Stress-induced expression in wheat of the *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions

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Abstract: One of the major environmental factors limiting plant productivity is lack of water. This is especially true for the major cereals maize, rice, and wheat, which demonstrate a range of susceptibility to moisture deficit. Although conventional breeding and marker-assisted selection are being used to develop varieties more tolerant to water stress, these methods are time and resource consuming and germplasm dependent. Genetic engineering is attractive because of its potential to improve abiotic stress tolerance more rapidly. Transcription factors have been shown to produce multiple phenotypic alterations, many of which are involved in stress responses. *DREB1A*, a transcription factor that recognizes dehydration response elements, has been shown in *Arabidopsis thaliana* to play a crucial role in promoting the expression of drought-tolerance genes. In our efforts to enhance drought tolerance in wheat, the *A. thaliana DREB1A* gene was placed under control of a stress-inducible promoter from the *rd29A* gene and transferred via biolistic transformation into bread wheat. Plants expressing the *DREB1A* gene demonstrated substantial resistance to water stress in comparison with checks under experimental greenhouse conditions, manifested by a 10-day delay in wilting when water was withheld.

Key words: Wheat transformation, MPB Bobwhite 26, *DREB1A*, *rd29* promoter, moisture stress tolerance.

Résumé : Une des contraintes environnementales les plus importantes B la productivité agricole est le manque d'eau. Ceci est particulièrement vrai pour les principales espèces de céréales cultivées, le maïs, le riz et le blé, puisque celles-ci montrent une grande gamme de sensibilité B un déficit hydrique. Bien que l'amélioration génétique conventionnelle et la sélection assistée soient mises B contribution pour développer des variétés plus tolérantes face B un stress hydrique, ces méthodes nécessitent temps et argent en plus de dépendre de la disponibilité de ressources génétiques adéquates. L'ingénierie génétique semble attrayante en raison de son potentiel d'augmenter plus rapidement la tolérance aux stress abiotiques. Des facteurs de transcription sont connus pour induire de nombreuses altérations phénotypiques et plusieurs de celles-ci sont impliquées dans les réponses face aux stress. Il a été démontré que *DREB1A*, un facteur de transcription qui reconnaît les éléments cis de réponse au stress hydrique, joue un rôle critique chez *Arabidopsis thaliana* pour stimuler l'expression des gPnes de tolérance B la sécheresse. Dans le cadre d'efforts visant B accroître la tolérance B la sécheresse chez le blé, le gPne *DREB1A* de l'*A. thaliana* a été placé sous le contrôle du promoteur inductible par le stress du gène *rd29A* et introduit chez le blé tendre par transformation biolistique. Les plantes exprimant le gène *DREB1A* ont montré une résistance accrue au stress hydrique (un flétrissement retardé de dix jours suite B l'interruption de l'arrosage) par rapport aux témoins lors d'expériences en serre.

Mots clés : transformation du blé, MPB Bobwhite26, *DREB1A*, promoteur du *rd29*, tolérance au sarss hydrique.

[Traduit par la Rédaction]

Received 5 August 2003. Accepted 21 November 2003. Published on the NRC Research Press Web site at http://genome.nrc.ca on 10 May 2004.

Corresponding Editor: P. Donini.

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 doi: 10.1139/G03-140

Introduction

Drought is one of the major factors contributing to severe yield loss of wheat grown in marginal lands and to significantly reduce yields in temperate areas (Morris et al. 1991; Trethowan et al. 2001). Several breeding strategies, including marker-assisted selection, are being used to improve crop yields under water stress. These have been somewhat successful, but they are limited by the time and other resources required. To improve the efficiency of such strategies and to develop new options, a better understanding of the physiological and molecular bases of drought tolerance in plants is required (Trethowan et al. 2001; Blum 1998). With respect to physiological aspects of drought tolerance in crop plants, a number of mechanisms have been studied that operate at the whole plant level (Blum 1998; Richards 1996; Edmeades et al. 1997). Although such traits are likely to be associated with improved productivity, they are frequently genetically complex and therefore require considerable effort to manipulate in a breeding program, where phenotypic expression is subject to interaction with the environment.

Molecular mechanisms of water stress response have been investigated primarily in the model plant species *Arabidopsis thaliana* (Liu et al. 1998; Iuchi et al. 2000). Although they are often associated with stress survival, they are relatively simple genetically and therefore lend themselves to genetic manipulation as well as potential manipulation of their level of expression via regulatory genes. In *Arabidopsis*, stress responses to limited water are reportedly mediated through abscisic acid (ABA) dependent and independent signals that regulate the expression of genes involved in tolerance to stress (Iuchi et al. 2000). In the last few years, several studies have analyzed the gene products that are believed to function in the accumulation of osmoprotectants (e.g., sugars) in stress-signaling pathways and in transcriptional regulation (Kleines et al. 1999; Miyazaki et al. 1999; Rotein et al. 2002; Scippa et al. 2000; Taji et al. 2002). These studies provide clues to understanding stress tolerance in plants. Analyses of the expression of dehydration-inducible genes have shown that at least four independent signaling pathways function in the induction of stress-inducible genes in response to dehydration: two are ABA dependent (Uno et al. 2001; Velasco et al. 1998) and two are ABA independent (Savoure et al. 1997). Several stress-induced genes, such as *rd29A* in *A. thaliana*, are induced through the ABA-independent pathway (Chak et al. 2000). Dehydration-responsive element binding gene 1 (*DREB1*) and *DREB*2 are transcription factors that bind to the promoter of genes such as *rd29A*, thereby inducing expression in response to drought, salt, and cold (Stockinger et al. 1997; Shen et al. 2003). *DREB1* and *DREB2* are members of the *AP2*/*EREBP* plant-specific family of transcription factors (Shinozaki and Yamaguchi-Shinozaki 2000; Yamaguchi-Shinozaki and Shinozaki 1993, 1994). The *DREB1* genes are believed to interact with the DRE and induce expression of stress tolerance genes and functions in response to cold, whereas *DREB2* genes are involved in drought-responsive gene expression (Liu et al. 1998; Seki et al. 2001). *DREB1A* has been overexpressed in transgenic *Arabidopsis* plants, and the resulting phenotype showed a strong induction of the expression of the target genes under unstressed conditions but also caused dwarfed phenotypes in the transgenic plants. These transgenic plants also revealed freezing and dehydration tolerance. In contrast, overexpression of *DREB2A* induced weak expression of the target genes under unstressed conditions and caused growth retardation of the transgenic plants (Liu et al. 1998; Kasuga et al. 1999). The stress-regulated expression of the *DREB1A* gene by the *rd29*A promoter produced plants with increased tolerance to freezing, salt, and drought stresses without a drastic change in the normal phenotype of the transformed plants (Kasuga et al. 1999).

To determine if transcriptional factors such as *DREB1A* could play a role in enhancing the tolerance of wheat and possibly other crops to water stress, transgenic bread wheat plants were developed that contain the *DREB1A* gene driven by the *rd29A* promoter, and the plants were studied for the effect(s) of this gene under water stress in the greenhouse.

Materials and methods

Recombinant plasmids

Two plasmids were used in the cotransformation experiments. The plasmid pAHC25 (Christensen and Quail 1996) contained the *gusA* reporter gene and the *bar* selectable marker (conferring resistance to DL-phosphinothricin (PPT)), with each gene under the control of the maize ubiquitin promoter. To make the construct containing the *A. thaliana rd29A*:*DREB1A* cDNA, a *Bam*HI fragment of the *DREB1A* cDNA was cloned into the *Bam*HI site of the plasmid pBI29ApNot. pBI29ApNot was constructed by ligation of the *Hin*dIII fragment of the *rd29A* promoter into the *Hin*dIII fragment of the pBI101 (Clontech, Palo Alto, Calif.), as described in Kasuga et al. (1999). This plasmid was predicted to express the complete *DREB1A* transcript only under stress conditions.

Transformation, selection, and regeneration procedure

Microprojectile-mediated transformation of wheat was carried out according to the procedure described in Pellegrineschi et al. (2002). After bombardment, the embryos were transferred to E3 medium (Pellegrineschi et al. 2002) supplemented with 2.5 mg 2,4-dichlorophenoxyacetic acid·L^{-1} (Duchefa) for somatic embryo induction. Twenty days later, the regenerating embryos were transferred to E3 selection medium containing 5 mg PPT·L^{-1} and grown in a growth chamber at 26 °C for 30 days. T_0 plantlets resistant to PPT were transferred again to E3 selection medium and allowed to grow as described in Pellegrineschi et al. (2002). After shoots had reached a height of 1–3 cm, the plantlets were transferred to rooting media containing MS medium (Murashige and Skoog 1962) plus 5 mg PPT·L^{-1}. After two weeks, the plantlets were transferred to soil and grown in a greenhouse to maturity.

Selection of transgenic lines and phenotype analysis

Plantlets were further selected for PPT resistance at the five- to six-leaf stage by spraying them with 0.3% (*w*/*v*) Basta™ solution. The surviving plantlets were then tested for the presence of the *DREB1A* and *bar* genes by PCR. All plantlets PCR positive for the *DREB1A* and *bar* genes were self-pollinated to produce the T_1 generation. All T_1 families were then subjected to water stress in the greenhouse. The water stress evaluation was first calibrated by subjecting

five- to six-leaf-stage plants of nontransgenic MPB-Bobwhite26 (the transformed variety) to various levels of water deficit in small square pots (5 cm × 5 cm) containing 25 g of a dry soil mix (one third peat, one third sand, and one third topsoil). Evaluations were conducted in the greenhouse with a photoperiod of 16 h light : 8 h dark, a temperature between 18 and 30 °C, and humidity maintained at 75%. Withholding water for 10–15 days gave the best discrimination between transgenic and control plants because all control plants died. Subsequent stress evaluations were conducted by subjecting 20 transgenic plants from each T_1 family and 20 control plants (nontransgenic MPB-Bobwhite 26), at the same developmental stage, to this water stress regime. The experiment was conducted in trays and the moisture level was measured with a soil moisture meter (U.S. Irrigation, Lincoln, Nebr.) with a scale from 0 to 10 (10 = 100% total moisture, 8–6 = 80–60% total moisture, 6–4 = 60–40% total moisture, 4–2 = 40–20% total moisture, and 2–0 = 20–0% total moisture). T_1 plants that were tolerant to water stress were grown to maturity, selfed (selfed T_2 families), and crossed to nontransgenic MPB-Bobwhite26 (crossed T_2 families). The T_2 homozygote lines (selected by progeny testing) at the four- to five-leaf stage were used to further evaluate drought tolerance in the *DREB1A* plants. Severe water stress was induced until the control plants reached their limit of survival (10–12 days), at which time the normal water regime was resumed and continued until the plants matured. Data were gathered on the total number of heads, sterile heads, fully developed heads, late development of heads, and the number of plants that died during the experiment.

PCR analysis

Plant DNA was extracted from young leaves using the Nucleospin DNA purification kit from Clontech following the manufacturer's protocol. The primers used to amplify the *bar* gene were forward primer 5′-GTCTGCACC-ATCGTCAACC-3′ and reverse primer 5′-GAAGTCCAG-CTGCCAGAAAC-3′ and for the *DREB1A* gene were forward primer 5′-ATGAACTCATTTTCTGCT-3′ and reverse primer 5′-GTCGCATCACACATCTC-3′ derived from the *DREB* primers described in Liu et al. (1998). The PCRs were carried out in a 25-μL solution comprising 10 ng of wheat genomic DNA, 50 mM KCl, 10 mM Tris–HCl buffer (pH 8.8), 3 mM $MgCl_2$, 0.1% (*w*/*v*) Triton X-100, 0.24 mM each dNTP, 1 U *Taq* DNA polymerase, and 0.16 μmol of each primer. The PCR profiles for both genes were an initial denaturation at 94 °C for 1 min followed by 30 amplification cycles (94 °C for 30 s, 64 °C for 2 min, and 72 °C for 2 min). The PCR amplification products were separated in 1% (*w*/*v*) agarose gels.

RNA extraction and RT–PCR analyses

T_2 plants leaf samples were ground in liquid nitrogen and total RNA was extracted using Trizol reagent (Life Technologies, Cleveland, Ohio) according to the manufacturer's instructions. To remove residual DNA, 1 μg of RNA was incubated with 1 unit of RNAse-free DNAse (Invitrogen) for 15 min at room temperature. cDNA was synthesized using 1 unit of Superscript II enzyme (Invitrogen) and oligo(dT) according to the provider's instructions. RT–PCRs were carried out in a 10-μL solution comprising 5 μL of Red *Taq* Readymix (Sigma, St. Louis, Mo.) with 4 pmol of each primer and 1 μL of the reverse transcription reaction. The PCR profile was 30 cycles at 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s.

Primer sequences for the *DREB1A* transcripts were forward primer 5′-CGAGTCTTCGGTTTCCTCAG-3′ and reverse primer 5′-CAAACTCGGCATCTCAAACA-3′. Wheat actin was used as a positive control for the RT–PCR with 5′-GACCCAGACAACTCGCAACT-3′ as the forward primer and 5′-CTCGCATATGTGGCTCTTGA-3′ as the reverse primer. The PCR and gel electrophoresis conditions were as described in the PCR analysis section.

Southern hybridization analysis

Total genomic DNA was isolated from 1 g fresh mass of leaf material using the Nucleon phytopure plant DNA extraction kit according to the manufacturer's protocol (Amersham Life Sciences). A 50-μg aliquot of DNA was digested overnight at 37 °C with an appropriate restriction enzyme. The digested DNA was fractionated in a 1.0% (*w*/*v*) agarose gel and transferred to a positively charged nylon membrane (Roche). The *DREB1A* gene (see Fig. 2) was labeled with digoxigenin-dUTP by nick-translation and hybridized according to the manufacturer's instructions (Roche). Detection was achieved using the digoxigenin luminescent detection kit (Boehringer Mannheim) and the hybridization signals detected following exposure to Kodak film at 37 °C for 40 min.

Results and discussion

Development of the transgenic material for evaluation

More than 20 independent transformation experiments (bombarded rings of embryos) were conducted using the MPB-Bobwhite26 wheat cultivars (Pellegrineschi et al. 2002). A total of 447 T_0 plantlets were obtained from the large number of PPT-resistant somatic embryos and transferred to the greenhouse. The majority of these plants were at least partially fertile and produced seeds. The molecular analyses to detect the presence of the *bar* gene, carried out independently, showed that 100% of the Basta-resistant plants contained the *bar* gene, while only 25% (113/447) were positive for the *DREB1A* gene.

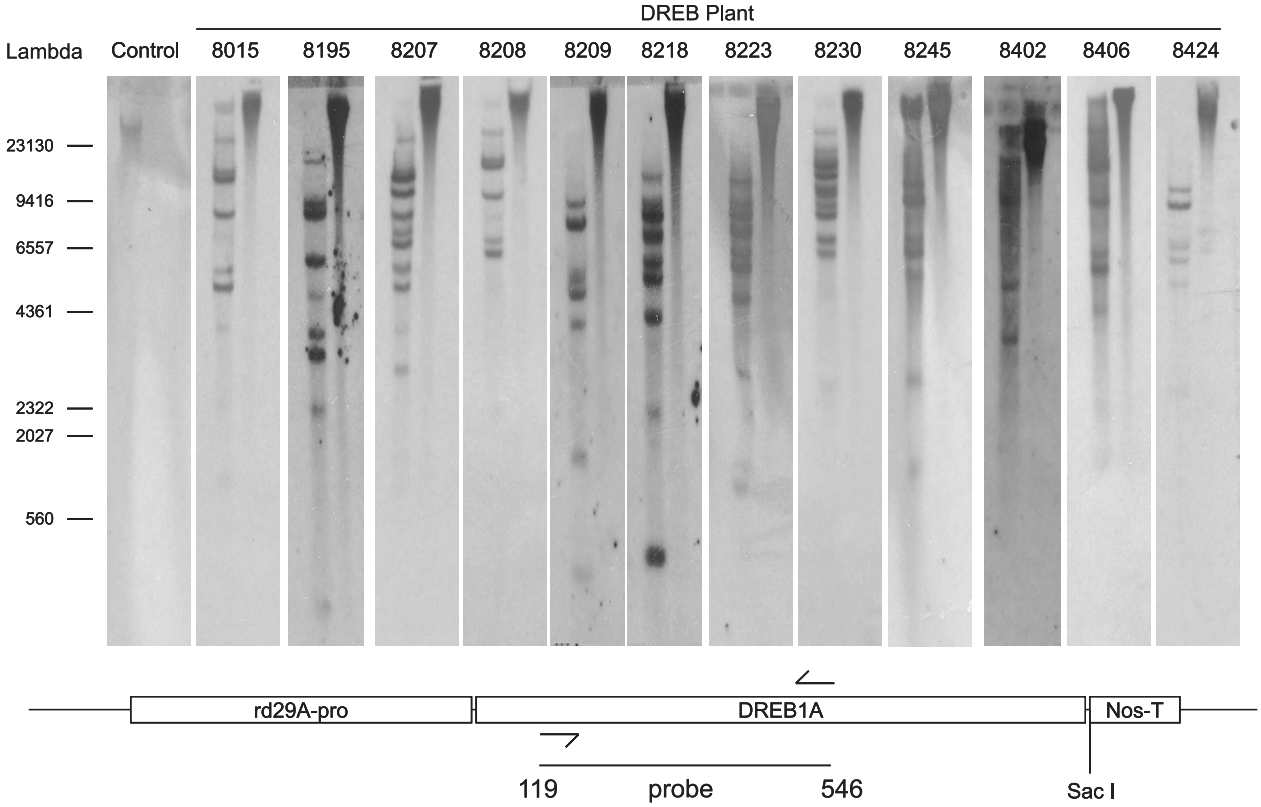
Southern blot analysis of T_1 individuals indicated that various copies of the *DREB1A* transgene were present and integrated into the genome of the transgenic plants. The phenotypic and genotypic segregation ratios of the *DREB1A* in the T_2 families derived from the stress-tolerant T_1 individuals were variable (data not shown). Among the 113 families derived from the T_0 individuals that were positive for *DREB1A*, 12 were selected based on the simple segregation patterns of the transgenes in the T_2 generation: 3:1 or 15:1 in progenies of selfed T_1 plants and 1:1 or 3:1 in progenies derived from T_1 plants crossed with the control (Table 1). All other families were discarded because of non-Mendelian segregation or because they did not show tolerance to water stress. This stringent selection process may have resulted in the selection of wheat phenotypes with greater adaptation to water stress, independent of the presence of the transgene. In the segregating progenies of the selected lines, however, the

Table 1. Segregation ratios of the T_2 families selected from T_1 individuals that were stress tolerant.

	Test crosses		Selfed	
Event No.	Phenotypic ratio (T:S)	Genotypic ratio (+:–)	Phenotypic ratio (T:S)	Genotypic ratio (+:–)
8015	12:14 (1:1)	8:5 (1:1)	24:8 (3:1)	20:5 (3:1)
8195	8:10 (1:1)	10:7 (1:1)	30:11 (3:1)	29:12 (3:1)
8207	14:3 (3:1)	12:5 (3:1)	28:2 (15:1)	30:2 (15:1)
8208	17:13 (1:1)	7:9 (1:1)	15:5 (3:1)	23:5 (3:1)
8209	10:9 (1:1)	8:8 (1:1)	20:7 (3:1)	32:8 (3:1)
8218	15:12 (1:1)	12:13 (1:1)	24:7 (3:1)	20:4 (3:1)
8223	10:4 (3:1)	9:1 (3:1)	35:2 (15:1)	37:3 (15:1)
8230	7:8 (1:1)	12: 8 (1:1)	12:3 (3:1)	25:6 (3:1)
8245	12:9 (1:1)	9:9 (1:1)	32:13 (3:1)	28:6 (3:1)
8402	13:15 (1:1)	11:10 (1:1)	23:6 (3:1)	22:5 (3:1)
8406	7:5 (1:1)	7:5 (1:1)	6:2 (3:1)	10:2 (3:1)
8424	16:12 (1:1)	14:10 (1:1)	27:9 (3:1)	27:8 (3:1)

Note: T, tolerant to drought stress; S, susceptible to drought stress; +, *DREB1A* gene present; –, *DREB1A* gene absent (based on PCR).

Fig. 1. Southern blot analysis of the 12 transgenic events selected. DNA cut with *Sac*I (single cut in the transgene) was placed in the first lane of each blot. The uncut plant DNA was placed in the second lane. Probe was a 428-bp fragment of the *DREB1A* gene.



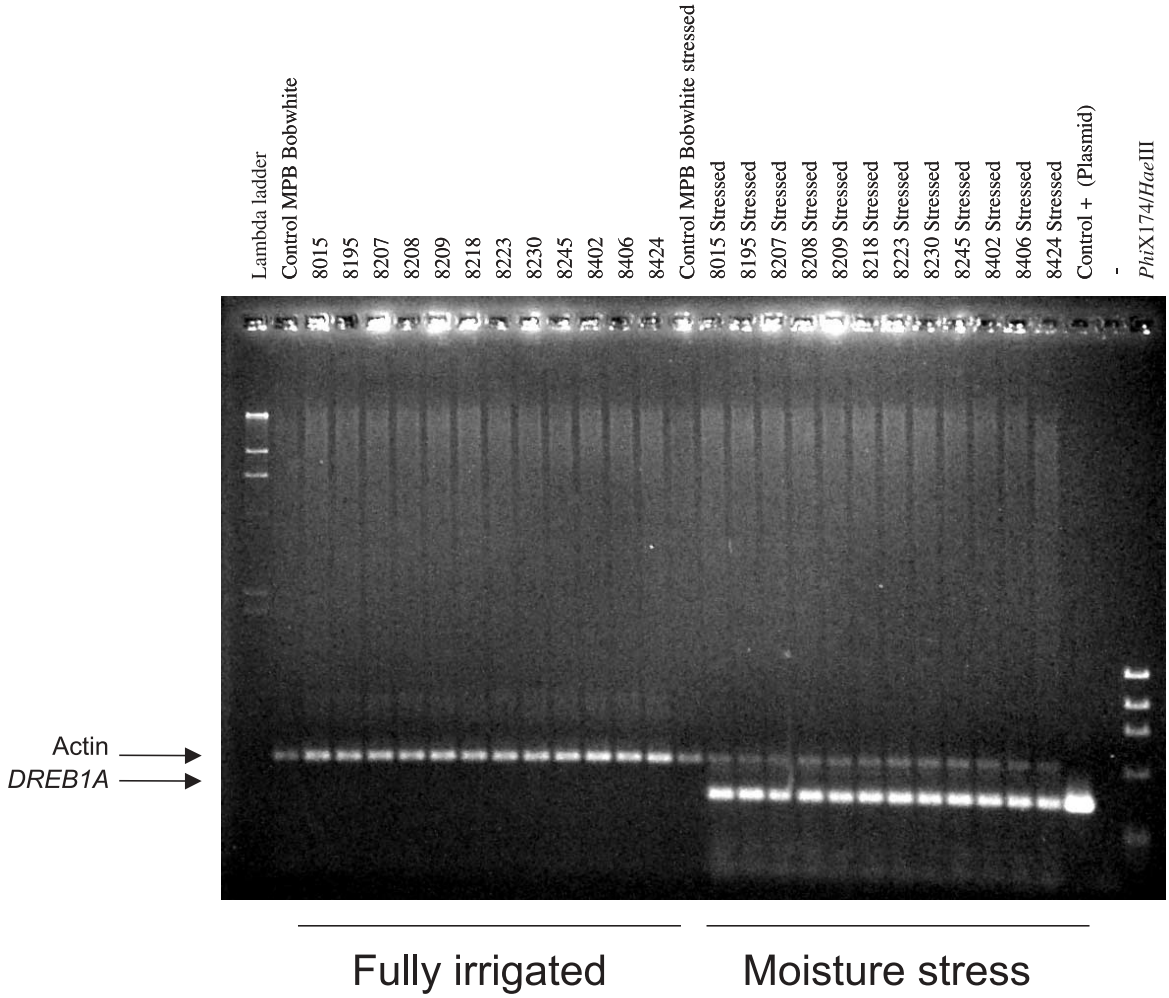
presence of the transgene was completely associated with the tolerant phenotype and its absence with the susceptible phenotype.

Characterization of transgene integration

Genomic DNA was isolated from individual plants in the 12 T_2 families selected for the presence of *bar* and *DREB1A* (PCR) and tolerant to low water stress. Each was digested with *Sac*I (single site in the *DREB1A* coding sequence) to give an estimate of the copy number of the transgene. The complexity of the transgene integration among the different plants was quite different (Fig. 1). The number of copies in the plants tested varied from an estimated minimum copy number of 5 to a maximum of 10 (Fig. 1).

The expression of the *actin* and *DREB1A* genes before and after water stress was determined in individual plants of

Fig. 2. RT–PCR analysis of *DREB1A* gene expression in control and transgenic plants before and after water stress. Lane 1, lambda molecular mass ladder; lanes 2–13, RNA isolated from plants grown under nonstress conditions; lanes 14–25, RNA isolated from plants after 2 days of stress; lane 26, PCR amplification of the plasmid used in the transformation; lane 28, *Phi*X174/*Hae*III molecular mass ladder.



the T_2 families by RT–PCR (Fig. 2). As expected, the *actin* gene was constitutively expressed in all plants before and after water stress. Transcripts of the *DREB1A* gene driven by the *rd29A* promoter were detected after only 2 days of water stress (Fig. 2). From these observations, we can conclude that the *A. thaliana rd29A* gene promoter is an effective water-stress-inducible promoter in wheat and that the *DREB1A* gene controlled by this promoter has a level of expression in wheat similar to that observed in *Arabidopsis*.

Evaluation of the response of the transgenic plants to water stress

After sowing, a delay in germination was observed in the transgenic lines, with the exception of events 8195 and 8424. Control MPB-Bobwhite26 plants germinated after 2–3 days, whereas the *DREB1A* plants showed nonuniform germination under both stressed and fully irrigated conditions. This delay was always associated with the presence of the *DREB1A* transgene, and the nonuniform germination was due to the segregation of the transgene (data not shown). However, no differences in plant growth or in plant morphology related to the presence of the *DREB1A* transgene were observed between the transgenic lines and controls during the first days of water stress, as reported in *Arabidopsis* (Kasuga et al. 1999). At the four to five-leaf stage, the plants were subjected to water stress by withholding water. The control plants and transgenic plants were randomly distributed on the trays. After 10 days without water, the control plants began to show water stress symptoms (loss of turgor and bleaching of the leaves). Severe symptoms (death of all leaf tissue) were evident in the controls after 15 days without water (Fig. 3A). The transgenic wheat lines started to show water stress symptoms only after 15 days. Tolerance to water stress was always associated with the presence of the transgene.

The second experiment was designed to assess if there was any yield penalty induced by the *DREB1A* gene. As shown in Table 2, the *DREB1A* plants consistently had a higher total number of heads and better head development. It remains to be determined whether this drought tolerance is attributable to the transgenic plants' reduced evapotranspiration or simply to an increase in the osmolarity of the cell. It was interesting to observe that the *DREB1A* plants developed a more branched root phenotype (Fig. 3B).

Fig. 3. (A) Phenotype of the *DREB1A* line containing event 8424 (left) and control plants (right) after 15 days of water stress and one watering (8 h). (B) Root phenotype of event 8424 (left) and control (right).

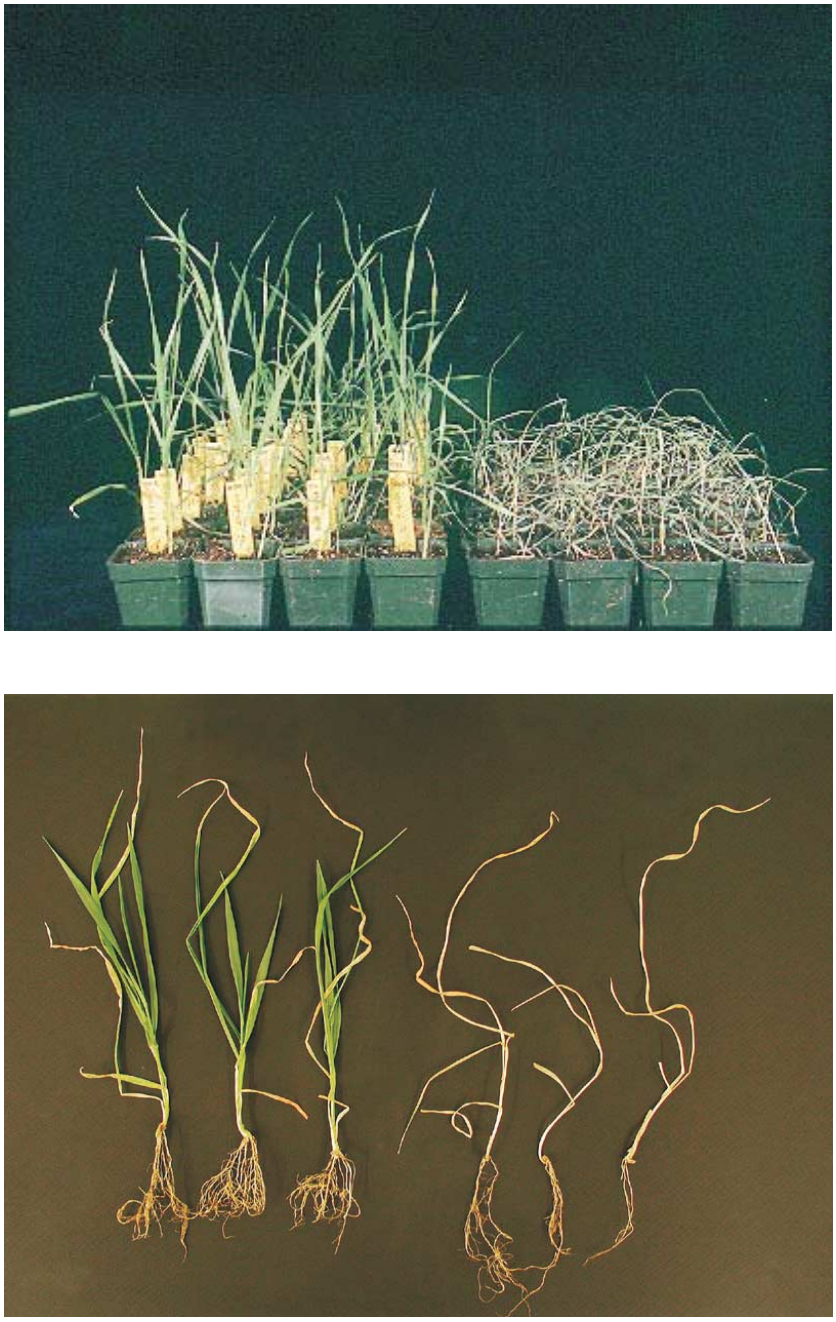


Table 2. Effects on controls and DREB1A plants (event 8424) after 10–12 days of water stress.

	Average no. of heads per plant	Average no. of sterile heads per plant	Average no. of fully developed heads per plant	Average no. of heads that developed late per plant
Controls	1.94±0.41	0.81±0.25	0.56±0.19	0.75±0.39
DREB1A plants	5.75±0.43	0.31±0.12	3.87±0.35	0.37±0.23

Note: Subsequently, the normal water regime resumed until plants reached maturity. At maturity, the plants were evaluated for the total number of heads, sterile heads, fully developed heads, and heads that developed late (average ± SE).

This root system could enable the *DREB* plants to use the moisture in the pots more efficiently, allowing them to survive longer.

The most likely causes of the "switching on" of this gene are abiotic stresses because the transcript has been detected only during moisture stress induction (Fig. 1). It is much more difficult, however, to understand if, after the first induction, the transgene is "switched off" when the plants return to nonstress conditions. This is because the DREB1A protein itself induces the *rd*29 gene's pathway, creating a "regulatory loop" that induces an amplification of the gene translation. Further experiments are underway to improve our understanding of the mechanism.

Two strategies are being pursued to further examine the effects of the *DREB1A* gene on induction of tolerance to water stress in transgenic wheat plants. First, the 12 transgenic lines selected in this work will be tested further under field conditions to confirm the observed phenotype and to determine any additional effects on growth and productivity. Second, additional constructs that constitutively express several *DREB* genes are being introduced into wheat. This procedure is expected to overregulate expression of the endogenous wheat *DRE* genes, which in turn would be expected to increase resistance to abiotic stresses (drought, salt loading, and freezing) while producing an "aberrant" phenotype that may promote a better understanding of drought adaptation in wheat.

Acknowledgements

The authors wish to thank the CIMMyT Applied Biotechnology Center greenhouse staff and José Louis Barrios for their technical support in biosafety greenhouse activities and physiology measurements, David Poland for editing the manuscript, and the CIMMyT Abiotic Stress Team for their constructive criticism of this paper. This research was funded in part by the Australian Cooperative Research Centre for Molecular Plant Breeding, of which CIMMyT is a core participant.

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