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ORIGINAL ARTICLE

Heterologous expression of the *Arabidopsis DREB1A/CBF3* gene enhances drought and freezing tolerance in transgenic *Lolium perenne* plants

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Abstract The dehydration-responsive element binding proteins (DREB1)/C-repeat (CRT) binding factors (CBF) function as transcription factors and play an important role in agricultural biotechnology and molecular biology studies of drought and freezing stress tolerance. We generated transgenic *Lolium perenne* plants containing the PCR-cloned *Arabidopsis DREB1A/CBF3* gene (*AtDREB1A/CBF3*) to study the function of this gene construct in drought and freezing tolerance in a species of turfgrass. Compared to the control, *AtDREB1A/CBF3* transgenic *L. perenne* plants showed enhanced drought and freezing stress tolerance. The activities of the enzymes superoxide dismutase (SOD) and peroxidase (POD) were higher in transgenic plants than in the non-transgenic plant control. These results demonstrate that the expression of the *AtDREB1A/CBF3* gene in transgenic *L. perenne* plants enhanced drought and freezing tolerance and that the increased stress tolerance was associated with the increased activities of antioxidant enzymes. These results are relevant to stress biology and biotechnology studies of turfgrass.

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Introduction

Drought and low temperature induce the expression of many plant genes through as-yet undefined signaling pathways (Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger et al. 1997; Thomashow 1999; Kasuga et al. 2004; Pellegrineschi et al. 2004). Many researchers have studied *Arabidopsis* mutants that exhibit altered regulation of low temperature-induced gene expression to gain insight into drought and cold signal transduction mechanisms (Thomashow 1999; Pellegrineschi et al. 2004). Wang et al. (2008) recently reported that members of the *Arabidopsis CBR* transcriptional factor family interact with both the dehydration-responsive element/C-repeat element (DRE/CRT) and low temperature-responsive *cis*-elements, thus conferring plants with the capability to acclimate to cold. The DRE/CRT is a *cis*-acting promoter element responsible for gene expression in response to drought, salt, and cold stress in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994).

Cold temperatures triggers the expression of the C-repeat binding factor (CBF) family of transcription factors, and these in turn activate many downstream genes that confer freezing tolerance to plants (Thomashow 1999; Agarwal et al. 2006; Dong et al. 2006). Gong et al. (2002) reported that an *Arabidopsis thaliana* mutant impaired in the cold-regulated expression of CBF genes and their downstream target genes is sensitive to chilling stress. The dehydration-responsive element binding proteins (DREB1)/CBF bind to the DRE/CRT *cis*-acting element

commonly present in cold-regulated (COR) genes and subsequently upregulate the expression of such genes in *Arabidopsis* (Thomashow 1999; Wang et al. 2008). The two CBFs DREB1A/CBF3 have been isolated and characterized in maize (Wang et al. 2008), tall fescue (*Festuca arundinacea* Schreb.), an important perennial cool-season grass (Zhao et al. 2007), perennial ryegrass (*Lolium perenne* L.; Xiong and Fei 2006), rice (*Oryza sativa*), a monocotyledonous plant that does not cold acclimate (Oh et al. 2005), and *Triticeae* [wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and rye (*Secale cereale*); Choi et al. 2002]. Over-expression of DREB2A was found to result in significant drought stress tolerance in transgenic *Arabidopsis* plants (Sakuma et al. 2006), with the region between DREB2A residues 136 and 165 playing a role in the stability of this protein in the nucleus, which is important for protein activation. Deletion of a region between residues 136 and 165 transformed DREB2A into a constitutive active form. These results suggest that the presence of a negative regulatory domain in the region between amino acids 136 and 165 which may negatively affect the activation of the DREB2A protein under unstressed control conditions (Sakuma et al. 2006).

Expression of the DREB1A/CBF3 gene enhances drought, salt, and cold tolerance in a number of plant species (Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger et al. 1997; Gilmour et al. 2000; Kasuga et al. 2004; Pellegrineschi et al. 2004). Gilmour et al. (2000) reported that overexpression of *CBF3* in *Arabidopsis* led to increased proline and total sugar levels and, in turn, enhanced freezing tolerance. Overexpression of *CBF3* in tobacco and rice also improved drought, salt, and freezing stress tolerances (Kasuga et al. 2004; Oh et al. 2005). The *Arabidopsis GIGANTEA* (*GI*) gene positively regulates freezing tolerance via a CBF-independent pathway (Cao et al. 2005), while the *HOS2* (high expression of osmotic stress-regulated gene expression 2) gene may act upstream of CBFs (Xiong et al. 2004), and SNF1-related protein kinase 2 (SRK2C) is capable of mediating signals initiated during drought stress (Umezawa et al. 2004). *HOS2* is a genetic locus that specifically controls cold signal transduction in the activation of stress-responsive genes. This gene is identical to the *FIERY1* gene that encodes a bifunctional enzyme with both nucleotidase and inositol polyphosphatase activities. *HOS2* protein negatively regulates cold signal transduction, and *hos2* mutant plants were found to show a specific cold-enhanced induction of stress-responsive genes. *SRK2C* improves drought tolerance by controlling stress-responsive gene expression in *A. thaliana* and is able to mediate signals initiated during drought stress, resulting in the appropriate gene expression (Xiong et al. 2004).

The genetic improvement of turf and forage species for enhanced tolerance to adverse environmental stress is of great importance in terms of agricultural and economical development. Although this strategy has been adopted in studies of other crop species, it has not used in economically important turf and forage crops. Perennial ryegrass (*Lolium perenne* L.) is one of the most important turf and forage grass species of the temperate regions. We have developed transgenic *L. perenne* plants that constitutively express the *Arabidopsis DREB1A/CBF3* gene. Enhanced drought and freezing tolerance were observed, as has been reported for *DREB1A/CBF3*-transgenic *Arabidopsis* plants (Kant et al. 2007). The enzymes superoxide dismutase (SOD) and peroxidase (POD) were also more active in transgenic *L. perenne* plants than in control plants. Our results demonstrate that enhanced drought and freezing tolerance is associated with increased activities of antioxidant enzymes and may be particularly relevant in stress biology studies of turfgrass.

Materials and methods

Plant materials and expression vector

Mature dry seeds of *L. perenne* were purchased from International Seeds, Inc. (USA). The seeds were stored in plastic bags at 4°C before they were used for germination. Prior to culture, seeds were disinfected by immersion in 70% ethyl alcohol for 30 s and then in 0.1% mercuric chloride for 15 min, followed by five rinses in sterile distilled water. Mature seeds were aseptically placed horizontally on solid MS medium (Murashige and Skoog 1962) in 125-mL Erlenmeyer flasks for callus induction. Embryogenic calli of *L. perenne* were used for *Agrobacterium*-mediated transformation. Plasmid pCAMBIA1301-*CBF3* carrying modified hygromycin phosphotransferase (*HPT*) and chimeric *β*-glucuronidase (GUS) coding sequences under the control of CaMV35S promoters and the *CBF3* gene under the control of the maize ubiquitin promoter (UBI) was used for *Agrobacterium*-mediated transformation, as described previously (Hiei et al. 1994).

Agrobacterium-mediated transformation and transgenic plant regeneration

Embryogenic calli were sub-cultured on MS medium for 5 days prior to infection. *Agrobacterium tumefaciens* strain LBA4404, containing the pC1301-CBF3 binary vector (Fig. 1), was used for transformation. A single plasmid-containing colony was picked into a flask with 2 mL YEB liquid medium (5 g Difco Bacto beef extract, 5 g tryptone, 5 g sucrose, 1 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$, and

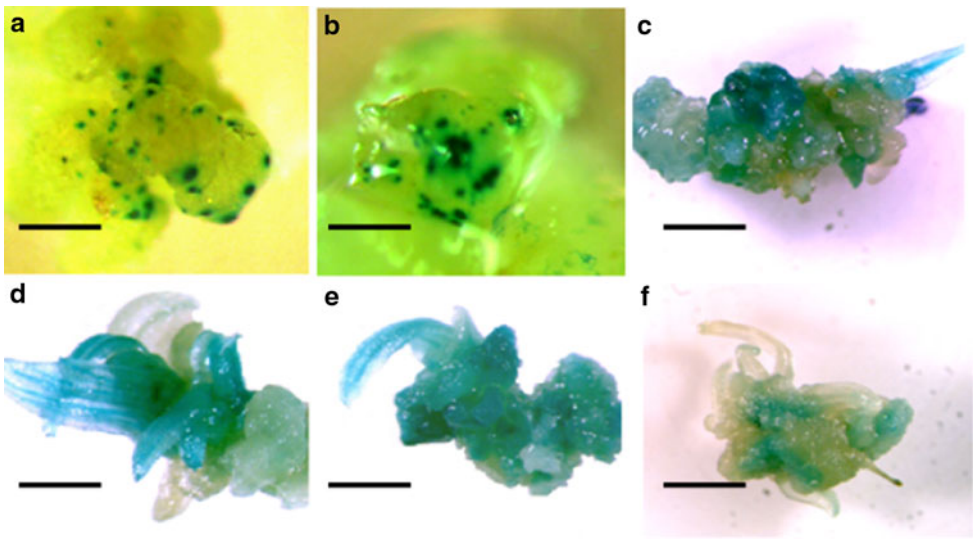

Fig. 1 Histochemical assays of β-glucuronidase activity (GUS). **a** Transient GUS expression 3 days after co-cultivation, **b** transient GUS expression 5 days after co-cultivation; **c–f** stable GUS expression of transgenic shoots at different developmental stages after selection. *Bars* **a**, **b** 0.5 cm, **c–f** 1 cm

125 mg streptomycin per liter) and incubated at 28°C with shaking (220 rpm) overnight. The culture were then transferred into 50 mL YEB liquid medium containing the antibiotic and incubated for 8 h until the density of the *Agrobacterium* (OD_{600}) cells reached 0.5–0.8. Thereafter, cultures were centrifuged at 12,000 rpm for 1 min and re-suspended in AAM liquid medium (Hiei et al. 1994), followed by the addition of 100 μmol/L acetosyringone (AS). Before transformation, the density of the *Agrobacterium* suspension was adjusted to $OD_{600} = 0.1$. Infiltration was performed in a vacuum chamber by immersing the fresh embryogenic calli in the *A. tumefaciens* solution and creating a vacuum of 650 mmHg for 2.5 min. Vacuum infiltration was repeated eight times within a period of 20 min. During the infection, the plate was shaken gently in order to ensure that the bacteria came into contact with the calli. The infected calli were then transferred into a plate containing sheets of sterile filter paper in order to removed excess bacteria. The calli were then placed onto co-cultivation medium (MS medium containing 100 μmol/L AS) at 25°C in the dark for 4 days. After co-cultivation, the infected calli were rinsed four times with sterile water containing 500 mg/L cefotaxime.

Co-cultivated calli were transferred into MS selection medium supplemented with 100 mg/L hygromycin and 300 mg/L cefotaxime and cultured in the dark at 25°C. Twenty days after selection on hygromycin-containing medium, hygromycin-resistant calli were transferred onto regeneration medium (MS supplemented with 0.2 mg/L 6-benzylaminopurine + 0.6 mg/L α-naphthaleneacetic acid + 0.5 mg/L zeatine + 6.4 mg/L Cu^{2+}) containing 50 mg/L hygromycin and 250 mg/L cefotaxime. Regenerated plantlets were then transferred to vessels containing MS medium without hygromycin. All of the regeneration cultures were kept at 25°C under a photoperiod of 16/8-h (light/dark) in the growth chamber. After 4–5 weeks, plants with well-developed roots were transferred to soil and grown under greenhouse conditions. Transgenic plants were transferred to the field under the permission of the State Forestry Administration, P. R. China.

Histochemical GUS assays

Histochemical GUS assays were conducted as described by Jefferson et al. (1987). Briefly, the histochemical analysis of GUS expression was performed after a 7-day co-cultivation of embryonic calli with *A. tumefaciens*. The hygromycin-resistant tissues or putative transgenic seedlings were subsequently tested by overnight incubation in X-Gluc staining buffer at 37°C (Jefferson et al. 1987). Stained plant materials were clarified with 70% ethanol for 48 h. A sample was scored as GUS positive if there was at least one discrete dark-blue region in the tissue.

Molecular characterization of transgenic plants

Total genomic DNA was extracted from putative transgenic perennial ryegrass and non-transformed control plants following the instructions in the manual of the Plant Genomic DNA Purification kit (DP305-03; Tiangen Biotech, Beijing, China). The presence of the *AtDREB1A/CBF3* gene was demonstrated by PCR amplification of a 750-bp DNA fragment amplified with CBF3-specific primers (CBF3-F: 5′-AAA GGA TCC TTA CCC GGG TTC TGA TCA ATG AAC TCA TTT TCT G-3′; CBF3-R: 5′-AAA GGT ACC AAT CCC GGG GTT TTA ATA ACT CCA TAA CGA TAC G-3′). A total of 300 ng genomic DNA was used as the template in a 50-μL PCR reaction mix containing 200 μM each of dNTP, 10 pmol of each primer, 2.5 U Taq DNA polymerase (Promega, Madison,

WI), 1.5 mM $MgCl_2$, and 5 mL 10× buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 at 25°C, 1% Triton X-100, 15 mM $MgCl_2$). The thermocycling conditions were 94°C for 4 min followed by 30 cycles of 94°C for 30 s (denaturation), 60°C for 30 s (annealing), 72°C for 45 s (extension), and a final extension at 72°C for 10 min. The PCR products were observed under UV after electrophoresis in a 1.2% agarose gel with 0.1% ethidium bromide. For Southern blot analysis, genomic DNA was isolated from PCR-positive transgenic plants. After 15 μg genomic DNA was digested with *Bam*HI and separated by electrophoresis, DNA was blotted onto a nylon membrane positively charged by capillary transfer with 20× SSC, hybridized with a digoxigenin (DIG)-labeled DNA probe at 58°C, and washed at high stringency [2× SSC, 0.1% sodium dodecyl sulfate (SDS), at 25°C for 10 min; then 0.5× SSC, 0.1% SDS, at 65°C for 30 min]. Immunological detection was according to the protocol of the DIG DNA Labeling and Detection kit (Roche Diagnostics, Indianapolis, IN).

For the northern blot analysis, total RNA was extracted from leaves of transgenic perennial ryegrass plants using TRN201 RNA Purification Reagent (Dp405-02; Tiangen Biotech). After 5 μg of total RNA were separated by electrophoresis, RNAs were blotted onto a membrane (Millipore, Bedford, MA), and hybridization was performed using the DIG High Prime DNA Labeling and Detection Starter kit I [nitro-blue tetrazolium/2,6-dichloroindophenol (NBT/BCIP); Roche Diagnostics]. The labeling of CBF3 probes, hybridization, and washing of the membrane were performed according to the manufacturer's protocol.

Drought and freezing treatment

Drought-tolerance experiments were carried out as described by Sugano et al. (2003). Plants were grown in plastic pots filled with a perlite/peatmoss/vermiculite (1:1:1 v/v) mixture located in a greenhouse. They were grown for 4 weeks at 25°C under a 16/8-h (light/dark) photoperiods before water was withheld. After 30 days without irrigation, plants were irrigated again for 1 week and then scored for recovery. Freezing-tolerance experiments were carried out as described by Yi et al. (2004).

Measurement of malondialdehyde and antioxidant enzyme activity

The content of malondialdehyde (MDA) was measured using 0.25 g fresh leaves skived with 5% (v/v) trichloroacetic acid (TCA). A mixture of 2 mL 0.67% 2-thiobarbituric acid (TBA) with the same volume of clear extract after centrifuging at 1,000 *g* for 10 min was heated in a boiling water bath for 30 min. After cooling to room temperature and centrifugation at 10,000 *g* for 10 min, the supernatant was read for absorbance at 450, 532 and 600 nm. The amount of MDA was calculated as described by Zhang (2001). Absorbance of the extractant at 663 and 645 nm was measured with a spectrophotometer (UV-7504c; Shanghai). The activity of SOD was measured as described by Chen (2001). Measurements of POD activity were according to Gong (2001).

Electrolyte leakage measurement

Electrolyte leakage tests were conducted essentially as described elsewhere (Yi et al. 2004; Warren et al. 1996) with minor modifications. Six-week-old plants were incubated in growth chambers at either 25°C (for non-acclimated plants) or 4°C (for cold-acclimated plants). After 7 days, young needles were harvested, washed, and four needles per plant were placed in 5-mL aliquots of 0.4 M sorbitol (Sigma, St. Louis, MO). Tubes were equilibrated to either −2 or 0°C in an 818-low temperature incubator (Precision Scientific, Winchester, VA) and allowed to remain there for 24 h. The cooled incubator temperature was then ramped down to −10°C at a decreased rate of 2°C per day. The cold-treated tubes were held at 4°C for 2 h and then warmed to room temperature. Electrical conductivity was measured (model 455C conductivity meter; Istek, Seoul, Korea), following which the tubes were autoclaved to release all electrolytes for the second determination of the total content of electrolytes in each sample.

Statistical analyses

Data obtained from the different experiments were analyzed using the General Linear Model (GLM) procedure of SAS (SAS, Cary, NC), employing analysis of variance (ANOVA) models. Differences between mean values obtained from at least three independent experiments were assessed with the least significant difference test at a significance probability level of 5%. Each value was presented as the means ± standard error of the mean (SEM), with a minimum of three replicates.

Results

Histochemical assays of GUS activity

After co-cultivation and callus regeneration, calli were randomly chosen from each transformation for histochemical assays of GUS activity. Histochemical assays of GUS activity demonstrated that transient GUS expression (blue spots) was present on the surface of calli (Fig. 1a) 3 days

after co-cultivation. Most of the proliferating calli survived on hygromycin-containing medium for 1–8 weeks following selection and showed GUS expression as blue-stained patches (Fig. 1b). Stable GUS expression was observed from transgenic shoots at different developmental stages after selection (Fig. 1c–f). Shoots generated from hygromycin-resistant calli grew well on medium containing 50 mg/L hygromycin and showed strong GUS expression (Fig. 1c–f). In contrast, GUS activity was never detected in control plants. Four days after co-cultivation, the average frequency of GUS expression was 45.9 and 55.9% at infection times of 10 and 20 min, respectively. Our results show that an *Agrobacterium* concentration at $OD_{600} = 0.1$, infection period of 20 min, and co-cultivation time of 4 days were the best conditions for *A. tumefaciens*-mediated genetic transformation in *L. preened* (data not shown).

The GUS-expressing vector was used in this study to monitor the transformation rate. To establish a stable genetic transformation system, we constructed an expression vector carrying the *HPT* and chimeric GUS coding sequences under the control of the CaMV35S promoter and the *CBF3* gene under the control of UBI and then used this vector for *Agrobacterium*-mediated transformation. Because the GUS gene can be transiently or stably expressed in plants, it is relatively easy to monitor the transgene expression and to identify an optimum concentration of *Agrobacterium*. The use of the GUS gene for transient and stable expression can optimize the factors that influence the transformation efficiency, including the concentration of *Agrobacterium*, time of infection of calli, time of co-cultivation, and developmental stage of embryogenic calli of *L. perenne*.

Selection and regeneration of transgenic plants

Putative transgenic calli were initiated 1 week after co-cultivation. All of the calli grew on medium with 50 mg/L hygromycin in the first week of selection, but the growth of the calli gradually decreased at the beginning of the second week. Some calli kept growing (buff to yellow color), while others did not grow or gradually turned brown due to necrosis. After the surviving, hygromycin-resistant calli had been cultured in regeneration selection medium for 2–4 weeks, shoots were regenerated (Fig. 2a). These shoots were transferred into rooting medium without hygromycin, and all shoots developed roots in 2–4 weeks (Fig. 2b). The plantlets grew well on medium containing 50 mg/L hygromycin, but control plantlets died within 20 days. Plantlets were adapted for 7 days by opening the lids of the containers (Fig. 2c) and then transplanted to soil. Transgenic *L. perenne* plants were established in soil 3 months after acclimatization (Fig. 2d, f).

Molecular analysis of the transgenic plants

Following transformation with *A. tumefaciens* strain LBA4404 containing the pC1301-*CBF3* binary expression vector carrying three genes [*uidA* (GUS), *hpt*, and *AtDREB1A/CBF3*; Fig. 3a], transgenic calli and plants were obtained. The presence of the *AtDREB1A/CBF3* transgene in transgenic plants was examined by PCR amplification and Southern blot analysis. The 750-bp *AtDREB1A/CBF3* DNA fragment was presente in all DNA samples extracted from putative transgenic plants (Fig. 3b, c). Northern blot hybridization of the *AtDREB1A/CBF3*

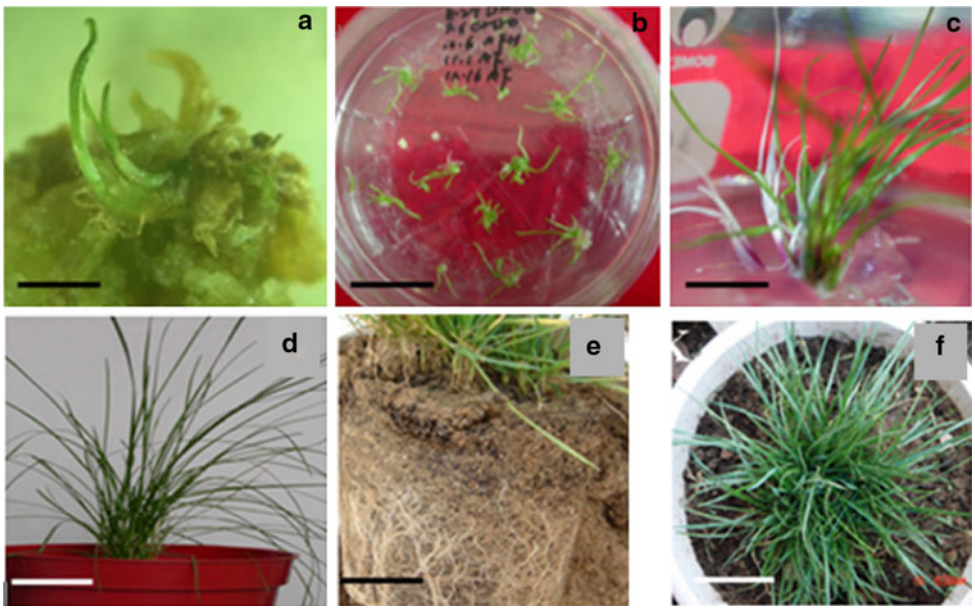

Fig. 2 Regeneration of transgenic *Lolium perenne* plants after inoculation with disarmed *Agrobacterium tumefaciens* strains. **a** Differentiation of hygromycin-resistant calli obtained after transformation with *A. tumefaciens* strains containing expression vector (*bar* 0.5 cm), **b**, **c** rooting of hygromycin-resistant shoots derived from calli (*bar* 0.9 cm). **d**, **f** transformed plants of *L. perenne* 3 months after acclimatization to culture room conditions (*bar* 2 cm)

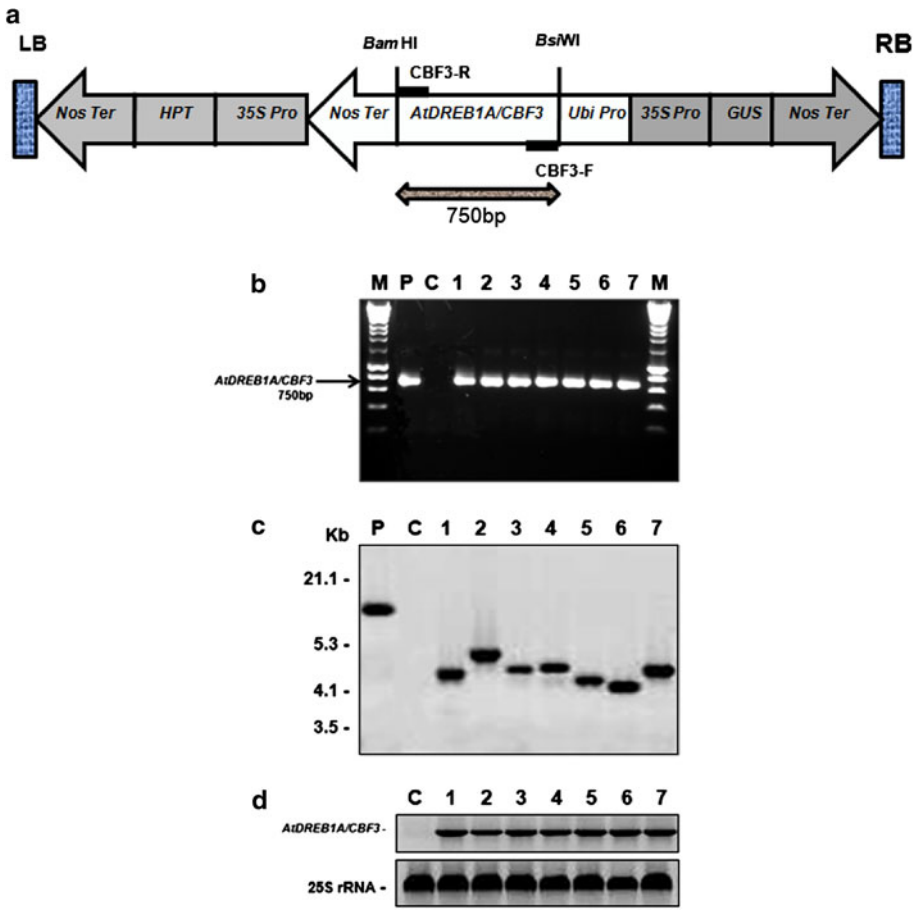

Fig. 3 Linear plasmid T-DNA map and molecular analysis of transgenic plants. **a** Plasmid vector pC1301-*CBF3* T-DNA indicating the localization of three genes [*uidA* (GUS); *hpt* (hygromycin phosphotransferase), and *CBF3*), two promoters [*35S Pro* (cauliflower mosaic virus 35S promoter) and ubiquitin (*Ubi Pro*)], a terminator [*nos Ter* (terminator from the nopaline synthase gene), and T-DNA borders (*LB* left border, *RB* right border). *Arrows* indicate gene translation orientation. The probe used in Southern and northern blot analyses of transgenic plants is the PCR products of the *CBF3* gene. **b** PCR analysis of DNA isolated from putative transgenic plants derived from stable transgenic lines L3, L5, L9, L12, L15, L18, and L19, respectively. *Lanes 1–7* Transgenic plants derived from transgenic lines L3, L5, L9, L12, L15, L18, and L19, respectively, *C* non-transgenic regenerated plantlet control, *P* pC1301-*CBF3* plasmid control. **c** Southern blot analysis of PCR-positive transformed plants. *Lanes*: *1–7* Transgenic plants derived from transgenic lines L3, L5, L9, L12, L15, L18, and L19, respectively, *C* non-transgenic regenerated plantlet control, *P* pC1301-*CBF3* plasmid control. **d** Northern blot analysis of PCR-positive transformed plants. *Lanes*: *1–7* Transgenic plants derived from transgenic lines L3, L5, L9, L12, L15, L18, and L19, respectively, *C* non-transgenic regenerated plantlet control. *Lower panel* Tobacco 25S rRNA

transgenic lines and non-transgenic control revealed that the transgene was expressed, as evidenced by the accumulation of *AtDREB1A/CBF3* mRNA in transgenic lines (Fig. 3d). No hybridization signal was detected in the non-transgenic control (Fig. 3d). We obtained seven stable transgenic lines (L3, L5, L9, L12, L15, L18, and L19), all confirmed by PCR, Southern, and northern blot analyses. As shown in Fig. 3d, the mRNA expression levels in the northern blots were consistent in all transgenic lines, without any positional effect of the transgene, mainly because the transgenic lines described here contain only one copy of T-DNA insertion. As such, in these transgenic lines, the positional effect of the transgene may be very low.

Drought and freezing tolerance

Under drought stress, most of non-transgenic *L. perenne* plants died, but transgenic *L. perenne* plants over-expressing *AtCBF3* grew well. Survival rates of plants derived from transgenic cell lines L3, L5, L9, L12, L15, L18, and L19 increased up to tenfold compared to those of the non-transgenic control 6 weeks after the dehydration treatments followed by re-watering (Table 1). Results from experiments of freezing tolerances of transgenic *L. perenne* plants over-expressing *AtCBF3* demonstrated that transgenic plants had increased freezing tolerance. Survival rates of plants derived from transgenic cell lines L3, L5, L9, L12, L15, L18, and L19 increased up to 11-fold

Table 1 Drought and freezing tolerances of transgenic *Lolium perenne* plants over-expressing *AtCBF3*

Treatment	Survival rate (%)							Control
	Transgenic lines							
	L3	L5	L9	L12	L15	L18	L19	
Drought treatment	82.2 ± 4.4 a	83.1 ± 1.9 a	84.2 ± 3.4 a	85.3 ± 2.7 a	86.2 ± 3.4 a	83.2 ± 3.7 a	81.2 ± 3.4 a	8.2 ± 1.4 b
Freezing treatment	81.6 ± 3.5 a	81.9 ± 2.8 a	82.7 ± 3.1 a	82.6 ± 4.2 a	80.6 ± 1.9 a	82.6 ± 3.3 a	80.6 ± 1.7 a	7.6 ± 1.5 b

Data represent the mean ± standard deviation. Values followed by different letters are significantly different ($\alpha = 0.05$) by the analysis of variance (ANOVA)

Survival rates of plants derived from transgenic cell lines L3, L5, L9, L12, L15, L18, and L19 and of non-transgenic control were determined 6 weeks after the dehydration treatments followed by re-watering for drought treatment and 6 weeks after plants were exposed to −6°C for 1 day (freezing treatment) followed by a return to 25°C. Experiments were repeated three times, and each replicate consisted of 30 plants

compared to the non-transgenic control 6 weeks after the freezing treatment (exposure of transgenic plants to −6°C for 1 day) followed by a return to 25°C (Table 1). These results showed that heterologous expression of the *Arabidopsis DREB1A/CBF3* gene enhances drought and freezing tolerance in transgenic *L. perenne* plants.

Physiological changes of transgenic plants

Following the drought treatments, each line showed wilting and drought-induced rolling of leaves with a concomitant increase in conductivity. In contrast to transgenic lines, control plants exhibited leaf rolling earlier and showed considerably more visual symptoms of drought stress, with leaf color changing from green-white to green-black and finally to yellow in the end of the drought period. Electrolyte leakage (EL) increased concommitantly with the enhanced drought stress and cold pressure (Fig. 4a). In the early days of the drought period, the EL of transgenic plants was a little higher than that of the control plants (Fig. 4a). The MDA content increased with increasing duration of the drought stress and remained lower in transgenic plants than in control plants (Fig. 4b). The activity of the antioxidant enzyme SOD in transgenic and control plants was equal at the beginning of the resistance treatments; it then increased slightly in the control plants, only to be followed by a sharp drop (Fig. 4c). POD activity increased erratically during the drought stress treatment (Fig. 4d). At the initial stage of the stress treatments, POD activity was equal in the transgenic and control plants. Only our results on EL, MDA content, SOD activity, and POD activity in transgenic lines L3 and L9 are presented in Fig. 4 because other transgenic cell lines, including L5, L12, L15, L18, and L19, showed similar results.

Discussion

The DREB/CBF transcription factors induce many of the genes involved in environmental stress tolerance in *A. thaliana* (Thomashow 1999; Wang et al. 2008; Maruyama et al. 2004). Using the full-length cDNA microarray and the Affymetrix GeneChip array, Maruyama et al. (2004) identified 38 *DREB1A* downstream genes, demonstrating that the DREB/CBF transcription factors may regulate the expression of a significant number of stress-inducible genes in Arabidopsis. In fact, transgenic plants over-expressing DREB1A showed activated expression of many stress-inducible genes and improved tolerance to not only drought, salinity, and freezing but also to growth retardation. Kasuga et al. (2004) reported that overexpression of *DREB1A* improved drought- and low-temperature stress tolerance in tobacco. A total of 145 DREB/ERF-related proteins are encoded in the *Arabidopsis* genome. These proteins have been classified into five groups: AP-2 subfamily, RAV subfamily, DREB subfamily, ERF subfamily, and others (Sakuma et al. 2002). Seki et al. (2001) constructed full-length *Arabidopsis* cDNA libraries to identify drought- and cold-inducible genes and target genes of DREB1A/CBF3 (Seki et al. 2001).

The results of our study show that overexpression of the transcription factor *AtDREB1A/CBF3* gene enhances drought and cold tolerance in transgenic *L. perenne* plants. Transgenic plants harboring a corn ubiquitin promoter driving the *AtDREB1A/CBF3* gene had higher SOD and POD activities than the wild-type controls. Determination of the electrolyte leakage and SOD and POD activity in transgenic and control plants demonstrated that AtDREB1A/CBF3 may be a regulator that is sufficient on its own to control gene expression involved in drought and cold tolerance in transgenic *L. perenne* plants without altering superficial morphology (Fig. 2). Therefore, the identification of transcription factors involved in environmental stress responses provides new targets for genetic engineering programs of turfgrass and other plants aimed at providing better multiple stress tolerance. The system described in this study is particularly suitable for the functional identification of novel genes derived from genomic research in species of turfgrass.

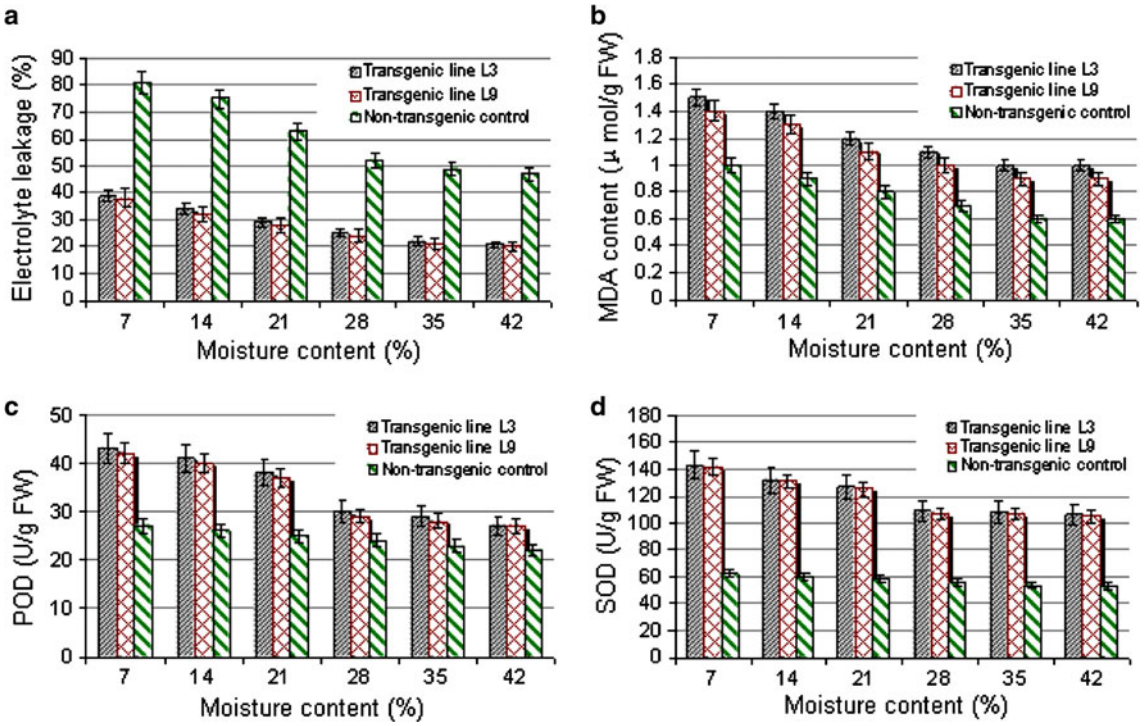

Fig. 4 Physiological changes in transgenic plants derived from transgenic lines L3 and L9, respectively, including effects of the drought stress on electrolyte leakage (**a**), malondialdehyde (*MDA*) content (**b**), peroxidase (*POD*) activity (**c**), superoxide dismutase (*SOD*) activity (**d**) of transgenic perennial ryegrass plants and control plants. Experiments were repeated three times, and each replicate consisted of three plants. Data represent the mean ± standard deviation

Oh et al. (2007) reported that transgenic rice plants over-expressing a barley gene HvCBF4 had an increased tolerance to drought, high-salinity, and low-temperature stresses without any stunting of growth. A comparison of 12 target rice genes of DREB1A/CBF3 revealed that five genes were common to both HvCBF4 and DREB1A/CBF3 and that ten and seven genes were specific to HvCBF4 and CBF3/DREB1A, respectively (Oh et al. 2007). These results suggest that the CBF/DREBs of barley may act differently in transgenic rice from those of the Arabidopsis system (Oh et al. 2007). Xin et al. (2007) reported that 312 genes regulated by ESK1 revealed a greater overlap with sets of genes regulated by salt, osmotic, and abscisic acid treatments than with genes regulated by cold acclimation or by the transcription factors CBF3 and ICE1, which have been shown to control genetic pathways for freezing tolerance. The *ESK1* gene encodes a 57-kDa protein and is a member of a large gene family of plant-specific genes whose function is unknown. The eskimo1 (*esk1*) mutation of Arabidopsis resulted in a 5.5°C improvement in freezing tolerance in the absence of cold acclimation. Following cold acclimation, esk1 plants are considerably more freezing tolerant than their cold-acclimated wild-type controls. *ICE1* (inducer of CBF expression 1), an upstream transcription factor that regulates the transcription of CBF genes in Arabidopsis, encodes a MYC-like bHLH transcriptional activator. *ICE1* binds specifically to the MYC recognition sequences in the CBF3 promoter. The *ice1* mutation blocks the expression of CBF3 and decreases the expression of many genes downstream of CBFs, which leads to a significant reduction in plant chilling and freezing tolerance. However, our results show that over-expression of the *Arabidopsis* transcription factor *DREB1A/CBF3* gene enhanced drought and cold tolerance in transgenic *L. perenne* plants through the pathway described in *Arabidopsis*. Determination of the SOD and POD activity in transgenic and control plants demonstrated that the activities of both of these enzymes decreased with increased soil moisture content. Compared to the non-transgenic control plants, transgenic plants had higher SOD and POD activities (Fig. 4c, d).

In conclusion, our results suggest that *AtDREB1A/CBF3* may be a regulator that is sufficient on its own to control the gene expression involved in antioxidant enzyme biosynthesis in transgenic *L. perenne* plants. Enhanced drought and freezing tolerance was associated with increased activities of antioxidant enzymes, and this result may prove to be useful in stress biology studies of turfgrass.

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