern blot hybridization results are shown in the lower part of Figure 1 (IGW-2971 background) and in Figure 2 (Bonnie Rock, Wyalkatchem and Magenta backgrounds). Very strong transgene expression was observed in four (Plants 1, 5, 7 and 9) from nine analysed F_1BC_3 progenies of IGW-2971xBW8-9-10-3 plants subjected to dehydration stress (Figure 1). Control samples collected from the same plants before dehydration treatment did not show transgene expression, demonstrating strong stress-inducible activation of the *TaDREB3* transgene driven by the *ZmRab17* promoter. Five other plants from the same cross failed to show transgene expression either in dehydrated or in control samples. The absence of the transgene in these lines was confirmed by semi-nested PCR (Data not shown).

A second hybrid (Bonnie RockxBW8-9-10-3) also demonstrated 1 : 1 segregation for the *TaDREB3* transgene, but the strength of transgene expression was variable between progenies (Figure 2a). Under dehydration stress the transgene was strongly expressed in Plant 2, and moderately expressed in three other plants (1, 4 and 7). Three of seven analysed plants did not show dehydration-inducible transgene expression. PCR on genomic DNA demonstrated that all plants with inducible expression of the *TaDREB3* contained the transgene, while those not showing transgene expression were confirmed as null-sergegants (Data not shown). No expression of the transgene was observed in the original cultivar, Bonnie Rock, which was used as a negative control in this experiment (indicated as WT in Figure 2a). Expression of the *TaDREB3* endogenous gene was too weak in all cultivars to be detected under these experimental conditions.

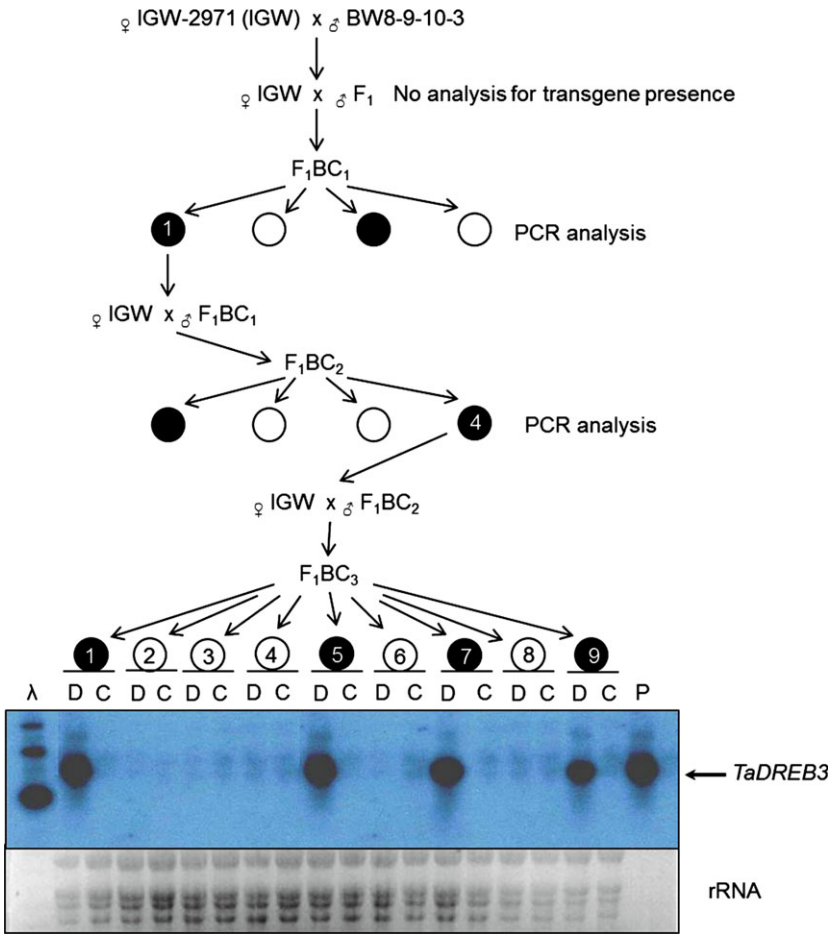

Figure 1 Schematic representation of the crossing strategy between bread wheat cv. IGW-2971 and T_3 progeny of transgenic line BW8-9-10-3, containing the construct *pZmRab17::TaDREB3*. Transgene-containing hybrid plants are shown in black circles and null-segregants in clear circles. Nine plants from the F_1BC_3 generation were used for Northern blot analysis, where both dehydrated (D) and control (C) leaf samples for each plant were analysed for transgene (*TaDREB3*) expression. λ, size marker; P, positive control of diluted DNA probe (coding region of *TaDREB3*). Ribosomal RNA (rRNA) bands indicate sample loading variability.

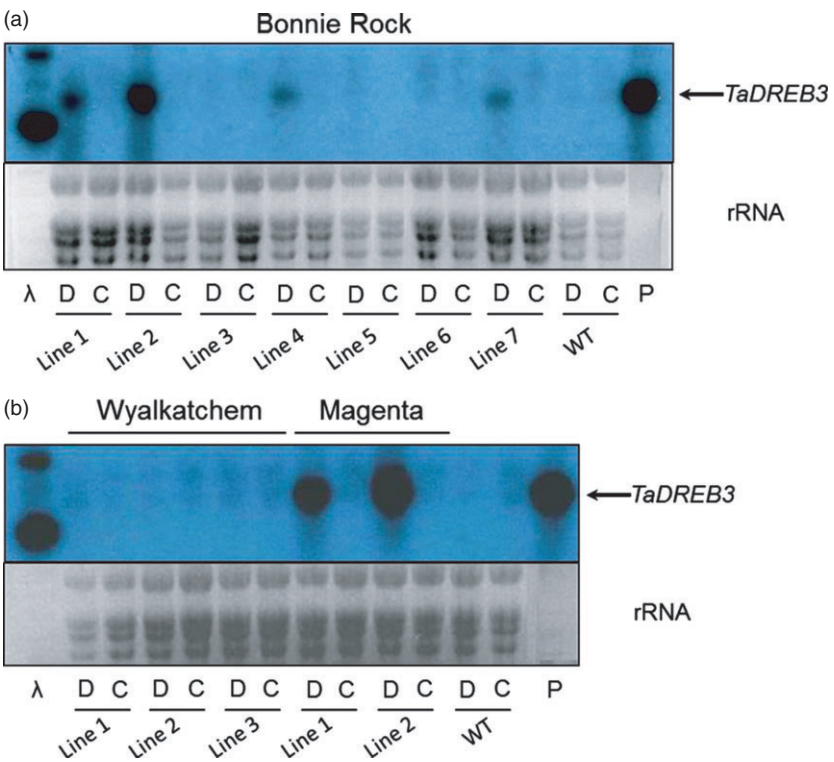

Figure 2 Transgene (*TaDREB3*) expression levels in leaves analysed by Northern blot hybridization. Leaves were collected from F_1BC_3 plants with: (a) Bonnie Rock, and (b) Wyalkatchem and Magenta. D, dehydrated leaves; C, control leaves; λ, size marker; P, positive control of diluted DNA probe (coding region of *TaDREB3*). Ribosomal RNA (rRNA) bands indicate sample loading variability.

Unexpected results were obtained for the third hybrid (WyalkatchemxBW8-9-10-3), where six plants of the F_1BC_3 progeny were analysed. Semi-nested PCR indicated that three plants (1, 2 and 3) contained the transgene, while plants 4, 5 and 6 were null-segregants (Data not shown). However, none of the plants displayed drought-inducible transgene expression (Figure 2b), and therefore, this hybrid was withdrawn from further analysis.

For the fourth hybrid between Magenta and BW8-9-10-3, only two plants were obtained in the F_1BC_3 generation. Semi-nested PCR confirmed the presence of the transgene in both plants, and both plants showed very high transgene expression levels in dehydrated leaves. Non-treated leaves and a control WT plant (original cultivar Magenta) showed neither transgene nor endogenous gene expression (Figure 2b).

Transgene copy number and segregation in F_2BC_3 plants

We performed analysis of transgene segregation in self-pollinated progenies of IGW-2971, Bonnie Rock and Magenta F_1BC_3 plants containing the *TaDREB3* transgene. Quantitative estimation of transgene copy number was also carried out in F_2BC_3 progenies (Table 1). The qPCR data confirmed our findings of the presence/absence of the transgene in segregating progeny using semi-nested PCR. Transgene copy number was estimated in 12 F_2BC_3 plants for each cross-combination and the data used to assess segregation ratios (Table 1).

The working hypothesis for transgene inheritance was that in the case of a single transgene insertion, segregation should follow simple Mendelian inheritance. Progeny from all four transgenic plants derived from the first hybrid (IGW-2971xBW8-9-10-3), and from both transgenic plants derived from a hybrid with Magenta, showed segregation according to Mendelian expectation, indicating the presence of a single copy of the transgene in F_1BC_3 plants. No significant deviation from the expected segregation ratio (1 : 2 : 1) was observed in these two cross-combinations, either for individual sibling lines or for the total progeny, as confirmed by Randomization tests for goodness-of-fit and χ^2 tests, respectively (Table 1). qPCR suggested that none of the progeny contained more than two copies (one on each homologous chromosome) of the transgene, confirming a single gene inheritance in these crosses. By contrast, there were multiple copies of the transgene in F_2BC_3 plants from the cross Bonnie RockxBW8-9-10-3, and transgene segregation ratios deviated significantly from that expected for a single gene inheritance hypothesis (Table 1).

Table 1 Transgene copy number determined by qPCR, in the progenies (12 plants) of transgene-containing F_1BC_3 lines from three cross-combinations

	Transgene copy number					Randomization	
Hybrid and lines	0	1	2	3	4	probability	χ^2
Hybrid 1: IGW-2971xBW8-9-10-3							
Line 1	4	4	4	0	0	0.60	
Line 5	3	7	2	0	0	0.93	
Line 7	5	6	1	0	0	0.27	
Line 9	4	6	2	0	0	0.71	
Total	16	23	9	0	0		2.13
Hybrid 2: Bonnie RockxBW8-9-10-3							
Line 1	3	5	3	1	0	-	
Line 2	0	1	3	5	3	-	
Line 4	4	0	5	1	2	-	
Line 7	3	0	5	1	3	-	
Total	10	6	16	8	8		15.17*
Hybrid 3: MagentaxBW8-9-10-3							
Line 1	3	4	5	0	0	0.39	
Line 2	4	6	2	0	0	0.71	
Total	7	10	7	0	0		0.67

Results of Randomization tests for goodness-of-fit (Randomization probability) for 10 000 replicates and χ^2 tests ($\chi^2_{0.05} = 5.99$; dF = 2), both for single gene segregation (1 : 2 : 1), are shown for each transgenic line independently ($n = 12$) and for F_2BC_3 progeny from all transgenic lines in each hybrid combination ($n = 24$–60), respectively.

*Significantly ($P < 0.05$) different from expected segregation for a single gene using χ^2 tests.

Growth and yield characteristics of transgene-containing backcross lines grown in pots under well-watered or drought conditions

Growth analysis of transgenic plants in the F_2BC_3 generation in favourable, well-watered conditions in pots revealed no significant differences compared to either corresponding WT plants or null-segregants (Table 2). Next, a drought survival test was conducted in pots. Each pot contained three backcross plants from the same line with a single copy of the transgene, and one WT plant as a control. Drought conditions were severe enough to kill 35–40% of WT plants before re-watering (Figure 3). The survival test indicated better drought survival rates in all four F_2BC_3 transgene-containing lines derived from the hybrid combination IGW-2971xBW8-9-10-3, with 12–18% higher survival rates compared with both WT (parental cultivar IGW-2971) and null-segregants (Figure 4a). The differences were moderate but significant for all four tested lines.

Drought survival rates were also higher than controls (corresponding WT and null-segregant) for lines derived from the hybrid combination Bonnie RockxBW8-9-10-3, although the differences were significant only for one of the four lines (Figure 4b; No. 7). Significant differences in survival were observed for both F_2BC_3 transgene-containing lines derived from the hybrid MagentaxBW8-9-10-3 compared with both the corresponding WT and null-segregants (Figure 4c).

Seed yields of transgene-containing backcross lines grown in large containers with either sufficient or insufficient watering

Under well-watered conditions, none of the tested transgene-containing backcross lines selected from three hybrid combinations showed significant differences in seed yield compared with the corresponding recurrent parents or null-segregants (Figure S1).

When grown in water-limited conditions, two of the transgene-containing backcross lines (IGW-2971xBW8-9-10-3, No. 5-8, and Bonnie RockxBW8-9-10-3, No. 4-1) demonstrated significant ($P < 0.05$) increase in seed yield compared to the corresponding recurrent parents (Figure 5). The yield increases for these two lines were 18.9% and 21.5%, respectively. Seed yields of two tested lines derived from the cross MagentaxBW8-9-10-3 were not significantly different compared to the yield of recurrent parent Magenta (Figure 5).

Table 2 Growth and yield components of F_2BC_3 lines of three hybrid combinations in pots under well-watered conditions with confirmed drought-inducible expression of the *TaDREB3* transgene. Corresponding wild-type (WT) and null-segregant plants (Nulls) were used as controls. Data represent means of eight biological replicates ± SE. A single-factor ANOVA test was applied for each hybrid combination

	Plant height (cm)	Seed weight per plant (g)	Number of seeds per spike	Single seed weight (mg)	ANOVA, $F_{critical}$
Hybrid 1: IGW-2971xBW8-9-10-3					
WT	60.5 ± 2.6	3.3 ± 0.5	27.7 ± 2.6	34.3 ± 1.3	
Nulls	62.0 ± 6.3	3.4 ± 0.4	27.9 ± 4.7	31.9 ± 3.3	
Line 1	57.0 ± 3.9	4.2 ± 1.1	28.9 ± 4.9	38.0 ± 1.6	
Line 5	62.0 ± 3.0	3.5 ± 0.5	29.4 ± 2.7	38.6 ± 1.3	
Line 7	66.9 ± 4.6	3.9 ± 0.4	29.5 ± 1.3	40.5 ± 2.2	
Line 9	60.6 ± 6.2	3.8 ± 0.7	27.7 ± 2.5	37.2 ± 3.2	
ANOVA	**0.44** (ns)	**0.57** (ns)	**0.29** (ns)	**1.96** (ns)	**2.84**
Hybrid 2: Bonnie RockxBW8-9-10-3					
WT	68.6 ± 2.6	5.8 ± 0.4	25.5 ± 2.4	43.1 ± 1.1	
Nulls	69.5 ± 2.4	5.6 ± 0.7	23.9 ± 2.1	41.5 ± 2.1	
Line 1	66.4 ± 3.2	5.4 ± 0.5	23.0 ± 1.4	44.3 ± 1.0	
Line 2	69.1 ± 2.6	5.8 ± 0.3	23.1 ± 2.9	43.4 ± 1.2	
Line 4	69.7 ± 1.7	5.7 ± 0.8	25.3 ± 2.5	39.2 ± 1.5	
Line 7	72.7 ± 3.5	4.9 ± 0.6	25.6 ± 2.6	41.2 ± 1.1	
ANOVA	**2.19** (ns)	**1.18** (ns)	**1.61** (ns)	**0.68** (ns)	**2.74**
Hybrid 3: MagentaxBW8-9-10-3					
WT	68.0 ± 4.5	5.2 ± 0.6	20.8 ± 2.8	45.2 ± 3.2	
Nulls	65.0 ± 5.5	5.0 ± 0.6	21.8 ± 3.0	45.9 ± 3.4	
Line 1	61.3 ± 3.8	4.8 ± 0.5	21.5 ± 3.1	43.6 ± 3.5	
Line 2	61.4 ± 6.5	4.9 ± 0.3	23.7 ± 3.3	41.3 ± 2.8	
ANOVA	**1.95** (ns)	**0.14** (ns)	**1.14** (ns)	**0.81** (ns)	**4.74**

ns, not significant difference.

F values and $F_{critical}$ are shown in bold.

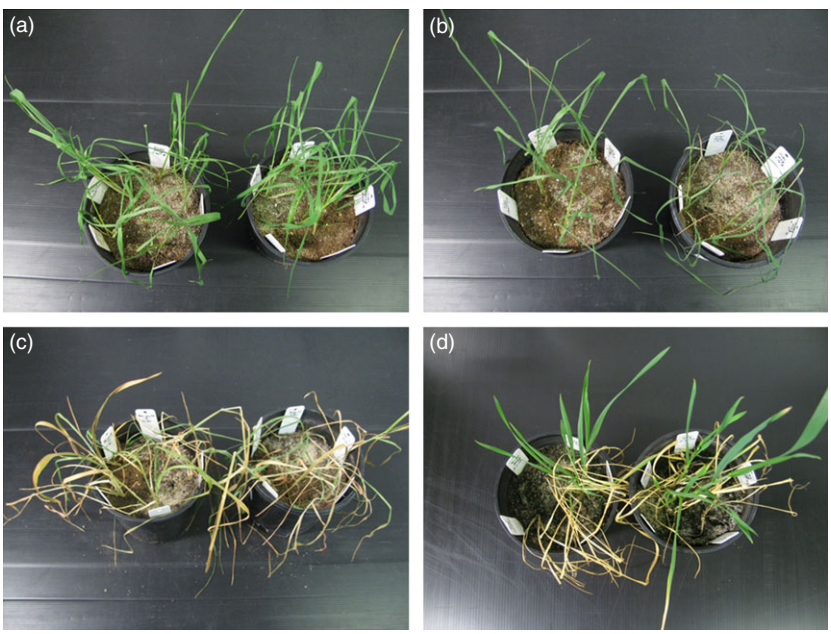

Figure 3 Images of plants at different stages of the drought survival test: (a) the day on watering was withheld, (b) day six of drought, (c) day 12 of drought, immediately before re-watering, (d) 1 week after re-watering. Each pot contained five plants including WT, a null-segregant and three plants from each of three randomly selected independent F_2BC_3 transgenic lines.

Frost tolerance of transgene-containing backcross plants

The two best-performing backcross lines from the drought experiment were chosen for assessment of their response to cold stress. There were no significant differences in survival rates following a frost treatment between transgene-containing backcross lines IGW-2971xBW8-9-10-3 and Bonnie RockxBW8-9-10-3 and their corresponding recurrent parents (Figure S2). Survival rates of all analysed plants were within the range 41.7–50.0%. Furthermore, no significant differences were found between *TaDREB3* expression levels of untreated leaves and leaves exposed to cold temperatures for either of the backcross lines (Figure S3),

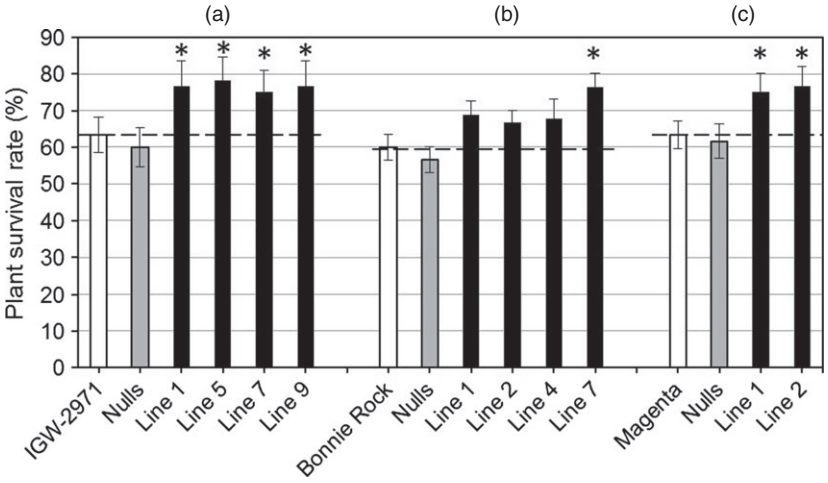

Figure 4 Percentage survival of F_2BC_3 plants in a pot-based drought tolerance test for lines derived from each of three hybrid combinations: (a) IGW-2971xBW8-9-10-3, (b) Bonnie RockxBW8-9-10-3 and (c) MagentaxBW8-9-10-3. Corresponding wild-type plants and respective null-segregants are also shown (white and grey bars, respectively). Bars represent means ± SE for five experiments, where 12 plants were used in each experiment. Significant differences ($P < 0.05$) between transgenic lines and corresponding WT/Nulls in each hybrid were calculated by Student's *t*-test and are indicated by asterisks.

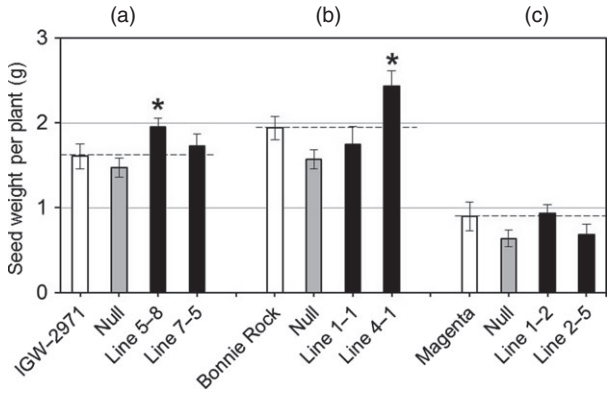

Figure 5 Seed yields obtained in water-limited growth conditions for transgene-containing lines derived from three cross-combinations: (a) IGW-2971xBW8-9-10-3, (b) Bonnie RockxBW8-9-10-3 and (c) MagentaxBW8-9-10-3. Two lines of each transgene-containing hybrid F_3BC_3 combination were used in an experiment conducted in large containers under a controlled watering regime. Parental cultivars and respective null-segregants were included for comparison. Bars represent means for 16 biological replicates ± SE. Asterisks indicate significant differences ($P < 0.05$) between transgenic lines and the corresponding recurrent parent, calculated using a Student's *t*-test.

indicating that the *TaDREB3* transgene in these lines was not cold-stress inducible and conferred no performance or survival advantage in cold conditions.

Discussion

Genetic transformation is often limited to those plant genotypes which are amenable to transformation and subsequent regeneration in tissue culture. The bread wheat cultivar Bobwhite is particularly favourable for regeneration after transformation by particle bombardment and provides high numbers of transformation events (reviewed in Sahrawat *et al.*, 2003; Vasil, 2007). Bobwhite is a generic name that refers to all sister-lines derived from the cross CM 33203 with a line containing Aurora, Kalyan, Bluebird and Woodpecker in its pedigree, made by the CIMMYT bread wheat program in the early 1970s (Warburton *et al.*, 2002). It is not grown commercially in Australia, but is used across the world for its relative amenability to genetic modification. Successful transgenic lines generated in the Bobwhite background have a low potential for commercial application. In this work, we: (i) successfully transferred a transgene from transgenic wheat cv. Bobwhite into modern elite Australian cultivars by conventional cross-hybridization and backcrossing; (ii) analysed transgene inheritance and stability of expression across several backcross generations; (iii) evaluated the backcross lines for tolerance to severe drought and performance under sufficient and limited watering; and (iv) assessed two selected backcross lines for frost tolerance during vegetative development.

A concerning consequence of transformation by particle bombardment is that DNA of the recipient genome can be damaged by random, uncontrolled insertions of construct-derived DNA fragments, resulting in mutations in endogenous genes or their regulatory regions (Ismagul *et al.*, 2014). This can lead to aberrant phenotypes of transgenic plants that have no relation to the phenotype produced as a result of transgene expression. One solution to this problem is the development of protocols with improved transformation efficiency, high regeneration capability and decreased DNA damage, optimized for specific target bread wheat cultivars. If achievable in a range of adapted genotypes, this would provide the most time and labour-effective pipeline for generation of transgenics (reviewed in Sahrawat *et al.*, 2003). Alternatively, the primary transgenic material obtained by current methods of biolistic bombardment of cv. Bobwhite may be used for hybridization and backcrossing with elite target bread wheat cultivars. This would facilitate transgene transfer into more suitable genetic backgrounds, and multiple rounds of backcrossing would dilute out any effects of undesirable T-DNA insertions. We have investigated the validity of this approach in the current study.

Backcrossing approaches have been used since the early days of systematic plant breeding and are routinely used in conventional plant breeding. However, there are only a few reports of the use of hybridization and backcrossing for transfer of a transgene. The approach was used for rice (Roy *et al.*, 2012) and tobacco (Lewis and Kernodle, 2009), and in both cases, transgenes were inherited as simple Mendelian traits. In wheat, the inheritance of transgenes during self-pollination over several generations has been studied (reviewed in Vasil, 2007), as well as the control of transgene flow following accidental hybridization of transgenic wheat with non-transgenic plants in the field (Rieben *et al.*, 2011). There are no reports of intentional transgene transfer by crossing of wheat plants.

In spite of Mendelian inheritance, both stable (Yao *et al.*, 2006; Zhang *et al.*, 2003) and unstable (Bahieldin *et al.*, 2005; Bourdon *et al.*, 2002; Rooke *et al.*, 2003) expressions of transgenes has been reported in subsequent generations after self-pollination of transgenic wheat plants. In addition, transgene silencing was observed either in some (Zhang *et al.*, 2003) or in all (Mitchell *et al.*, 2004) studied transgenic lines. Such differences in transgene inheritance and in the frequency of gene silencing were linked to numbers of insertions per transformation event and differences in genotypes used for transformation. It is still not absolutely clear where and how transgenes are introduced into the genome during biolistic transformation, and this largely remains an uncontrolled process. However, transgene inheritance is expected to be directly related to its status (hemi-, homo- or heterozygous) in the transgenic plants, and to recombination events occurring in areas surrounding the transgene.

In this study, we have shown that inheritance of the transgene in F_1BC_3 and F_2BC_3 progenies of two hybrids (IGW-2971 and Magenta crossed with the line BW8-9-10-3, containing multiple copies of a *ZmRab17::TaDREB3* transgene) was generally in agreement with classic Mendelian segregation as a single locus. A third hybrid combination (Bonnie RockxBW8-9-10-3) showed a more complicated inheritance pattern, probably as a result of the segregation of several transgene copies.

Failure to detect gene expression in any progeny derived from the hybrid combination WyalkatchemxBW8-9-10-3, despite the presence of the transgene, may suggest transgene silencing in this particular cultivar. Alternatively, there may have been transfer of only a partial fragment of the transgene to this case. A third, although less likely reason could be that there was insufficient leaf dehydration of the backcross lines derived from the Wyalkatchem background, resulting in failure to activate the *ZmRab17* promoter. However, the drought stress imposed for gene expression analysis on all cultivars was severe, and Wyalkatchem is not reported to be outstandingly drought-tolerant relative to the other chosen recipient wheats. Further analysis of transgene integrity and methylation status, identification of transgene position in the wheat genome and/or optimization of stress conditions will be required to understand the absence of transgene expression in the Wyalkatchem background.

We found that under well-watered conditions, all transgene-containing backcross lines had growth and grain yield characteristics similar to those of corresponding WT plants and null-segregants. These results were consistent in all experiments, regardless of whether plants were grown in pots (Table 2) or in large containers (Figure S1). We also found that the *TaDREB3* transgene driven by the stress-inducible *ZmRab17* promoter is expressed only in response to drought / leaf dehydration and had very low basal levels of expression in the absence of stress (Figures 1 and 2). Therefore, any differences in plant growth or seed production under well-watered conditions between F_2BC_3 lines and control plants would be attributable to the presence of a small portion of the Bobwhite genome (approx. 6%), which still remains in the genome of backcross plants after three rounds of backcrossing.

All F_2BC_3 transgene-expressing hybrid lines derived from two hybrid combinations (IGW-2971xBW8-9-10-3 and MagentaxBW8-9-10-3) and one transgenic line derived from the hybrid combination Bonnie RockxBW8-9-10-3 demonstrated higher drought tolerance than WT plants and null-segregants by showing significantly higher (12–18%) drought survival rates after strong drought stress (Figure 4). We then evaluated the lines by growing them in large containers in conditions of limited water at flowering, allowing a more precise comparison of yields of backcross and control plants in conditions more related to water deficit in the field. Only two transgenic lines (Line 5–8 from IGW-2971 background and Line 4–1 from Bonnie Rock background) demonstrated significantly higher seed yield in drought conditions than control plants (Figures 4 and 5). Inconsistent results were obtained for another transgenic line from the first hybrid combination. The Line 7–5 (IGW-2971 background) did not have improved seed yield compared with control plants under limited watering (Figure 5) but had better drought survival rate than other well-performing lines (Figure 4). Conflicting results were also obtained for lines of the hybrid combination MagentaxBW8-9-10-3. Two transgenic lines of this hybrid were evaluated for both drought survival rates and performance under limited watering (Figures 4 and 5). Despite good drought survival rates, the same lines showed no yield improvement under moderate drought conditions compared with WT plants (cv. Magenta). The results of our drought survival test do not necessary correlate with seed yield under moderate drought conditions, and this observation needs more attention in further research. A number of recent reviews emphasizes that strong drought conditions used in glasshouse experiments can lead to results that are very different from those obtained in the field, where drought usually has a moderate or cyclical character. Disparate outcomes have been observed in studies with Arabidopsis (Skirycz *et al.*, 2011) and crop species (Langridge and Reynolds, 2015; Lawlor, 2013; Tuberosa, 2012). Nevertheless, in our study, transgenic Line 5–8 (IGW-2971 background) and Line 4–1 (Bonnie Rock background) demonstrated promising results in drought survival test and also produced significantly higher yield in moderate drought conditions. It is important to note that the yield assay system used would only allow detection of major differences in yield; the two positive lines both showed around 20% increased yield under drought relative to controls. More extensive trials, particularly under normal field conditions, will be needed. With the aim of better understanding the physiological differences between transgenic and parental plants, these trials should include the collection of data on a range of drought physiological parameters, such as membrane thermo-stability, Fv/Fm ratios, chlorophyll measurements, osmotic adjustment, photosynthetic rate, canopy temperature and carbon isotope discrimination. F_3BC_3 progeny of Line 5–8 (IGW-2971 background) and Line 4–1 (Bonnie Rock background) are currently being multiplied for field trials.

The *TaDREB3* gene has been demonstrated to confer a significant enhancement of frost tolerance in transgenic barley when over-expressed using stress-inducible promoters (Kovalchuk *et al.*, 2013; Morran *et al.*, 2011). However, in this study, comparison of the frost tolerance of two hybrid lines (IGW-2971xBW8-9-10-3 and Bonnie RockxBW8-9-10-3) with those of control plants revealed no significant differences. There was also no detectable increase in transgene expression in response to cold treatment (Figure S3). These data correlate with earlier findings for *Rab17*-driven expression of *TaDREB3* in wheat (cv. Bobwhite; Morran *et al.*, 2011). Our findings indicate that the *ZmRab17* promoter behaves in the same way in all tested wheat backgrounds: namely, no basal constitutive component of activity, no induction by cold, and strong activation in four from five tested backgrounds (including cv. Bobwhite) by the dehydration component of drought.

It is important to emphasize that the four recipient cultivars chosen for this study are modern, elite Australian varieties of bread wheat that already have excellent yield and stress tolerance characteristics. Yet our results indicate that further improvement of drought tolerance and performance of such cultivars by transfer of a single transgene is possible. These findings support the potential for further gains in drought tolerance and improved yield under limited watering regimes by application of genetic engineering technologies and their incorporation into conventional breeding programs.

Experimental procedures

Plant material

Four bread wheat (*Triticum aestivum* L.) cultivars (Bonnie Rock, IGW-2971, Magenta and Wyalkatchem) were kindly provided by Robyn McLean, Department of Agriculture and Food, Western Australia (Australia).

The preparation of *pZmRab17::TaDREB3* construct and generation of transgenic wheat cv. Bobwhite plants using biolistic bombardment was described previously (Ismagul *et al.*, 2014; Kovalchuk *et al.*, 2009; Morran *et al.*, 2011). The progeny of the transgenic line BW8-9-10-3 (T_3 generation) were used for crossing. This transgenic line had multiple transgene insertions, stable transgene expression across three generations and significantly enhanced drought tolerance compared with WT plants.

Plant hybridization

Progeny of transgenic line BW8-9-10-3 and four recipient bread wheats were grown together in a greenhouse for crossing. One plant from each of four recipients was used as a maternal parent for hybridization. Florets were emasculated and isolated with paper bags. Pollen was collected from several T_3 plants of BW8-9-10-3 and used for pollination. F_1 hybrid plants were grown to flowering, and a single plant from each cross was randomly selected as a pollen producer for backcrossing with the respective recurrent parent. Backcrosses were repeated three times using the recipient bread wheat cultivar as the maternal parent, and a single plant for each backcross and combination was selected as a pollinator, based on PCR analysis of transgene presence. Four F_1BC_3 sib-lines were represented by 7, 9, 2 and 6 plants after manual pollination of each cross-combination with pollen of recurrent parents: Bonnie Rock, IGW-2971, Magenta and Wyalkatchem, respectively. Transgenic lines in four F_2BC_3 cross-combinations were produced as a result of paper bag-controlled self-pollination of each individual plant from the F_1BC_3 progenies.

Detection of transgene presence using semi-nested PCR

DNA was extracted from young leaf tissue using a freeze-dry method (Shavrukov *et al.*, 2010). The conditions of semi-nested PCR, which was used to determine the presence of the transgene, are described in Data S1.

Analysis of transgene expression

Two leaves were collected from well-watered 3 week-old plants. One leaf was immediately frozen in liquid nitrogen and used as a control. The second leaf was subjected to 5 h of dehydration at room temperature (about 23 °C) until the first visual symptoms of dehydration (wilting) became obvious. For cold/frost treatment experiments, a single leaf was collected from each plant at room temperature before application of cold/frost, and a second leaf collected after a slow temperature decrease (three hours) from room temperature to 4 °C followed by 4 h of incubation at 4 °C. After sampling, these plants were immediately exposed to a frost treatment as described by Morran *et al.* (2011). RNA was extracted from leaf samples using TRIzol-like reagent and a modified method (Chomczynski and Sacchi, 1987), as described by Shavrukov *et al.* (2013). Transgene expression was analysed using Northern blot hybridization as described by Sambrook and Russell (2001). For the frost tolerance experiment, quantitative RT-PCR (qRT-PCR) analyses of *TaDREB3* gene expression in leaves were performed using transgene specific primers (Table S1). Two housekeeping genes, *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) and *Cyclophilin* (*Cyc*), were used for normalization. The conditions for qRT-PCR have been described previously (Burton *et al.*, 2008).

Estimation of transgene copy number

Quantitative PCR on genomic DNA was used for estimation of transgene copy number, essentially as described by Kovalchuk *et al.* (2013). For template loading normalization, PCRs were performed using primers and probes complimentary to the single-copy endogenous reference gene *Puroindoline-b* (*Pin-b*) (Li *et al.*, 2004; Zhiwu *et al.*, 2004). The oligonucleotide sequences of primers *Pin-b*-F and *Pin-b*-R are given in Table S1. The TaqMan probe was dual-labelled with 5′-CAL-fluor-Gold540 and BHQ1-3′, and a portion of the *nos* terminator was used as the target sequence (primers given in Table S1).

Characterization of growth and yield under well-watered conditions

Eight biological replicates for each line in the F_2BC_3 generation, their corresponding null-segregants and WT plants, were grown to maturity in a greenhouse, in 10-cm square pots (one plant per pot) filled with a coco-peat-based potting mix. Plants were grown under well-watered conditions, at 24 °C/16 °C, day/night temperature, and 16 h long day. Measurements of plant height and yield components (e.g. seed weight per plant, seed number per spike and number of spikes) were recorded at harvesting.

Assessment of survival rates under stringent drought and frost conditions

Plants of the F_2BC_3 generation were tested for transgene presence and expression, and selected lines were used in drought tolerance tests. Seeds were germinated in Petri dishes, and seedlings were grown for 1 week. During this time, transgene presence was confirmed by semi-nested PCR, and plants containing the transgene were transferred to 15 cm diameter pots filled with coco-peat. Each pot contained three randomly selected transgene-positive plants from a single line, one confirmed null-segregant from the same cross and one WT plant of the maternal cultivar used in the initial cross. Twelve biological replicates from each F_2BC_3 family were used in the experiment. Plants were grown in a growth room, using conditions previously described by Morran *et al.* (2011). After 1 month of growth under well-watered conditions, water was withheld for 12 days. When the volumetric water content (VWC) in soil decreased to 2–3% and severe wilting symptoms were observed, the plants were re-watered. Numbers of surviving plants were recorded 12 days after re-watering. The experiment was repeated five times.

For frost survival stress, 1-month-old transgenic and WT plants were grown in pots as described for the drought tolerance test. Two stable non-segregating transgenic lines (F_3BC_3 generation) were selected for the experiment, from different hybrid combi-

nations: Line 5–8 from IGW-2971xBW8-9-10-3 and Line 4–1 from Bonnie RockxBW8-9-10-3. Two transgenic plants from a single line and two plants of the corresponding WT were grown in each pot. Twelve biological replicates of each transgenic line and WT in six pots were used in each of three survival tests. In total, 36 plants for each transgenic line and the same number of WT plants were used in these experiments. The frost survival test was performed according to Morran *et al.* (2011), except here the frost treatment was set to −7 °C for 4 h. After the frost treatment, plants were returned to the growth room and numbers of surviving plants were recorded after 2 weeks of recovery.

Seed yield analysis for plants growing in large containers under sufficient and limited watering

Two transgenic lines (F_3BC_3 generation) were selected from each cross-combination with wheat cultivars IGW-2971, Bonnie Rock and Magenta, and their phenotypes and yields were compared to those of the corresponding recurrent parents and null-segregants. The experiment was conducted using a controlled watering regime, in two large containers (190 × 68 × 60 cm) filled with a 1 : 1 : 1 mix of coco-peat soil, river sand and clay soil collected near Adelaide (South Australia). Plants were grown in rows, eight plants per row for each backcross line, null-segregant and WT. Two randomized blocks were used in each container comprising in total 16 biological replicates for each hybrid line, null-segregant and WT. Experimental plants were flanked by a border row of WT plants on each short side of the container. The experimental design was identical for the two containers, one of which was used for growing plants under well-watered conditions and the other for growing with restricted watering. Containers were watered every second day using an automatic dripping system. Watering was withheld in the drought treatment container when the majority of plants started tillering. The soil water potential in this container reached -0.3 ± 0.05 MPa a short time before flowering. Plants were subject to slowly increasing drought until 10 days after the end of flowering, when water potential reached -0.5 MPa. Watering was then recommenced, and the soil water content restored to a level similar to the well-watered container. Soil water content was monitored using the system, Magpie-3 (Measuring Engineering Australia, www.mea.com.au), where data for water content in the soil were regularly collected and automatically recorded with sensors positioned in each container at three soil depths (15, 30 and 45 cm). Measurements of yield components were taken at harvest. Leaf material was sampled from all plants and used for genotyping using PCR and analysis of gene expression using Northern blot hybridization. The absence of the transgene in WT plants and null-segregants was confirmed using PCR analysis. As no significant differences were found in plant growth between the two blocks in preliminary experiments (Data not shown), all replicates for each line, the null-segregants and WT plants were used to calculate means and standard errors for the measurements.

Statistical treatment of data

Single-factor ANOVAs and Student *t*-tests (unpaired, two-tails) from Microsoft Excel software were applied for statistical analyses of data from the plant phenotyping experiment and drought and frost tests. Both the Chi-square (χ^2) test and a Randomization test for goodness-of-fit were used for comparative analyses of experimental and expected data for segregations of transgene copy numbers. The χ^2 test was used manually for plants (n = 24–48) in lines derived from each hybrid combination. A Randomization test for goodness-of-fit with 10 000 repeats was used for each of the transgenic lines because numbers of analysed plants (12 per line) were too few for the χ^2 test (http://udel.edu/~mcdonald/statrand.html).

Acknowledgements

We thank Ursula Langridge and Alex Kovalchuk for assistance with growing plants, Tatiana Pyvovarenko for Northern blot hybridization and Julie Hayes for critically reading the manuscript. This work was supported by the Australian Research Council, the Grains Research and Development Corporation, and the Government of South Australia. Authors declare no conflict of interest.

References

Agarwal, P.K., Agarwal, P., Reddy, M.K. and Sopory, S.K. (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Rep.* **25**, 1263–1274.

Bahieldin, A., Mahfouz, H.T., Eissa, H.F., Saleh, O.M., Ramadan, A.M., Ahmed, I.A., Dyer, W.E., El-Itriby, H.A. and Madkour, M.A. (2005) Field evaluation of transgenic wheat plants stably expressing the *HVA1* gene for drought tolerance. *Physiol. Plant.* **123**, 421–427.

Bourdon, V., Ladbrooke, Z., Wickham, A., Lonsdale, D. and Harwood, W. (2002) Homozygous transgenic wheat plants with increased luciferase activity do not maintain their high level of expression in the next generation. *Plant Sci.* **163**, 297–305.

Burton, R.A., Jobling, S.A., Harvey, A.J., Shirley, H.J., Mather, D.E., Bacic, A. and Fincher, G.B. (2008) The genetics and transcriptional profiles of the cellulose synthase-like *HvCslF* gene family in barley. *Plant Physiol.* **146**, 1821–1833.

Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162**, 156–159.

Cui, M., Zhang, W., Zhang, Q., Xu, Z., Zhu, Z., Duan, F. and Wu, R. (2011) Induced over-expression of the transcription factor OsDREB2A improves drought tolerance in rice. *Plant Physiol. Biochem.* **49**, 1384–1391.

Fu, D., Huang, B., Xiao, Y., Muthukrishnan, S. and Liang, G.H. (2007) Overexpression of barley *hva1* gene in creeping bentgrass for improving drought tolerance. *Plant Cell Rep.* **26**, 467–477.

Huang, D., Wu, W., Abrams, S.R. and Cutler, A.J. (2008) The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. *J. Exp. Bot.* **59**, 2991–3007.

Ismagul, A., Iskakova, G., Harris, J.C. and Eliby, S. (2014) Biolistic transformation of wheat with centrophenoxine as a synthetic auxin. In *Crop Breeding. Methods in Molecular Biology*, Vol. **1145** (Fleury, D. and Whitford, R., eds), pp. 191–202. New York: Springer.

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant Cell Physiol.* **47**, 141–153.

James, V.A., Neibaur, I. and Altpeter, F. (2008) Stress inducible expression of the DREB1A transcription factor from xeric, *Hordeum spontaneum* L. in turf and forage grass (*Paspalum notatum* Flugge) enhances abiotic stress tolerance. *Transgenic Res.* **17**, 93–104.

Kovalchuk, N., Smith, J., Pallotta, M., Singh, R., Ismagul, A., Eliby, S., Bazanova, N., Miligan, A.S., Hrmova, M., Langridge, P. and Lopato, S. (2009) Characterization of the wheat endosperm transfer cell-specific protein TaPR60. *Plant Mol. Biol.* **71**, 81–98.

Kovalchuk, N., Jia, W., Eini, O., Morran, S., Pyvovarenko, T., Fletcher, S., Bazanova, N., Harris, J., Beck-Oldach, K., Shavrukov, Y., Langridge, P. and Lopato, S. (2013) Optimization of *TaDREB3* gene expression in transgenic barley using cold-inducible promoters. *Plant Biotechnol. J.* **11**, 659–670.

Langridge, P. and Reynolds, M.P. (2015) Genomic tools to assist breeding for drought tolerance. *Curr. Opin. Biotechnol.* **32**, 130–135.

Lata, C. and Prasad, M. (2011) Role of DREBs in regulation of abiotic stress responses in plants. *J. Exp. Bot.* **62**, 4731–4748.

Lawlor, D.W. (2013) Genetic engineering to improve plant performance under drought: Physiological evaluation of achievements, limitations, and possibilities. *J. Exp. Bot.* **64**, 83–108.

Lewis, R.S. and Kernodle, S.P. (2009) A method for accelerated trait conversion in plant breeding. *Theor. Appl. Genet.* **118**, 1499–1508.

Li, Z., Hansen, J.L., Liu, Y., Zemetra, R.S. and Berger, P.H. (2004) Using real-time PCR to determine transgene copy number in wheat. *Plant Mol. Biol. Rep.* **22**, 179–188.

Li, J., Sima, W., Ouyang, B., Wang, T., Ziaf, K., Luo, Z., Liu, L., Li, H., Chen, M., Huang, Y., Feng, Y., Hao, Y. and Ye, Z. (2012) Tomato *SlDREB* gene restricts leaf expansion and internode elongation by downregulating key genes for gibberellin biosynthesis. *J. Exp. Bot.* **63**, 6407–6420.

Mitchell, R.A.C., Joyce, P.A., Rong, H., Evans, V.J., Madgwick, P.J. and Parry, M.A.J. (2004) Loss of decreased-rubisco phenotype between generations of wheat transformed with antisense and sense *rbc*S. *Annals Appl. Biol.* **145**, 209–216.

Morran, S., Eini, O., Pyvovarenko, T., Parent, B., Singh, R., Ismagul, A., Eliby, S., Shirley, N., Langridge, P. and Lopato, S. (2011) Improvement of stress tolerance of wheat and barley by modulation of expression of DREB/CBF factors. *Plant Biotechnol. J.* **9**, 230–249.

Ni, F.T., Chu, L.Y., Shao, H.B. and Liu, Z.H. (2009) Gene expression and regulation of higher plants under soil water stress. *Curr. Genomics*, **10**, 269–280.

Pellegrineschi, A., Reynolds, M., Pacheco, M., Brito, R.M., Almeraya, R., Yamaguchi-Shinozaki, K. and Hoisington, D. (2004) Stress-induced expression in wheat of the *Arabidopsis thaliana DREB1A* gene delays water stress symptoms under greenhouse conditions. *Genome*, **47**, 493–500.

Rieben, S., Kalinina, O., Schmid, B. and Zeller, S.L. (2011) Gene flow in genetically modified wheat. *PLoS ONE*, **6**, e29730.

Rooke, L., Steele, S.H., Barcelo, P., Shewry, P.R. and Lazzeri, P.A. (2003) Transgene inheritance, segregation and expression in bread wheat. *Euphytica*, **129**, 301–309.

Roy, S., Banerjee, A., Tarafdar, J. and Senapati, B.K. (2012) Detection of probable marker-free transgene-positive rice plants resistant to rice tungro disease from backcross progenies of transgenic Pusa Basmati 1. *J. Genet.* **91**, 213–218.

Sahrawat, A.K., Becker, D., Lütticke, S. and Lörz, H. (2003) Genetic improvement of wheat via alien gene transfer, an assessment. *Plant Sci.* **165**, 1147–1168.

Sambrook, J. and Russell, D. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd edn. New York: Cold Spring Harbor Laboratory Press.

Shanker, A.K., Maheswari, M., Yadav, S.K., Desai, S., Bhanu, D., Attal, N.B. and Venkateswarlu, B. (2014) Drought stress responses in crops. *Funct. Integr. Genomics* **14**, 11–22.

Shavrukov, Y., Gupta, N.K., Miyazaki, J., Baho, M.N., Chalmers, K.J., Tester, M., Langridge, P. and Collins, N.C. (2010) *HvNax3* – a locus controlling shoot sodium exclusion derived from wild barley (*Hordeum vulgare* ssp. *spontaneum*). *Funct. Integr. Genomics*, **10**, 277–291.

Shavrukov, Y., Bovill, J., Afzal, I., Hayes, J.E., Roy, S.J., Tester, M. and Collins, N.C. (2013) *HVP10* encoding V-PPase is a prime candidate for the barley *HvNax3* sodium exclusion gene: evidence from fine mapping and expression analysis. *Planta*, **237**, 1111–1122.

Singh, A., Sengar, K. and Sengar, R.S. (2013) Gene regulation and biotechnology of drought tolerance in rice. *Inter. J. Biotechnol. Bioengineer. Res.* **4**, 547–552.

Skirycz, A., Vandenbroucke, K., Clauw, P., Maleux, K., De Meyer, B., Dhondt, S., Pucci, A., Gonzalez, N., Hoeberichts, F., Tognetti, V.B., Galbiati, M., Tonelli, C., van Breusegem, F., Vuylsteke, M. and Inzé, D. (2011) Survival and growth of *Arabidopsis* plants given limited water are not equal. *Nature Biotechnol.* **29**, 212–214.

Terzi, V., Pastori, G., Shewry, P.R., Fonzo, N.D., Stanca, A.M. and Faccioli, P. (2005) Real-time PCR-assisted selection of wheat plants transformed with HMW glutenin subunit genes. *J. Cereal Sci.* **41**, 133–136.

Tsai, Y.T., Chen, P.Y. and To, K.Y. (2012) Plant regeneration and stable transformation in the floricultural plant *Cleome spinosa*, a C_3 plant closely related to the C_4 plant *C. gynandra*. *Plant Cell Rep.* **31**, 1189–1198.

Tuberosa, R. (2012) Phenotyping for drought tolerance of crops in the genomics era. *Frontiers Physiol.* **3**, 347.

Vasil, I.K. (2007) Molecular genetic improvement of cereals: transgenic wheat (*Triticum aestivum* L.). *Plant Cell Rep.* **26**, 1133–1154.

Vilardell, J., Mundy, J., Stilling, B., Leroux, B., Pla, M., Freyssinet, G. and Pages, M. (1991) Regulation of the maize *Rab17* gene promoter in transgenic heterologous systems. *Plant Mol. Biol.* **17**, 985–993.

Vilardell, J., Martinezzapater, J.M., Goday, A., Arenas, C. and Pages, M. (1994) Regulation of *Rab17* gene promoter in transgenic *Arabidopsis* wild-type, ABA-deficient and ABA-insensitive mutants. *Plant Mol. Biol.* **24**, 561–569.

Warburton, M.L., Skovmand, B. and Mujeeb-Kazi, A. (2002) The molecular genetic characterization of the 'Bobwhite' bread wheat family using AFLPs and the effect of the T1BL.1RS translocation. *Theor. Appl. Genet.* **104**, 868–873.

Xue, G.P., Way, H.M., Richardson, T., Drenth, J., Joyce, P.A. and McIntyre, C.L. (2011) Overexpression of *TaNAC69* leads to enhanced transcript levels of stress up-regulated genes and dehydration tolerance in bread wheat. *Mol. Plant* **4**, 697–712.

Yamaguchi-Shanozaki, K. and Shinozaki, K. (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Ann. Rev. Plant Biol.* **57**, 781–803.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (2009) DREB regulons in abiotic-stress-responsive gene expression in plants. In *Molecular Breeding of Forage and Turf. The Proceedings of the 5th International Symposium on the Molecular Breeding of Forage and Turf* (Yamada, T. and Spangenberg, G., eds.), pp. 15–28. New York: Springer.

Yao, Q., Cong, L., Chang, J.L., Li, K.X., Yang, G.X. and He, G.Y. (2006) Low copy number gene transfer and stable expression in a commercial wheat cultivar via particle bombardment. *J. Exp. Bot.* **57**, 3737–3746.

Zhang, X., Liang, R., Chen, X., Yang, F. and Zhang, L. (2003) Transgene inheritance and quality improvement by expressing novel HMW glutenin subunit (HMW-GS) genes in winter wheat. *Chinese Sci. Bull.* **48**, 771–776.

Zhiwu, L., Hansen, J.L., Ying, L., Zemetra, R.S. and Berger, P.H. (2004) Using real-time PCR to determine transgene copy number in wheat. *Plant Mol. Biol. Rep.* **22**, 179–188.

Supporting information

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

Figure S1 Seed yields of plants grown in large containers under well-watered conditions.

Figure S2 Survival rates of two transgene-containing F_3BC_3 backcross lines that performed well in the drought tolerance experiment, and their corresponding parental cultivars.

Figure S3 Expression levels of *TaDREB3* in plants of two WT and two transgene-containing backcross lines under normal growth conditions (+24 °C) and under cold stress (+4 °C), as determined by qRT-PCR.

Table S1 Primers and probes used in this study.

Data S1 The conditions of semi-nested PCR, which were used to determine presence of the transgene.