



ORIGINAL ARTICLE



Over-expression of the *Brachypodium* dehydrin gene, BdDHN3, enhances drought tolerance in *Brachypodium distachyon* (Poaceae)

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Abstract

Drought is the main abiotic stress that impacts biomass productivity and grain yield in crops. Dehydrins are part of the Late Embryogenesis Abundant protein family. They are typically upregulated under water-limited conditions, playing a key role in plant response and adaptation to stressful environments. However, the specific function of dehydrins and their relationship with plant functional traits and drought-related physiology remain poorly understood. *Brachypodium distachyon* L. is a well-studied monocot model plant for temperate grasses, yet DHN proteins in *Brachypodium* have not been extensively researched. To our knowledge, this is the first functional validation in *Brachypodium* of a dehydrin gene (BdDHN3) demonstrating a causal improvement in drought tolerance, together with a comparative analysis of BdDHN3 expression across 11 genetically distinct lines. Thus, we first examined variations in the expression level of the BdDHN3 gene among 11 genetically distinct *B. distachyon* inbred lines to assess the genetic consistency of gene expression. Second, we generated transgenic plants with overexpression constructs in the Bd21 line to analyze drought response at both the functional and genetic expression levels. On average, BdDHN3 expression was 447.2 times higher under drought conditions compared to the control across *B. distachyon* lines. Overexpression of BdDHN3 resulted in enhanced drought tolerance, as plants overexpressing BdDHN3 retained more water, exhibited lower cell damage, and had higher proline contents under water stress conditions compared to wild-type (WT) plants. These findings suggest that BdDHN3 may play an important role in the plant response to drought stress. Given its proximity and gene synteny to other significant temperate cereal crops, our results indicate that DHN3 proteins may also be relevant for temperate grasses with socio-economic importance.

Key message

Overexpression of the BdDHN3 gene in *Brachypodium distachyon* enhances drought tolerance by improving water retention, reducing cell damage, and increasing proline content, providing valuable insights for the development of drought-resistant crops.

Keywords *Brachypodium distachyon* · Dehydrins · Drought · Gene expression · Plant transformation

Introduction

Drought is a major constraint on crop productivity. Plants deploy coordinated physiological, biochemical, and molecular responses, including large transcriptional reprogramming, to tolerate water deficit (e.g., antioxidant defense, osmotic adjustment, and membrane protection (Hossain et al., 2016; Reddy et al., 2004). Different genes are induced by drought stress, and the functions of such gene products include not only protecting plant cells from dehydration but also regulating specific genes involved in signal transduction in response to drought (Hossain et al., 2016). Among

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drought-responsive genes, dehydrins (LEA-II) are highly hydrophilic proteins typically induced by dehydration, salinity, and freezing and proposed to protect macromolecules and membranes via water binding, metal/ROS sequestration, and chaperone-like activity (reviewed in Graether & Boddington, 2014; Hanin et al., 2011; Liu et al., 2017). Evidence from cereals and grasses indicates that dehydrin accumulation associates with improved drought tolerance and, in several cases, transgenic overexpression enhances stress tolerance (Decena et al., 2021; Graether & Boddington, 2014; Kosová et al., 2014; Liu et al., 2017; Yu et al., 2018). Yet, the specific functional role of individual dehydrins and their links to plant functional traits during drought remain incompletely understood.

Brachypodium distachyon L. (Poaceae) is a tractable model for temperate grasses closely related to small-grain cereals. It is well-suited to functionally validate candidate stress-tolerance genes relevant to crops (Catalan et al., 2014; Scholthof et al., 2018; Vogel, 2015; Watt et al., 2009). Previous expression work across *Brachypodium* suggested that dehydrin orthologs are up-regulated by drought, highlighting BdDHN3 as a strong candidate (Decena et al., 2021; Martínez, 2020). However, functional validation of dehydrins in *Brachypodium* has been limited. In this study, we focus on BdDHN3 (Bradi1g37410). We first assess drought-induced BdDHN3 expression across 11 inbred lines to test for consistency among genotypes. We then generate BdDHN3 overexpression (OE) lines in the Bd21 genotype background and quantify drought responses in key readouts: leaf water content (LWC) as a whole-plant water-status proxy, electrolyte leakage (EL) as a membrane injury indicator, and free proline as a canonical osmoprotectant and molecular chaperone. These complementary traits provide a concise, mechanistically grounded picture of drought tolerance in grasses (e.g., Des Marais et al., 2017; Martínez et al., 2018; Verbruggen & Hermans, 2008).

Here, we hypothesize that BdDHN3 overexpression enhances drought tolerance by improving leaf water status and membrane integrity and promoting osmotic adjustment. We test this prediction by comparing OE and Bd21 (WT) plants under well-watered and drought conditions, and by profiling BdDHN3 expression responses.

Materials and methods

Plant materials and growth conditions

We used 11 *B. distachyon* inbred lines differing in drought response (Bd21, Bd30, CANU14, CASIB15, CER5, JHIN13, RON11, SCA5, SOBRE7, VILLA13, VILLA3; Table S1; see also Martínez et al., 2018). Seeds were

stratified (4 °C, 2 d), sown in soil:perlite:sand: substrate (0.5:0.5:1, v/v), and grown at 21 °C, 65% RH, 16 h light. Details on the substrate and light intensity are provided in the Supplementary Methods. After 21 d, plants were maintained well-watered (control) or subjected to drought by withholding irrigation, following our previous protocol (Martínez et al., 2018). Briefly, Three-week-old plants (with three biological replicates per line) were subjected to control conditions—no watering restrictions for 21 days, with watering twice weekly—or to drought conditions, by withholding water after 21 days of growth.

Expression profiles of BdDHN3 between *B. distachyon* lines

Total RNA was isolated from the leaves (three replicates per line and treatment) using TRIzol® Reagent (Gibco-BRL, Life Technologies, Paisley, UK) according to the manufacturer's protocol. For cDNA synthesis, the First-Strand cDNA Synthesis Kit for RT-PCR (AMV, Roche) was used. Real-time quantitative PCR amplification was performed in 10 µl of reaction mixture per well containing 1 µl of cDNA, 0.4 µM of each gene-specific primer, and 5 µl of 2× SsoFast™ EvaGreen® Supermix (Bio-Rad). The BdDHN3 primers were designed using the Oligo Primer Analysis software v. 6.65, and their sequences are listed in Table S2. Full details are given in the Supplementary Materials. The ubiquitin genes BdUBC18 and BdUBI10 were used as internal reference genes to normalize gene expression values (Chambers et al., 2012; Hong et al., 2008). For each gene, calculation of normalized relative quantities (i.e., relative mRNA expression data) from Cq values was computed following the procedures described in Hellemans et al., (2007). Three technical measurements were conducted.

BdDHN3 cloning, transformation and transgenic lines

The transgenic *Brachypodium* lines (35S: BdDHN3), which constitutively overexpress BdDHN3 under the control of the CaMV35S promoter, were generated to examine the functions of BdDHN3 (Fig. 1). To confirm and assess the stress tolerance of transgenic lines, seeds of transgenic *Brachypodium* overexpressing BdDHN3 and wild-type (WT) plants were germinated, grown, and subjected to control and drought conditions as described above. Thus, a specific BdDHN3 region was amplified from Bd21 cDNA and cloned using Gateway recombination into pH7WG2D under CaMV 35S. The construct was introduced into *Agrobacterium tumefaciens* (AGL1) and Bd21 calli were transformed following Alves et al. (2009) with hygromycin selection (Fig. 1). Two independent OE lines (OE1, OE2) were

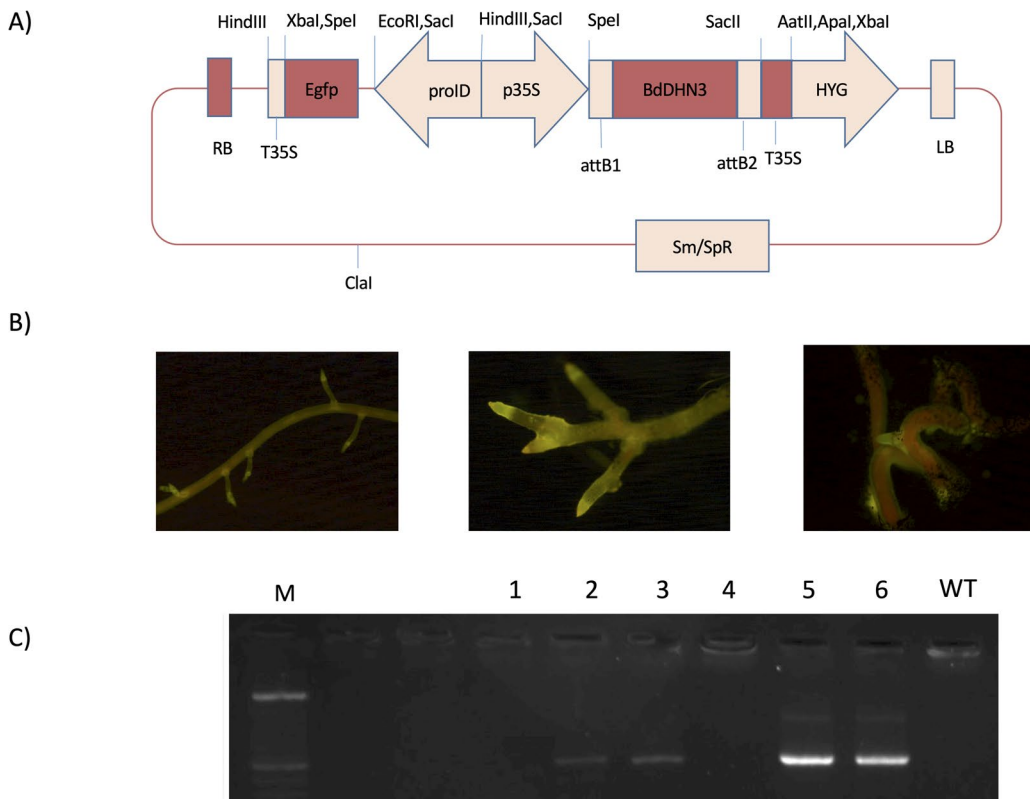


Fig. 1 **A** Diagram of the T-DNA regions of the binary vector (pH7WG2D-BdDHN3) used for *B. distachyon* transformation. The plasmid contain two expression units within the T-DNA region bordered by the right and left border sequences, RB and LD respectively. The first unit contain the Enhanced green fluorescent protein (Egfp) driven by rolD root loci D (rolD) promoter and terminated by the T35S CaMV 35S sequence (T35S). The second unit contain the hygromycin phosphotransferase gene (HYG) conferring hygromycin resistance driven by 35S cauliflower mosaic virus (CaMV) 35S promoter, attB1 and attB2 LR reaction site and terminated by the T35S CaMV 35S

sequence (T35S). A bacterial gene for Spectinomycin resistance (Sm/SpR) is also present in the vector backbone. Examples of single and double cutter restriction sites are indicated. Identification of transgenic *Brachypodium* plants with BdDHN3 expression. **B** Identification of constitutively expressing 35 s:BdDHN3 transgenic *Brachypodium* by BdDHN3-GFP fusion proteins visualization. **C** Identification of constitutively expressing 35 s:BdDHN3 transgenic *Brachypodium* by genomic PCR. M represents DL2000 DNA marker, 1 empty, 2, 3, 4, 5 (OE1), 6 (OE2) represent different transgenic lines and WT wild type Bd21

recovered. Reagent lists, media composition and selection steps are summarized in Supplementary Methods; primer sequences are in Table S2.

Molecular confirmation and expression analysis

To verify the integration of the transgene in *B. distachyon* transformants, leaf tissues from two-week-old transgenic and WT *Brachypodium* plants were used to extract genomic DNA using the CTAB method. PCR was then performed with a set of primers (Table S2) on the extracted genomic DNA (Fig. 1C). The PCR products were separated by electrophoresis on a 1% agarose gel and stained with RedSafe Nucleic Acid Stain Solution (iNtRON Biotechnology). PCR. BdDHN3 expression in WT and OE lines (control vs. drought) was quantified by RT-qPCR using BdUBC18 and BdUBI10 as references. Normalized relative quantities were calculated as in Hellemans et al. (2007). Technical

replication and thermal profiles are briefly described in the Supplementary Methods.

Stress tolerance assay on leaf functional traits

For the drought stress tolerance assay on leaf traits, transgenic and WT lines were subjected to control and drought conditions. We selected only the OE1 transgenic line for tolerance assays because it exhibited a high expression level of BdDHN3 (see Results). We measured Leaf Water Content (LWC), Electrolyte Leakage (EL), Specific Leaf Area (SLA), and Proline content under normal conditions and drought stress conditions. (1) To assess leaf water status, LWC was analyzed by harvesting leaf samples under control and drought conditions. The fresh weight (FW) was recorded immediately after harvest, and the dry weight (DW) was determined after 16 h incubation at 80 °C. LWC was measured according to the previously described method using the following formula: $LWC (\%) = (FW - DW) /$

FW $\times 100$ (Martínez et al., 2018). (2) EL was determined to evaluate cell membrane integrity by measuring electrical conductivity (Martínez et al., 2018). For this analysis, 0.1 g of leaves was incubated in 20 mL of deionized water and then shaken at 150 rpm and room temperature for five hours. The initial conductivity (C_i) was measured using a conductometer (HI9033 Multi-Range Conductivity Meter, Hanna® Instruments). The solution was then boiled for 20 min, cooled at room temperature, and the conductivity of the tissues (C_{max}) was determined again. We calculated the equation EL (%) as $(C_i/C_{max}) \times 100$. (3) The specific leaf area (SLA) of the largest leaf was leaf area/DW ($m^2 kg^{-1}$). Leaf area was assessed using a leaf area meter (LI-3000C Portable Area Meter from LI-COR® Biosciences). (4) Free proline, proline hereafter, was extracted by grinding 100 mg of dried plant material with 600 mL of 3% (w/v) sulfosalicylic acid. The extract was centrifuged at 13,000 rpm for 15 min at room temperature. A 0.3 mL aliquot of the extract was mixed with 1.5 mL of a solution of acidic ninhydrin [40% acidic ninhydrin (8.8 mM ninhydrin, 10.5 M glacial acetic acid, 2.4 M orthophosphoric acid), 40% glacial acetic acid and 20% of 3% sulfosalicylic acid]. The samples were then incubated for 60 min at 96 °C and the reaction was terminated by incubating the samples on ice for 5 min. Samples were then extracted by adding 3 mL of toluene and vortexing for 20 s. Absorbance at 520 nm was measured using toluene as a reference. A standard curve was created using L-proline in a range of 0–57.5 mg/mL. Free proline content was calculated according to (Bates et al., 1973): Free proline = $(mg \text{ proline } mL^{-1} \times mL \text{ toluene}) / 115 \times 5 \text{ mg } mmol^{-1} / (g \text{ sample}/5)$.

Statistical analysis

An ANOVA analysis was performed to examine variation in BdDHN3 gene expression among *B. distachyon* lines, with line, treatment, and their interaction as fixed factors. Similarly, variation in BdDHN3 gene expression and leaf functional traits between transgenic plants (BdDHN3 over-expression construct) and WT plants was analyzed using ANOVA in the same manner as described above. Analyses were conducted using JMP software (SAS Institute Inc).

Results

Expression profiles of BdDHN3 gene in *B. distachyon* lines

Drought treatment significantly affected the expression of the BdDHN3 gene ($F_{1,10}=26.4$, $P<0.0001$), which was consistently upregulated under drought stress conditions

across *B. distachyon* lines. However, the magnitude of this expression varied ($F_{1,10}=2.23$, $P=0.058$; Fig. 2A). Specifically, the expression of BdDHN3 was stronger in Bd30, CASIB15, SCA5, and VILLA13, while it was weaker in Bd21, CANU14, and SOBRE7. The remaining lines exhibited intermediate levels of drought-induced gene expression (Fig. 2A). On average, BdDHN3 expression was 447.2 times higher under drought conditions compared to the control.

Generation and confirmation of transgenic *Brachypodium distachyon* plants

The expression of the BdDHN3 gene in transgenic plants was verified using RT-qPCR. As shown in Fig. 2B, under control conditions, BdDHN3 mRNA levels were barely detectable in WT plants, while expression was significantly higher in the two transgenic lines tested. The OE1 and OE2 transgenic lines showed 35.5- and 6.1-fold increases in BdDHN3 expression compared to WT, respectively (Fig. 2B).

Over-expression of BdDHN3 enhances the drought tolerance of transgenic *Brachypodium* plants

Transgenic *Brachypodium* plants overexpressing BdDHN3, especially the transgenic line OE1, along with WT Bd21 plants, are shown in Fig. 3. The plants were photographed five weeks after they were sown. During a 21 day water stress drought, WT plants wilted, while transgenic plants remained less dehydrated and greener (Fig. S1). Under control conditions for five weeks without water restrictions, WT plants exhibited greater vigor than transgenic plants (Fig. S1).

Except for SLA, drought affected the expression of traits, although its magnitude depended on the specific line (Table 1; Table S3). Thus, transgenic plants showed higher LWC (7.4-fold) and less EL (2.3-fold) under water stress than WT plants (Fig. 3A, B). Similarly, proline drought induction was significant only in transgenic plants compared to WT ones, 3.6-fold (Fig. 3C). Finally, drought did not affect the expression of SLA in either OE or WT plants (Fig. 3D).

Discussion

Our results indicate that overexpressing the gene BdDHN3 in the model grass *B. distachyon* enhances drought stress tolerance. Similarly, the expression profiles of the BdDHN3 gene across 11 *B. distachyon* lines consistently show up-regulation during drought stress conditions, although some variation exists between lines. Previous studies have

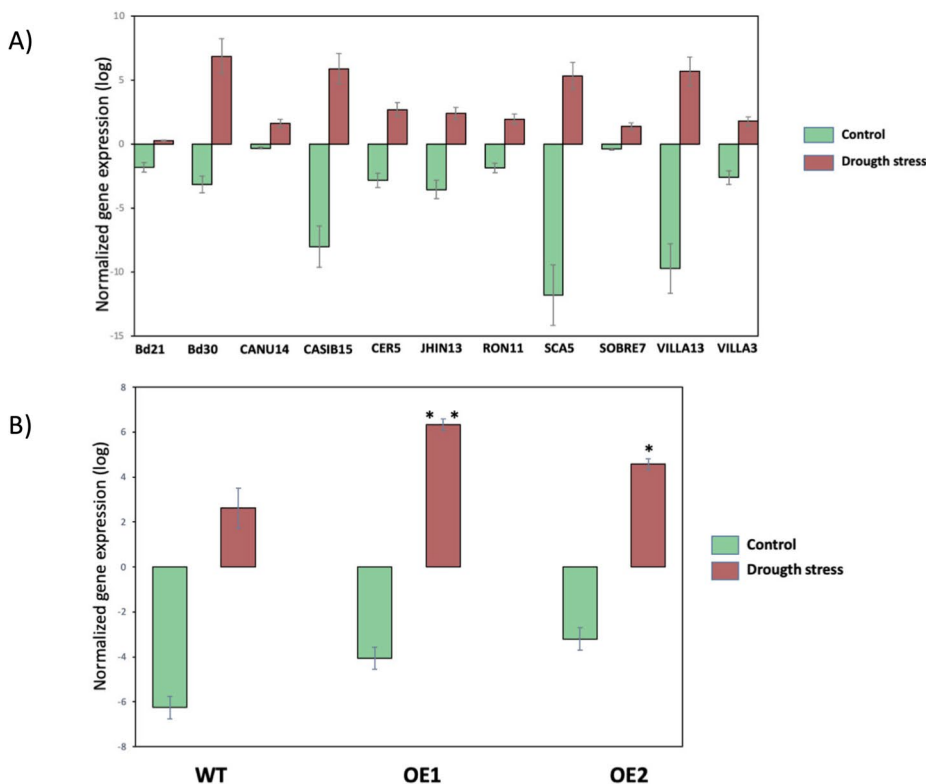


Fig. 2 **A** Variation in the normalized expression levels of BdDHN3 gene between 11 *B. distachyon* lines in control (well-watered, green bars) and after 21 days of experimental water restriction (25% of soil water content, red bars) estimated by RT-qPCR. Two-week-old *Brachypodium* plants were subjected for treatments, after which the leaves were collected and used for BdDHN3 expression analysis. Relative expression values are normalized to the geometric mean of BdUBC18 and BdUBI10 calibration genes and calibrated against WT control (Bd21)=1 (log2 scale). The values were computed using qBase (see methods). Values less than 1 (negative log2) indicate lower

expression than the calibrator genes, not “downregulation of controls.” **B** BdDHN3 overexpression level in 35 s: BdDHN3 transgenic (OE1 and OE2) and WT *Brachypodium* plants under control and drought stress. Total RNA from leaves of WT and transgenic plants were isolated and used for Real-time PCR employing gene-specific primers with actin as an internal control. Values are means $\pm$ SE ($n=5$ or 3 groups) from three independent experiments. Each group contained 10 plants. Asterisks indicate significant differences between the transgenic and WT plants under drought stress (* $P < 0.05$, ** $P < 0.01$)

reported differences in DHN3 gene expression between tolerant and sensitive lines in the annual grass *Hordeum vulgare* (Karami et al., 2013), documenting stronger drought induction in the tolerant line. In contrast, here we observed that the most drought-sensitive *B. distachyon* lines SCA-5, showed stronger BdDHN3 induction when exposed to water restriction, whereas the most tolerant ones, Sobre-7 and Bd21, exhibited relatively lower drought-induced expression of DHN3 (Fig. 3). Our findings are consistent with current DHNs literature, showing that overexpressing DHNs improves drought tolerance by enhancing water retention and protecting membranes. In many cases, this also results in increased proline levels, such as in the *Arabidopsis* line AnDHN-OX, which had higher RWC, less ion leakage, and changes in ROS-related enzymes (e.g., Sun et al., 2021). Recent reviews discuss the roles of DHNs, their structural diversity, subcellular localization—including organellar protection—and their ongoing relevance in engineering stress tolerance (Szlachetowska et al., 2023; Sun et al., 2021).

A recent study on barley supports the role of DHNs in drought survival within monocots and provides context for our *Brachypodium* findings (Zaidi et al., 2024). Our study advances this area by offering, to our knowledge, the first functional validation of a specific dehydrin (BdDHN3) in *Brachypodium*. It connects gene manipulation to whole-leaf functional metrics—such as LWC, EL, and proline—under standardized drought conditions, effectively linking gene to trait in a grass model closely related to cereals.

On the other hand, physiological analyses conducted here revealed that BdDHN3-overexpressing transgenic *Brachypodium* plants exhibited higher leaf water content (LWC), less cell damage, and increased proline levels under water stress conditions, which are indicators of improved drought tolerance in this system (Martínez et al., 2018). Whereas DHNs have been associated with water-deficit stress tolerance in many plants (Graether & Boddington, 2014; Liu et al., 2017; Yu et al., 2018), including cereals (Kosová et al., 2014; Park et al., 2006; Suprunova et al., 2004), here we

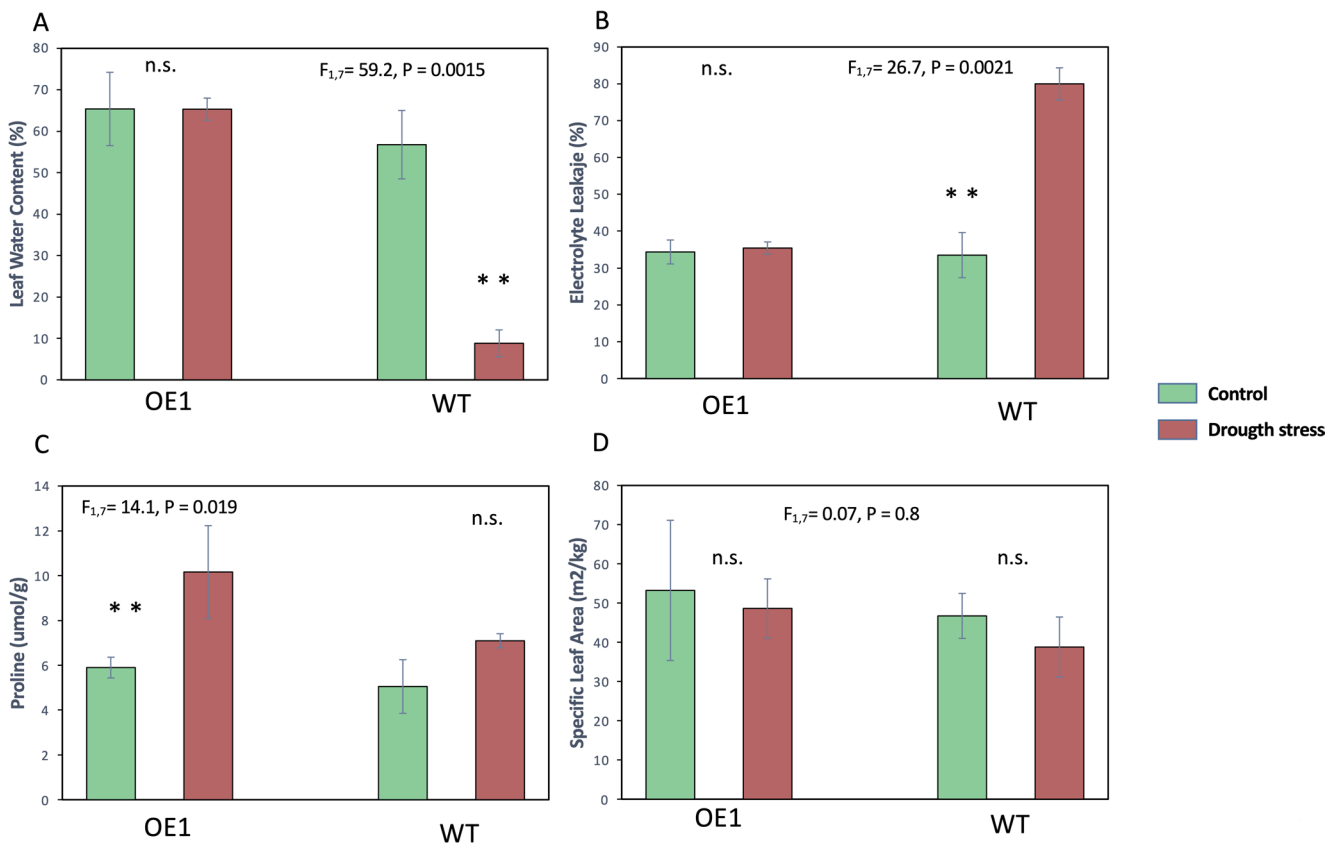


Fig. 3 Analysis of leaf functional traits in transgenic and WT plants under normal and drought conditions. **A** Leaf Water Content (LWC). **B** Electrolyte leakage (EL). **C** Proline content and **D** Specific Leaf Area (SLA). For each trait, data represent mean $\pm$ SD ($n=3$) from three rep-

licates. Two-way ANOVA (Treatment $\times$ Line) p values are shown in the graphs. Asterisks indicate significant difference between treatments for the transgenic and WT plants (* $P<0.05$, ** $P<0.01$; n.s. = non-significance α -level of 0.05)

Table 1 Summary results of ANOVA conducted to test the effect of drought treatment, line (WT vs. OE1), and their interaction in the expression of four drought-related traits. In bold, significant P values ($P<0.05$)

Effect	Trait							
	Electrolyte leakage		Water Content		Proline		SLA	
	F	P	F	P	F	P	F	P
Treatment	64.95	0.0013	26.7	0.0021	1.33	0.317	0.95	0.363
Line	54.82	0.0018	49.16	0.0004	22.38	0.0091	1.65	0.245
Treatment $\times$ Line	59.22	0.0015	26.7	0.0021	14.11	0.019	0.07	0.8

report the characterization of a specific drought stress tolerance dehydrin from *B. distachyon*. Previous in silico studies have revealed the significance of DHNs in *B. distachyon*, identifying dehydrin genes and conserved promoter motifs. This is consistent with gene expression analyses, which have shown that dehydrin orthologs are more highly expressed in plants under drought conditions across the *Brachypodium* species (Decena et al., 2021). Moreover, our transcriptome study using RNAseq in the *B. distachyon* complex showed that drought-induced expression of DHNs, including BdDHN3, was prominent in all three species of the complex (Martinez 2018). Thus, together, these results confirm the importance of DHNs regulation under drought stress in *B. distachyon*. LWC reflects the responses of the

entire plant, making it a relevant tool for measuring drought stress tolerance in *B. distachyon*. The value of LWC in our transgenic *Brachypodium* was higher than in WT when they were subjected to drought stress (Fig. 3A). This indicates that BdDHN3 overexpression contributes to maintaining the leaf water status of the plant at the same level that was detected in plants without stress treatment, although the specific physiological mechanism is unknown. As a significant indicator of cellular membrane injury, the EL value was significantly higher in the WT than in the transgenic lines, indicating that the transgenic plants suffered less membrane damage than the WT under drought treatment (Fig. 3B). The contribution of LEA proteins and dehydrins to transgenic plants maintaining higher water content and less membrane

damage under drought stress has been previously reported in *Nicotiana tabacum* (Sun et al., 2015), *Hordeum vulgare* (Karami et al., 2013) and *Ipomoea pes-caprae* (Zhang et al., 2018), and has been suggested to be related to inhibition of the ROS response and/or improvements of antioxidative activity (Karami et al., 2013; Sun et al., 2015). While we did not assay antioxidant enzymes here, dehydrin-mediated protection is often associated with attenuated ROS damage (Liu et al., 2017; Saibi et al., 2015); our EL and proline profiles in OE plants are compatible with this mechanism and warrant direct quantification of ROS and antioxidant enzyme activities in future work. Cellular accumulation of free proline is considered a protective cellular mechanism against environmental stresses, including drought (Verbruggen & Hermans, 2008). Proline acts as both an osmoprotectant and a molecular chaperone, maintaining cell viability and preventing oxidative damage caused by dehydration (Verbruggen & Hermans, 2008). Although the main effect of treatment on proline was not significant, the significant Treatment $\times$ Line interaction reflects a drought-induced proline increase in OE but not in WT (Fig. 3C), consistent with genotype-specific osmotic adjustment. Thus, a higher accumulation of proline was detected in the BdDHN3 overexpression transgenic lines (Fig. 3C), indicating that under drought treatment, BdDHN3 overexpression provided better protection by regulating proline metabolism to maintain plant water status and growth. Similar results to those reported here have been observed in studies examining the functional characterization, where the overexpression of dehydrin genes has improved drought tolerance in transgenic plants. Thus, transgenic alfalfa overexpressing a dehydrin, CsLea, from a desert grass *Cleistogenes sgorica* showed higher free proline content, higher relative water content (RWC), decreased membrane injury, and higher content during drought and salt stresses (Zhang et al., 2016). Transgenic Arabidopsis overexpressing a dehydrin, IpDHN from *Ipomoea pes-caprae*, showed higher content of proline, higher RWC, and decreased membrane damage when exposed to different abiotic stresses, including drought (Zhang et al., 2018). Because SLA reflects leaf thickness, reduced SLA is presumed to be a way to improve water-use efficiency in drought stress, as photosynthetically active tissue that is more tightly packed allows for more efficient use of a relatively minor surface (Wellstein et al., 2017). However, the SLA values did not differ significantly between transgenic and WT plants (Fig. 3D), indicating that BdDHN3 overexpression did not affect SLA in our constructions. Despite improved LWC and reduced EL in OE plants, the absence of SLA response suggests that BdDHN3 mainly provides short-term osmoprotection rather than altering leaf structure, which often takes longer to develop or requires stronger selection histories (Navarro et al., 2022). This

separation between structural and physiological traits during drought has been observed in grasslands and aligns with meta-analytic evidence on how SLA responds to extreme drought in various contexts (e.g., Wellstein et al., 2017).

Conclusion

Functional analyses showed that BdDHN3 is vital for drought stress tolerance in *B. distachyon*. Similarly, the consistent drought gene expression pattern across *B. distachyon* lines with different water stress tolerances (Martínez et al., 2018) suggests that the up-regulation of the BdDHN3 gene is crucial for the species' drought response, aligning with findings from transcriptome analysis. Together, our data position BdDHN3 as a downstream effector that confers short-term osmoprotection (improved LWC, reduced EL, increased proline), complementing upstream regulator studies. In line with motif analyses reported for plant DHN promoters, which commonly harbor ABRE/DRE elements (e.g., Nakasima et al., 2014), this suggests a tractable ABA/DREB control of BdDHN3 expression in grasses that can be tested by promoter analyses and TF perturbations in future work. Given its proximity and gene synteny to other key temperate cereal crops (Scholthof et al., 2018), our results suggest that DHN3 proteins may also be important for temperate grasses, which have economic and social significance. This work thus provides a foundation for further research and the potential use of the DHN3 gene in genetic engineering of other crops. In summary, our study confirmed the significant value of the continued investigation into the function and mechanisms of the BdDHN3 gene in *B. distachyon* for the further development of stress-tolerant crops in genetic breeding.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s40502-025-00905-9>.

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Author contributions Luis María Martínez, Antonio José Manzaneda, Pedro J Rey and Ana María Fernández Ocaña all contributed to the study's conception and experimental design. Luisa M. Martínez handled material preparation, data collection, and analysis. Luisa M. Martínez and Antonio J. Manzaneda drafted the initial version of the manuscript, with all authors providing comments on subsequent drafts. All authors reviewed and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors declare that they have no competing interests.

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