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ORIGINAL PAPER

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

Victoria A. James · Isaac Neibaur · Fredy Altpeter

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Abstract The dehydration-responsive element binding proteins (DREB1)/C-repeat (CRT) binding factors (CBF) function as transcription activators and bind to the DRE/CRT *cis*-acting element commonly present in the promoters of abiotic stress-regulated genes. A *DREB1A* transcription factor ortholog was isolated from a xeric, wild barley (*Hordeum spontaneum* L.) accession, originating from the Negev desert. Sequence comparison revealed a very high degree of sequence conservation of *HsDREB1A* to the published barley (*Hordeum vulgare* L.) *DREB1A*. Constitutive expression of the *HsDREB1A* gene was able to trans-activate a reporter gene under transcriptional control of the stress-inducible *HVA1s* and *Dhn8* promoters. *HsDREB1A* was subcloned under transcriptional control of the stress-inducible barley *HVA1s* promoter and introduced into the apomictic bahiagrass (*Paspalum notatum* Flugge) cultivar 'Argentine'. *HsDREB1A* integration and stress inducible expression was detected in primary transgenic bahiagrass plants and apomictic seed progeny by Southern blot, RT-PCR and northern blot analysis respectively. Transgenic bahiagrass plants with stress-inducible expression of *HsDREB1A* survived severe salt stress and repeated cycles of severe dehydration stress under controlled environment conditions, in contrast to non-transgenic plants. The observed abiotic stress tolerance is very desirable in turf and forage grasses like bahiagrass, where seasonal droughts and irrigation restrictions affect establishment, persistence or productivity of this perennial crop.

Keywords DREB1A · transcription factor *Hordeum spontaneum* · bahiagrass · turfgrass · forage · transgenic plants · freezing tolerance · drought tolerance · salt tolerance

V. A. James · I. Neibaur · F. Altpeter (✉)
Agronomy Department, Plant Molecular and Cellular Biology Program, Genetics Institute, University of Florida, PO Box 110300, Gainesville, FL 32611, USA
e-mail: faltpeter@ifas.ufl.edu

Introduction

Bahiagrass is an important turf and forage grass in the Southeastern US and in subtropical regions around the world. The persistence and productivity of bahiagrass is compromised by environmental stresses such as drought, freezing and high salinity. This causes regional and seasonal limitations of forage production (Blount et al. 2001) and turf persistence. The popular turf and forage bahiagrass cultivar Argentine is an obligate apomict. Its asexual seed production, providing uniform seed progeny, makes it an attractive target for genetic engineering of stress tolerance.

Plants respond to environmental stresses, such as salinity, dehydration and freezing, by induction of numerous genes at the transcriptional level to increase survival (Hughes and Dunn 1996; Bray 1997; Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). The expression analysis of stress-inducible genes in ABA-deficient (*aba*), ABA-insensitive (*abi*) and other mutants have established that signal transduction from the perception of stress to the expression of stress-inducible genes is mediated by ABA-dependent and ABA-independent pathways (Gilmour and Thomashow 1991; Nordin et al. 1991, 1993; Shinozaki and Yamaguchi-Shinozaki 1997; Grill and Himmelbach 1998; Leung and Giraudat 1998). ABA-responsive elements (ABRE) and their *trans*-acting factors that regulate the expression of genes in the ABA-dependent pathway have been extensively analyzed (Leung and Giraudat 1998) and several components involved in the signal transduction pathway have been identified (Giraudat et al. 1992; Leung et al. 1994; Meyer et al. 1994; Gosti et al. 1999; Finkelstein and Lynch 2000). In the ABA-independent pathway, the C-repeat/dehydration response element (C/DRE), containing a core sequence of –A/GCCGAC–, has been shown to be essential for transcriptional activation in response to cold, drought, and/or high salt treatments (Yamaguchi-Shinozaki and Shinozaki 1994). The transcription factors that bind to the C/DRE have been isolated; CBF (*C*/DRE *b*inding *f*actor)s, also called DREB (*DRE b*inding protein)s, containing a DNA binding motif found in EREBP1 and AP2 transcriptional activators (Stockinger et al. 1997; Liu et al. 1998).

Over-expression of a single stress-inducible transcriptional activator of the C/DRE stress response pathway, *CBF1* (*DREB1B*) or *DREB1A* (*CBF3*), in transgenic *Arabidopsis* enhanced freezing, drought and salt tolerance with concomitant expression of downstream DREB1 target genes (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999). Recent data also indicate that the C/DRE stress response pathway is conserved in grasses, with *DREB/CBF* homologs being isolated from rice (*Oryza sativa* L.; Dubouzet et al. 2003), wheat (*Triticum aesticum* L.; Jaglo et al. 2001; Shen et al. 2003), barley (*Hordeum vulgare* L.; Choi et al. 2002; Xue 2002), rye (*Secale cereale* L.; Jaglo et al. 2001), oat (*Avena sativa* L.; Bräutigam et al. 2005), maize (*Zea mays* L.; Qin et al. 2004) and perennial ryegrass (*Lolium perenne* L.; Xiong and Fei 2006). Numerous genes have been identified by microarray analysis as potential downstream genes of *DREB1* (Seki et al. 2001; Fowler and Thomashow 2002; Rabbani et al. 2003; Maruyama et al. 2004). In addition, inducible target genes have been identified by RNA gel-blot analysis of transgenic plants over-expressing *DREB1/CBF* transcription factors (Jaglo-Ottosen et al. 1998; Kasuga et al. 1999; Gilmour et al. 2000; Dubouzet et al. 2003; Lee et al. 2004; Qin et al. 2004; Oh et al. 2005; Ito et al. 2006).

DREB/CBF genes have been over-expressed in rice and wheat resulting in improved stress responses under controlled environment conditions (Pellegrineschi et al. 2004; Oh et al. 2005; Ito et al. 2006). In several instances, however, under normal conditions, *DREB/CBF* over-expression was associated with a reduction in growth (Shen et al. 2003; Lee et al. 2004; Ito et al. 2006). Combination of the transcription factor gene with a stress-inducible promoter element might be important to avoid growth suppression during non-stress conditions (Kasuga et al. 1999, 2004).

The *HVA1s* promoter from barley (Xiao and Xue 2001) was used to drive stress inducible expression of a *DREB1A* transcription factor ortholog isolated from xeric, wild barley in bahiagrass. Controlled environment data describing salinity and dehydration stress tolerance of the transgenic bahiagrass are presented.

Materials and methods

Plasmid construction and bahiagrass transformation

The expression vector *HVA1s:HsDREB1A* contains the *nptII* gene under control of the maize ubiquitin 1 promoter with its first intron (*Ubi1*; Christensen and Quail 1996) and the Cauliflower Mosaic Virus 35S 3′ region. The *HsDREB1A* coding sequence was amplified by PCR from genomic DNA isolated from wild barley ecotype

22-12, originating from Sede Boqer in the Negev desert, using primers 5′-AAATATGTCTCCCA-CACTCTCG -3′ and 5′- CACAGTCAAAACA GAGCAGAATC-3′. The barley *HVA1s* promoter, kindly provided by Dr. G.-P. Xue (CSIRO Plant Industry, Australia) was used to drive stress-inducible expression of the *HsDREB1A* transgene in combination with the nopaline synthase 3′ region (*nos*). The plasmid backbone was removed by restriction digest, gel electrophoresis and purification prior to transformation via particle bombardment. Transformation of Argentine bahiagrass was performed as described previously, using the antibiotic paromomycin as a selective agent (Altpeter and James 2005). For functional analysis of the *HVA1s* promoter, the *HsDREB1A* gene was replaced by the *β-glucuronidase* (*gusA*) gene (Jefferson et al. 1987). For transactivation assays, the *Dhn8* promoter was amplified by PCR from wild barley genomic DNA using primers 5′-GCGGTACCGGAGGCGTTTTGATCCGTCG-3′ and 5′-TTGGCGCGCCGAGATCGGTCG GTGCAGC-R-3′ and placed upstream of the *gusA* gene. The Cauliflower Mosaic Virus enhanced *35S* promoter was used to drive expression of the *HsDREB1A* gene to evaluate constitutive transactivation of target promoter-reporter gene constructs.

Transactivation assay

Argentine bahiagrass callus was transformed by particle bombardment, as previously described (Altpeter and James 2005), with 200 μg gold and 22.2 μg plasmid DNA per shot. Four plates, containing 30 calli each, were bombarded with each DNA combination: *35S:HsDREB1A* alone; *35S:HsDREB1A* + *Dhn8:gusA*; *Dhn8:gusA* alone; *35S:HsDREB1A* + *HVA1s:gusA*; *HVA1s:-gusA* alone. In addition, one plate of callus was transformed with *Ubi1:gusA* to confirm the functionality of the reporter gene assay. Histochemical staining of callus material was performed three days after bombardment according to Jefferson et al. (1987) and relative GUS activity determined by counting the number of blue foci on callus pieces with the aid of a stereomicroscope.

Southern and northern blot analysis

For genomic DNA extraction, young leaves were homogenized in liquid nitrogen, immediately transferred to a tube containing pre-heated (65°C) extraction buffer (0.1 M Tris–HCl, pH 8.0, 0.02 M EDTA, 1.4 M NaCl, 2% CTAB and 0.2% β-mercaptoethanol) and incubated at 65°C for 1 h. Upon cooling to room temperature, an equal volume of chloroform/isoamylalcohol (24:1) was added and samples were mixed well by gentle inversion for 15 min. Phase separation was achieved by centrifugation at 3000*g* and the supernatant was transferred to a new tube. Genomic DNA precipitated following addition of two-thirds volume of cold isopropanol. After rinsing with 70% ethanol, DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and treated with RNaseA overnight. Samples were extracted with phenol/chloroform/isoamylalcohol (25:24:1) followed by chloroform/isoamylalcohol (24:1) and genomic DNA was precipitated by adding one tenth volume of sodium acetate, pH 5.2 and two volumes of ethanol. DNA samples were rinsed with 70% ethanol and resuspended in TE buffer.

Total RNA was isolated by homogenizing young leaves in liquid nitrogen and immediately transferring to a tube containing equal volumes pre-heated (55°C) extraction buffer (0.1 M LiCl, 0.1 M Tris–HCl, pH 8.0, 1% SDS, 10 μM EDTA) and equilibrated phenol pH 4.5. Following 5 min incubation at 55°C, samples were incubated for 5 min at 28°C with gentle inversion before addition of one third volume chloroform and continued inversion for a further 20 min. Phase separation was achieved by centrifugation at 20,000*g* and the aqueous phase extracted with an equal volume of chloroform. RNA was precipitated from the aqueous phase by addition of one-third volume 8 M LiCl and incubation overnight at 4°C. RNA was recovered by centrifugation at 12,000*g* and washed with 2 M LiCl, followed by 70% ethanol. RNA was resuspended in DEPC-treated distilled water.

For Southern blot analysis, 15 μg genomic DNA was subjected to restriction digestion with

*Hin*dIII (New England Biolabs Inc., Ipswich, MA), cutting once within the transgene expression unit, separated on a 1% agarose gel and transferred to Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ). For northern blot analysis, 10 μg total RNA was resolved on a formaldehyde agarose gel (1.2% agarose) and transferred to Hybond-N+ membrane. Membranes were hybridized with a probe representing 779 nt of the *HsDREB1A* gene. Probes were labelled with [α-^{32}P] dCTP by random priming using the Prime-a Gene Labeling System (Promega, Madison, WI). Following hybridization, membranes were washed with 0.1× SSC, 0.1% SDS and hybridization signals were detected by autoradiography. Equal loading was evaluated by methylene blue staining of the membranes.

PCR and RT-PCR analysis

Frozen leaf material was homogenized under liquid nitrogen and RNA extracted using the RNeasy kit with on-column DNase treatment (Qiagen, Valencia, CA) according to the manufacturer's instructions. One microgram of total RNA was used for first strand cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). An internal 161 bp fragment of the *HsDREB1A* gene was amplified using primer pair BF1 (5′-CTGGCGCTCCTTCACTATCT-3′) and QBR4 (5′-GTAGTACGAGCCCAGGTCCA-3′). Cycling parameters were: 95°C 4 min initial denaturing, 30 cycles of 95°C 30s, 52°C 30s, 72°C 30s, and 72°C final extension.

The same primer pair and cycling parameters were used for PCR of bahiagrass genomic DNA.

Stress tolerance of plants grown under hydroponic conditions

Evaluation of stress tolerance was conducted under hydroponic growth conditions in a growth chamber maintained at 28°C with a 16 h photoperiod. Single tiller plants were supported, in a completely randomized design, in trays of nutrient solution (8 ml BC Boost, 3 ml BC Grow Technaflora, BC, Canada per liter distilled water) in a 20-L tray with constant aeration. For dehydration stress experiments, plants were removed from solution with roots exposed to the air. For salt stress experiments, nutrient solution was supplemented with 100 mM NaCl for one week, followed by 200 mM NaCl for 2 weeks.

Statistical analysis

Statistical analysis was performed according to the randomization structure using the ANOVA-procedure of SAS version 9.1 (SAS Institute Inc. 2005). Means were compared by the t-test (LSD, $p < 0.05$). Standard error is shown in figures as vertical bars.

Results

Vector construction, promoter induction by stress and trans-activation of stress responsive promoters by HsDREB1A

The *HsDREB1A* transcription factor ortholog was isolated from genomic DNA of a wild barley accession, originating from Sede Boqer in the Negev desert, by PCR, using primers designed against the published *DREB1A* sequence from cultivated barley (Choi et al. 2002). The *HsDREB1A* gene contains no introns and analysis of the deduced amino acid sequence revealed 99% homology to the published barley *HvDREB1A* (Choi et al. 2002; Fig. 1a). The 0.9 kb PCR product was subcloned under control of the stress-inducible barley *HVA1s* promoter (Xiao and Xue, 2001) (Fig. 1b). Functionality of the *HVA1s* promoter in bahiagrass was confirmed using transient expression of the *gusA* reporter gene. Bahiagrass callus was subjected to cold treatment (2°C for 24 h) 24 h after bombardment with a *HVA1s:gusA* expression unit. Histochemical staining (Jefferson et al. 1987) of the callus material immediately following cold treatment revealed *gusA* expression in the cold-treated callus (Fig. 2a), in contrast to callus incubated at 25°C. Furthermore, *HVA1s:gusA* and *Dhn8:gusA* expression units were activated *in-trans* by a co-transferred constitutive barley *35S:HsDREB1A* expression cassette (Fig. 2b). Histochemical GUS

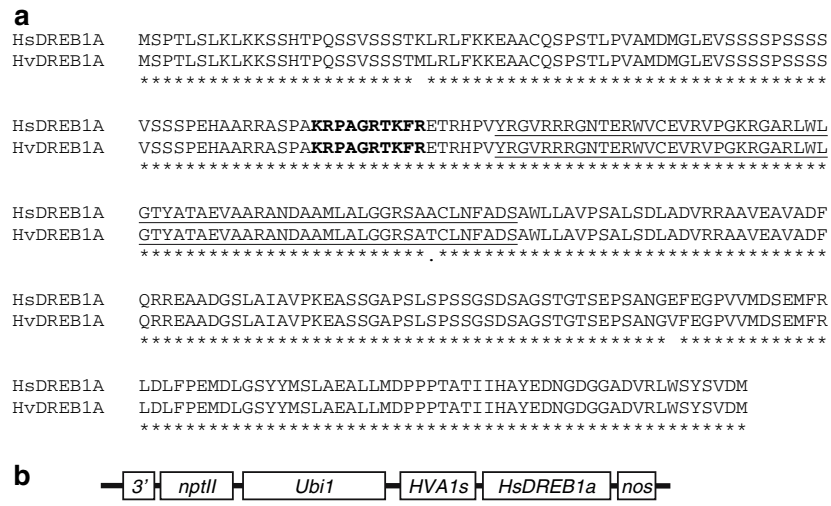


Fig. 1 Sequence analysis and cloning of the *HsDREB1A* gene. (**a**) Comparison of the deduced amino acid sequence of HsDREB1A with HvDREB1A (Genbank accession no. AF298231; Choi et al. 2002). Amino acids are designated in single letter code. An asterisk indicates an identical amino acid. The nuclear localization sequence is shown in bold type and the AP2 domain is underlined. The alignment was performed by BLAST search against the PIR-NREF database. (**b**) The *HVA1s:HsDREB1A* expression cassette consists of the *HsDREB1A* gene linked to the barley HVA1s promoter and the 3′ region of the nopaline synthase gene (nos) and an expression cassette containing the neomycin phosphotransferase II gene (*nptII*) gene under control of the maize ubiquitin 1 promoter with its first intron (*Ubi1*) and the Cauliflower Mosaic Virus 35S 3′ region

assay was performed three days after biolistic co-transformation of *35S:HsDREB1A* and *HVA1s:gusA* or *Dhn8:gusA* expression units in comparison to *35S:HsDREB1A, Ubi1:gusA, HVA1s:gusA* or *Dhn8:gusA* alone as controls, according to Jefferson et al. (1987). As expected, transformation with the *35S:HsDREB1A* expression unit alone resulted in no detectable GUS activity in bahiagrass callus (Fig. 2b). Low levels of GUS activity were observed when bahiagrass callus was transformed with either *Dhn8:gusA* or *HVA1s:gusA* alone (Fig. 2b). The *HsDREB1A* transgene activated both the *Dhn8* and *HVA1s* promoters, resulting in a 5.8-fold or 8.25-fold increase in relative GUS activity, respectively. This was significantly higher ($p < 0.05$) than GUS activity driven by each promoter without *HsDREB1A* (Fig. 2b).

Generation of transgenic bahiagrass plants with stable integration and expression of HVA1s:HsDREB1A

The *HVA1s:HsDREB1A* expression cassette was introduced into callus of Argentine bahiagrass via biolistic gene transfer, following removal of the vector backbone by restriction digest, gel electrophoresis and purification. Twenty-two independent transgenic lines were regenerated under selection with paromomycin sulfate (Altpeter and James 2005) following biolistic gene transfer to approximately 1400 calli. Supplementation of the culture medium with paromomycin fully suppressed the regeneration of non-transgenic plants escaping the selection process. Southern blot analysis confirmed independent transformation events containing multiple *HsDREB1A* transgene copies in addition to the endogenous *DREB1A* homolog (Fig. 3a). Transgene expression was examined by RT-PCR (Fig. 3b) and northern blot analysis (Fig. 3c) of non-stressed or stressed T_0 or T_1 plants. Constitutive transgene expression under non-stress conditions was detected from the inducible *HVA1s:HsDREB1A* construct in most of the bahiagrass lines and their seed progeny by RT-PCR (Fig. 3b,). However, this constitutive expression was below the detection limit of the northern blot analysis for most lines (Fig. 3c) and only line C2, displayed a very week signal in northern blot analysis of non-stressed plants. Following six hours

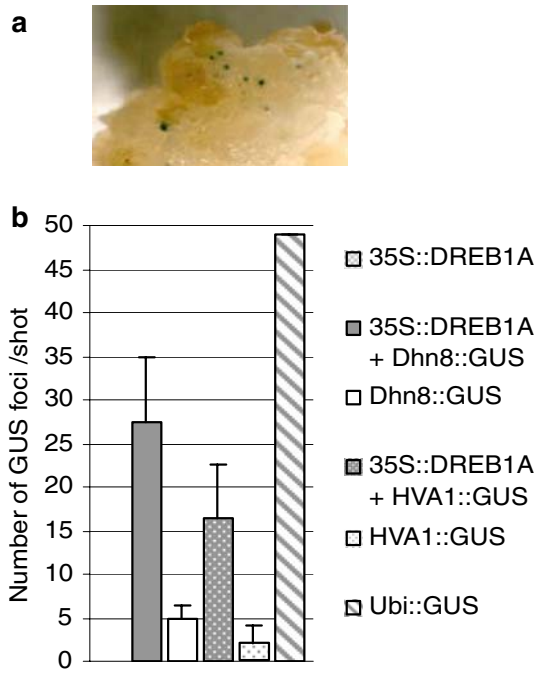
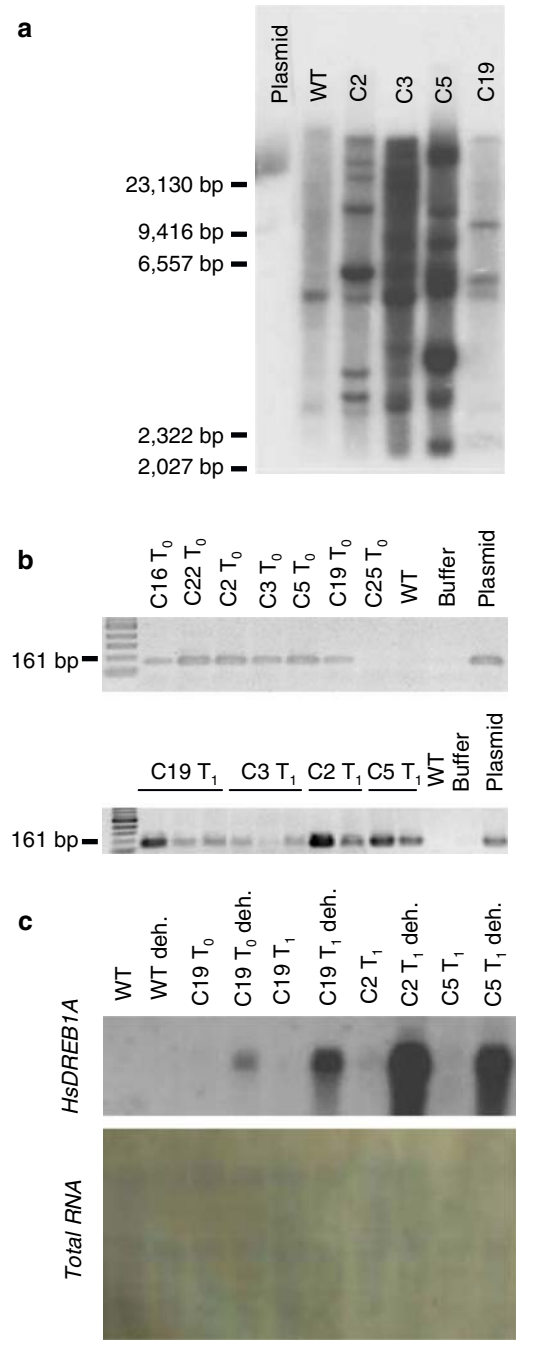


Fig. 2 Activation of *HVA1s:gusA* and *Dhn8:gusA* by abiotic stress or *HsDREB1A*. (**a**) Transient *gusA* expression in bahiagrass callus subjected to cold treatment (2(C for 24 h), 24 h after bombardment with a *HVA1s:gusA* expression unit. (**b**) Transactivation of *HVA1s:gusA* or *Dhn8:gusA* expression units by a co-transformed *35S:HsDREB1A* expression unit. *35S:HsDREB1A, Ubi1:-gusA, HVA1s:gusA* or *Dhn8:gusA* were introduced alone as controls. Histochemical GUS assay was performed three days after biolistic transformation. Each data point represents the mean number of blue foci / shot from four independent experiments, except *Ubi1:gusA*, which was the result of a single experiment. Bars represent standard error. No GUS activity was observed in callus transformed with *35S:HsDREB1A* alone



dehydration stress, *HsDREB1A* expression was clearly detectable in northern blot analysis of transgenic bahiagrass seed progeny plants in contrast to wild-type plants, indicating that the *HVA1s* promoter supports inducible transgene expression in bahiagrass under abiotic stress conditions (Fig. 3c).

All of the transgenic lines produced seed under greenhouse conditions. Seed from four independent lines was germinated to prove viability and analyze transgene expression in progeny plants. T_1 plants expressed the *HsDREB1A* transgene, as detected by RT-PCR and northern blot (Fig. 3b and c). Analysis of seed progeny plants from four transgenic Argentine bahiagrass lines indicated transgene expression in all progeny plants without segregation, suggesting apomictic inheritance of the transgenic trait.

◀ **Fig. 3** Analysis of HsDREB1A transgene expression. a. Southern blot analysis of transgenic bahiagrass lines. The blot was hybridized with a radiolabelled *HsDREB1A* probe. WT denotes wild-type bahiagrass control. (**b**) RT-PCR of non-stressed T_0 and T_1 plants (upper and lower panels, respectively) to amplify an internal 161bp fragment of the *HsDREB1A* transgene. WT denotes wild-type bahiagrass control. (**c**) Northern blot analysis of total RNA from T_0 and T_1 plants under non-stress and dehydration stress. The blot was hybridized with a radiolabelled *HsDREB1A* probe (upper panel) and the membrane stained with methylene blue (lower panel) to evaluate equal loading of total RNA. WT denotes wild-type bahiagrass control

Performance and persistence of transgenic bahiagrass plants under controlled environment conditions

To assess the survival of transgenic lines under severe salt stress, four independent transgenic lines expressing *HsDREB1A* (lines C2, C3, C5, C19) were grown alongside wild-type Argentine bahiagrass under hydroponic conditions. Each hydroponic tray contained five replicates of each genotype in a completely randomized design. Nutrient solution in one hydroponic tray was supplemented with 100 mM NaCl for one week, followed by 200 mM NaCl for two weeks. Plants were then allowed to recover for 10 days without NaCl. The second, control tray was not supplemented with NaCl. Total biomass of each plant was recorded and compared to the mean of non-stressed plants from the same line. Under non-stressed hydroponic growth conditions, none of the four transgenic bahiagrass lines differed significantly in the amount of biomass to wild-type Argentine bahiagrass (Fig. 4a). Relative to the non-stressed plants, transgenic lines C2, C3 and C5 showed a trend towards higher biomass production under salt-stress than wild-type plants (Fig. 4b), but this difference was not significant. However, none of the wild-type plants recovered when salt was removed from the nutrient solution, whereas one to three plants from each transgenic line showed new growth of leaves and roots after 10 days (Fig. 4c).

To evaluate the performance of transgenic lines under dehydration stress, independent transgenic lines expressing *HsDREB1A* (lines C2, C3, C5, C19) plus wild-type Argentine bahiagrass were grown under hydroponic conditions (Fig. 5a). Six plants per genotype were evaluated in a completely randomized design in each of two independent experiments. Plants were removed from nutrient solution and allowed to dry at room temperature for increasing lengths of time (Fig. 5b), up to 36 h, with at least 12 h recovery time between treatments (Table 1). In total, 11 successive dehydration and re-hydration treatments were applied to all plants (Table 1). This simulates natural conditions typical for sandy soils with multiple cycles of dehydration and re-hydration. Total plant biomass was measured 22 days after the 24 h dehydration treatment (Fig. 5c). Biomass was significantly higher ($p < 0.05$) in lines C3 and C19 than in the wild-type plants. Measurements in both independent experiments reflected faster recovery and increased survival of transgenic plants over wild-type plants, following repeated cycles of dehydration and rehydration (Fig. 5d). In both experiments transgenic plants recovered more quickly from dehydration than wild-type plants due to more rapid growth of new roots (Fig. 5e). None of the wild-type plants recovered from 24 h dehydration, whereas plants from all transgenic lines produced new leaves and roots (Fig. 5d, e). One plant from each of two transgenic lines, C19 and C3, survived 36 hours dehydration, in contrast to wild-type plants.

Discussion

The improvement of drought stress tolerance in the commercially important, apomictic turf and forage grass cultivar Argentine bahiagrass is very desirable to reduce irrigation needs and enhance productivity and persistence of this perennial species during seasonal periods of drought stress. Transcription factors play a critical role in regulation of the plants stress response. Increased drought tolerance has been reported in transgenic *Arabidopsis* over-expressing rice or maize orthologs of the transcriptional activator *DREB1A* (Dubouzet et al. 2003; Qin et al. 2004), transgenic rice over-expressing rice or *Arabidopsis DREB1A* orthologs (Oh et al. 2005; Ito et al. 2006) and wheat over-expressing *Arabidopsis*

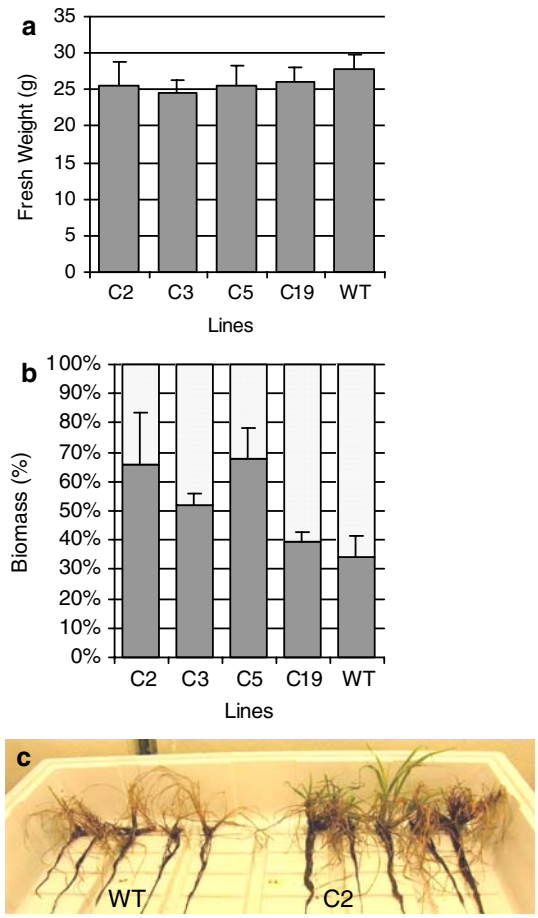


Fig. 4 Response of bahiagrass over-expressing *HsDREB1A* to severe salt stress. (**a**) Mean fresh weight of transgenic and wild-type bahiagrass plants grown under non-stressed hydroponic conditions. Each data point represents the mean fresh weight of five plants, vertical bars represent standard error. b. Mean fresh weight of transgenic and wild-type bahiagrass plants under salt-stress (100 mM NaCl for one week, followed by 200 mM NaCl for 2 weeks). Each data point represents the mean fresh weight of five plants, shown as fresh weight measured in stressed plants relative to non-stressed plants (100%). Vertical bars represent standard error. (**c**) Wild-type and transgenic plants from line C2, 10 days after salt-stress

DREB1A (Pellegrineschi et al. 2004). Gene mining in plants adapted to extreme environments is a promising tool to identify beneficial mutations or highly conserved sequences of critical relevance for plant performance under extreme conditions. A *DREB1A* ortholog was successfully isolated from a xeric *Hordeum spontaneum* ecotype from the Negev desert. Surprisingly, the sequence was almost (99%) identical to the published *DREB1A Hordeum vulgare* sequence. The very high degree of sequence conservation across these two related species (Fig. 1) suggests that this ortholog is important for plant performance and persistence indifferent environments.

Expression of *HsDREB1A* in bahiagrass is expected to enhance expression of stress-protective target genes. Several candidates have been identified as potential downstream genes of *DREB1A* in *Arabidopsis* and rice (Seki et al. 2001; Fowler and Thomashow 2002; Rabbani et al. 2003; Maruyama et al. 2004). Members of the dehydrin gene-family are among the many gene products believed to function in stress protection. *HvDhn8* is homologous to the rice dehydration induced protein 1 (Dip1) or low temperature induced protein 9 (Lip9) previously shown to be up-regulated in transgenic rice plants over-expressing *DREB1A* or *DREB1B* (Lee et al. 2004; Oh et al. 2005). To investigate the potential interaction between *HsDREB1A* and the promoters of candidate target genes, trans-activation experiments using a *gusA* reporter gene fused to either the barley *Dhn8* or *HVA1s* promoter were performed. Both promoters contain DRE/CRT motifs. Under non-stress conditions, the constitutive *35S:HsDREB1A* transgene was able to activate both the *Dhn8* and *HVA1s* promoters, resulting in a significant 5.8-fold or 8.25-fold increase in relative GUS activity, respectively, compared to GUS activity driven by each promoter in the absence of *HsDREB1A*. Whilst the precise mechanism by which *DREB1A* is able to increase transcript levels of target genes remains unclear, it appears that an interaction between *DREB1A* and the specific DRE elements of these promoters is essential to initiate this process. Previously, Dubouzet et al. (2003) showed that *OsDREB1A* was able to activate expression from the *Arabidopsis Rd29A* promoter, with two DRE elements, during similar reporter gene trans-activation studies. In contrast, Oh et al. (2005) found that *Arabidopsis DREB1A* did not elevate expression of the *Wsi18* promoter in rice. The *Wsi18* promoter contains only one DRE element, like the barley *HVA1s* promoter used in the present study, which was still up-regulated by *HsDREB1A*. Although DRE

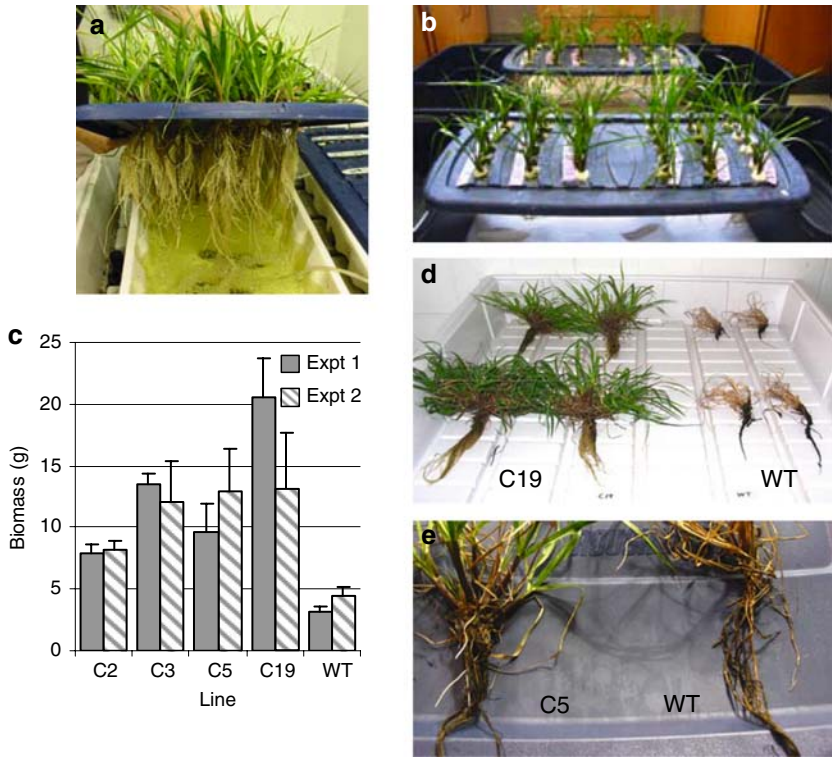


Fig. 5 Response of bahiagrass over-expressing *HsDREB1A* to progressive dehydration stress. (**a**) Transgenic and wild-type bahiagrass plants propagated in aerated hydroponic nutrient solution. (**b**) Plants removed from hydroponic system for dehydration treatment. (**c**) Mean biomass (g) of plants from each line after a 24-h dehydration treatment plus 22 days recovery. Each data point represents the mean biomass of six plants, vertical bars represent standard error. (**d**) Survival of wild-type (WT) bahiagrass and plants from line C19 following 24 h dehydration-stress plus 22 days recovery. (**e**) Root regrowth in transgenic plants (C5, left) compared to wild-type plants (WT, right)

elements are known to be found in the promoters of genes induced under stress conditions, it remains possible that over-expression of the *DREB1A* transcription factor might also activate genes in distinct pathways. Putative *DREB1A* interactions with other transcription factors include the STZ zinc-finger transcription factor, which suppresses genes involved in photosynthesis and carbohydrate metabolism and has been suggested to be involved in the growth retardation of transgenic plants over-expressing DREB1A (Maruyama et al. 2004).

Bahiagrass lines expressing the *DREB1A* ortholog from *Hordeum spontaneum* under control of the abiotic stress-inducible *HVA1s* promoter were generated via particle bombardment of seed derived callus from the apomictic cultivar Argentine (Altpeter and James 2005). The selection for

Table 1 Dehydration treatments applied to hydroponic bahiagrass plants over-expressing *HsDREB1A*

Dehydration cycle	Stress (Hours)	Recovery (Days)
1	4	10
2	4	7
3	4	4
4	4	1
5	4	1
6	7.5	0.5
7	12	4
8	20	4
9	22	7
10	24	22
11	36	

Plants were removed from nutrient solution and allowed to dry at room temperature for increasing lengths of time, up to 36 h, with at least 12 h recovery time before the next dehydration treatment

expression of the linked *nptII* expression cassette was very efficient in the presence of paromomycin and non-transgenic plants escaping the selection process were not identified. Activation of the *HVA1s* promoter in barley, under conditions of low temperature, dehydration and salt stress, was previously demonstrated in transient reporter gene assays (Xiao and Xue 2001). In stably transformed bahiagrass lines, constitutive *HsDREB1A* expression was clearly detectable by RT-PCR under non-stress conditions. However, these constitutive expression levels under non-stress conditions were hardly detectable by northern blot hybridization. Northern blot analysis clearly indicated drought-inducible *DREB1A* expression under control of the *HVA1s* promoter in primary transgenic bahiagrass plants and their apomictic progenies (Fig. 3c).

High-level, constitutive over-expression of *Arabidopsis*, rice and perennial ryegrass *DREB1A* genes in *Arabidopsis* led to growth retardation under non-stress conditions (Liu et al. 1998; Dubouzet et al. 2003; Xiong and Fei 2006). In addition, dwarf phenotypes were observed in transgenic rice constitutively over-expressing rice or wheat *DREB1A* genes (Shen et al. 2003; Ito et al. 2006). The use of the inducible *Rd29A* promoter to drive *DREB1A* expression in both *Arabidopsis* and wheat alleviated the growth retardation associated with over-expression under non-stress conditions (Kasuga et al. 1999; Pellegrineschi et al. 2004). Similar results were observed in the current study, where expression of the *HsDREB1A* transcription factor under control of the stress inducible *HVA1s* promoter did not compromise plant performance and fertility under non-stress conditions. In both transgenic *Arabidopsis* (Kasuga et al. 1999) and bahiagrass, a low level expression of *DREB1A* was detected under non-stress conditions despite the use of an inducible promoter. This *DREB1A* expression level did not affect plant growth under non-stress conditions and transgenic bahiagrass plants produced a similar amount of biomass to wild-type Argentine bahiagrass plants in non-stress hydroponic culture (Fig. 4a). The obligate apomictic nature of Argentine bahiagrass resulted in uniform seed progeny with stable transgene expression (Fig. 3).

Over-expression of *HsDREB1A* in bahiagrass enhanced survival and biomass production under severe dehydration and salt-stress compared to wild-type plants, under controlled environment conditions (Figs. 4 and 5). Following repeated cycles of dehydration, transgenic plants recovered more quickly than wild-type plants due to more rapid growth of new roots, which was monitored non-destructively in a hydroponic growth system. These results point to the potential of stress-induced *HsDREB1A* to enhance drought tolerance and performance of a commercially important, perennial, monocotyledonous plant.

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