

Communication

CRISPR-Editing *AsDREBL* Improved Creeping Bentgrass Abiotic Stress Tolerance

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Abstract

Cool-season creeping bentgrass (*Agrostis stolonifera* L., *As*) is extensively used on golf courses worldwide and is negatively affected by several fungal diseases and abiotic stresses including drought and salinity. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated) gene editing technology was employed in this project to knock out the *AsDREBL* (*dehydration responsive element binding-like factor*) gene, a potential negative regulator in stress tolerance. With our established single guide RNA (sgRNA)-based CRISPR-editing vector and optimized creeping bentgrass tissue culture system using mature seed-derived embryogenic calli of cv. Crenshaw as explant, more than 20 transgenic plants were produced by gene gun bombardment. Fifteen confirmed *AsDREBL* mutant plants were tested for drought and salinity tolerance by withholding water and applying salt spray in greenhouse settings. Some of the mutants were shown to be more tolerant of drought and salinity stress compared to the non-edited, wild type Crenshaw plants. Our results demonstrate that CRISPR-gene editing technology can be successfully applied to improve the agronomical traits of turfgrass.

Keywords: CRISPR-gene editing; *DREB-like*; creeping bentgrass; drought tolerance; salinity tolerance



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1. Introduction

Cool-season creeping bentgrass (*Agrostis stolonifera* L., *As*) is extensively used on golf courses throughout the northern and central parts of the U.S. and in the world. Besides being affected by several fungal diseases, including brown patch caused by *Rhizoctonia solani*, anthracnose by *Colletotrichum cereale*, gray snow mold, *Microdochium* patch, *Pythium* foliar blight and root dysfunction, and dollar spot caused by *Clarireedia jacksonii* (formerly *Sclerotinia homoeocarpa* F.T. Bennet) [1], creeping bentgrass is affected by drought, heat, and cold stresses similar to other turfgrass species. Drought-tolerant cultivars are becoming particularly important as water becomes a more limited resource [2]. Great progress has been made in turfgrass breeding including improving turf quality, stacking disease resistance, and stress tolerance in turfgrass cultivars [1]. Functional genomics is becoming increasingly important in studying the mechanisms of stress tolerance and disease resistance in grasses. It was reported in 2010 that many genes are differentially expressed in perennial ryegrass under drought stress [3]. These genes were classified into 13 groups with known functions, some of which encode mitogen-activated protein kinases (MAPKs), in association with abscisic acid (ABA) accumulation and superoxide dismutases (SODs), leading to reactive oxygen species (ROS), heat-shock proteins (HSPs) and dehydrins. The differential

accumulation of dehydrins has been characterized in hybrid and common bermudagrass genotypes under drought stress [4]. Elevation of antioxidant defense protein levels was also found to be important for drought tolerance in C_4 perennial *Cynodon* species [5]. It was shown that microRNA319 positively regulates salinity and drought stress responses in creeping bentgrass [6]. Transcription factors (TFs) have been found to play central roles in the regulation of genes contributing to stress tolerance. Although very few stress-related TFs have been described in grasses such as the *BdDREB2* (dehydration responsive element binding 2) in buffalograss (*Buchloe dactyloides*) [2], many TFs have been characterized in rice (*Oryza sativa*) [7–10].

Since the publication of the CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated) system in *Science* in 2012 [11], this amazing technology has been used to mutate and engineer genes in many different plants, including our own works [12–14]. It has been demonstrated that *CBF2/DREB1C* (C-repeat-binding factor/dehydration responsive element-binding factor 1) is a negative regulator of *CBF1/DREB1B* and *CBF3/DREB1A* expression and plays a central role in stress tolerance in *Arabidopsis* [15]. It was shown that mutations in *AtDREB1C* enhanced *Arabidopsis* tolerance to freezing, dehydration and salinity [15]. We hypothesize that knocking out (KO) the homologous *DREB1C* gene in creeping bentgrass would lead to a loss of function for the negative regulator, resulting in positive response, i.e., tolerance to drought and other abiotic stress. We developed the single guide RNA (sgRNA)-based CRISPR-editing platform for monocotyledonous plants and mutated the *DREB1C-like* (*DREBL*) in the cultivar Crenshaw. Our results showed that the *AsDREBL* mutant Crenshaw plants were more tolerant of drought and salinity compared to the non-edited, wild type (WT) plants.

2. Materials and Methods

2.1. Cloning of *Agrostis stolonifera* (*As*) *DREB1C-like* (*AsDREBL*) Genomic DNA (gDNA) by PCR

The amino acid sequence of *Arabidopsis CBF2/DREB1C* [15] gene (accession #FJ169308) was used to search for the equivalent gene in *Brachypodium distachyon* (*Bd*). The *BdDREB* peptide sequence was then used to search for the *A. stolonifera* cv. Crenshaw EST (expressed sequence tag) library constructed by Rutgers [16] via the NCBI (National Center for Biotechnology Information) tblastn function. The cDNA of *AsDREB1C-like* (named as *AsDREBL* hereafter) mRNA, DY543604, was identified and based on which primers were designed and used to PCR-amplify the gDNA of Crenshaw. Total DNA of Crenshaw was isolated from a greenhouse-grown grass plant using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma, Burlington, MA, USA) following the manufacturer's instruction. The PCR protocol was as follows: one cycle of 95 °C for 5 min; thirty-five cycles of 95 °C, 1 min, 55 °C, 1 min, 72 °C, 1 min; and one cycle of 72 °C, 5 min. The PCR product was cloned into pGEMT-easy (Promega, Madison, WI, USA) and sequenced by Sanger sequencing.

2.2. Construction of *AsDREBL* CRISPR-Editing Vector

The 553 bp partial sequence of *AsDREBL* gDNA was scanned for the target site for CRISPR-editing with focus in the region encoding the 58 amino acids that align to that of *BdDREB*. The 20 nucleotide (nt) sequence, GCCGATGCCGATGCCGATAT with a starting “G”, was identified as the potential target sequence (CRISPR RNA, crRNA) that encompasses the *EcoRV* restriction cutting site at the immediate 5' upstream of the “CGG” PAM (protospacer adjacent motif) site. With a pair of mutated oligos, these 20 nucleotides were incorporated into our existing sgRNA cassette upstream of the synthesized 82 bp tracrRNA (CRISPR RNA) scaffold with nine Ts [17] for the binding of *Streptococcus pyogenes* Cas9 nuclease with the wheat U6 promoter [18]. The crRNA/scaffold cassette that would

produce the single guide RNA (sgRNA) was cloned into our vector pRD297, containing the synthesized, codon-optimized Cas9 driven by the intron-containing maize ubiquitin promoter, and terminated by the nopaline synthase terminator [19]. The sgRNA and Cas9 cassettes were subsequently cloned into pCambia1300 (Addgene, Watertown, MA, USA) with hygromycin resistance gene as the plant selective marker, resulting in the *AsDREB*-editing vector pRD303.

2.3. Transformation of Creeping Bentgrass cv. Crenshaw Embryogenic Calli with pRD303

The published tissue culture and gene gun bombardment protocols for creeping bentgrass were followed with modifications [20]. Essentially, seeds of cv. Crenshaw were disinfected by 30% bleach for 15 min, rinsed with sterile water, and placed on Murashige and Skoog (MS) medium (PhytoTech Labs, Lenexa, KS, USA), containing 6.6 mg/L dicamba to induce embryogenic calli. Freshly induced calli were transformed by gene gun bombardment with *AsDREBL*-editing vector pRD303 by the BioRad Biolistic® PDS-1000 system. Transformed calli were selected on MS medium containing 6.6 mg/L dicamba and 200 mg/L hygromycin. The surviving calli were transferred to MS medium with 1 mg/L 6-benzylaminopurine (6-BA) and 100 mg/L hygromycin to induce transgenic shoots, which were then moved to MS medium without hormones but containing 50 mg/L hygromycin to induce root formation. Regenerated plantlets were acclimatized to grow in soil in greenhouse conditions.

2.4. Identification of *AsDREBL*-Edited Crenshaw Mutants

Restriction fragment length polymorphism (RFLP) method was used to identify *AsDREBL*-edited Crenshaw mutant plants. The gDNA from each regenerated plant and the non-transformed, wild type (WT) plant was isolated using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma, Burlington, MA, USA) following the manufacturer's instruction. PCR was used to amplify a 277 bp-fragment spanning the 20 nt target sequence with the following protocol: one cycle of 95 °C for 5 min; thirty-five cycles of 95 °C, 30 s, 55 °C, 30 s, 72 °C, 30 s; and one cycle of 72 °C, 5 min. The PCR products were purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). The purified PCR products were digested with *EcoRV* restriction enzyme that constitutes part of the 20 nt target sequence. The digested PCR products were electrophoresed on 1.2% agarose gel. The different banding patterns of the digested PCR products from the putative mutant plants were compared to the WT plant. The mutated, undigested PCR products were purified from the agarose gel with the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) and sequenced by Sanger sequencing (Genewiz now Azenta, Burlington, MA, USA). Some of the undigested, purified PCR products were cloned into pGEMT-easy (Promega, Madison, WI, USA) and the clones were sequenced by Sanger sequencing using the built-in T7 promoter primer.

2.5. Drought Tolerance Testing of *AsDREBL* Mutant Crenshaw Plants

Fifteen confirmed mutants and fifteen WT plants were grown in the greenhouse, and individual tillers were separated into single pots filled with Promix soil mix. Each mutant and WT lines had three replicates. After the establishment of the vigorous growth of the plants for two months with weekly fertilization of 20-20-20 (N-P-K), they were trimmed and transferred to 2.5" × 2.5" plastic square pots filled with sand. Plant pots were placed in the controlled growth chamber with 16/8 light/dark cycle and constant 25 °C. Plants were irrigated with lab-prepared Hogland solution once per day for two weeks, with daily randomization of the plant pot position in the growth chamber. The drought testing started by withholding the irrigation of Hoagland solution. Plants were visually examined daily and photographed by putting the potted plants into a dark box with a camera fitted onto

the top without light leakage until all plants died on Day 5. The images were analyzed by the open-source ImageJ (Version 1.54p) software developed by the National Institute of Health (NIH). The greenness of the plants after withholding water from Day 1 to Day 5 was compared to that of the plants at Day 0, which was set as 100%. The Normalized Difference Vegetation Index (NDVI) of each plant was measured three times and averaged with Spectrum TCM500 (Spectrum Technologies Inc., Aurora, IL, USA). The weight of the pot, label, and plant was measured and recorded for each plant every day from Day 0 to Day 5.

2.6. Salinity Tolerance Testing of *AsDREBL* Mutant *Crenshaw* Plants

The 15 confirmed mutant lines and 15 WT plant lines were grown in the greenhouse. Then, three individual tillers from each line were transferred into 6-inch long, circular (1.5 in diameter) conical cones filled with sand. Plants were grown in our salt tent under 16/8 light/dark cycle, fertilized weekly with 20-20-20 (N-P-K) and trimmed regularly for two months. For salt treatment, the plants were sprayed with 50% homemade Hoagland solution plus Instant Ocean Sea Salt every day. The salt concentration increased from 1 decisiemens (DS) for one week to 2, 3, 4, 5, 6 DS in six weeks. Plants were trimmed every week, and their ratings of “tip burn” (1 to 9, with 1 = full leaf burn; 9 = no leaf burn) were recorded before each trimming.

3. Results

3.1. Identification of the *AsDREBL* Gene in Creeping Bentgrass *Crenshaw* and the Construction of *AsDREBL* CRISPR-Editing Vector

The cDNA of *AsDREB-like* (named as *AsDREBL* hereafter) (GFJH01020221) was identified from the *Crenshaw* EST library constructed by Rutgers [16] in NCBI by tblastn function using the amino acid sequence of Arabidopsis *CBF2/DREB1C* [15] (accession #FJ169308) and the *Brachypodium distachyon* homolog gene. The translated peptide sequence of *AsDREBL* has a stretch of 58 residues that are identical to that of the *B. distachyon* homolog. Primers were designed and used to PCR-amplify the partial gDNA. A 553 bp-PCR product was obtained and sequenced. The 20-nucleotide sequence GCCGATGCCGATGCCGATATCGG was chosen as the target gene editing site that starts with the “G” for the transcription by wheat U6 promoter, contains the *EcoRV* site (GATATC) and ends with the “CGG” PAM site. The eventual integrating CRISPR-editing vector pRD303 produced the sgRNA and monocot-optimized Cas9 driven by the intron-containing maize ubiquitin promoter (Figure 1).

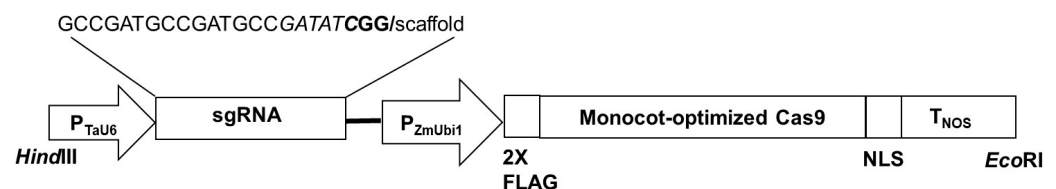


Figure 1. Schematic representation of pRD303, the *AsDREBL*-editing CRISPR vector. The sgRNA with the 20 nt target sequence and the scaffold is driven by the wheat U6 promoter (P_{TaU6}). The *EcoRV* restriction site is italicized, and the PAM site is in bold. The monocot codon-optimized Cas9 is under maize intron-containing ubiquitin promoter, with the 2X FLAG (DYKDDDDK) peptide at the N-terminus and the nucleol localization signal (NLS) at the C-terminus, and terminated by the nopaline synthase terminator (T_{Nos}). The sgRNA and Cas9 cassettes were cloned into the HindIII and EcoRI sites in plant expression vector pCambia1300 with the hygromycin resistance as the selectable marker for transgenic plants.

3.2. Production of *AsDREBL*-Edited Mutant *Crenshaw* Plants

Out of 12 plates (4 plates/batch, totaling three batches) with *Crenshaw* embryogenic calli, plated in a circle with a diameter of 3 cm on a filter paper that was bombarded by gene gun, 26 individual shoots were regenerated after hygromycin selection and transferring to MS medium containing 6-BA and hygromycin. Twenty-one (21) shoots readily produced roots on MS medium with 25 mg/L hygromycin. Some of the representative images are shown in Figure 2. These 21 plantlets (namely 303-1 to 21) were successfully acclimated to grow in soil in the greenhouse.

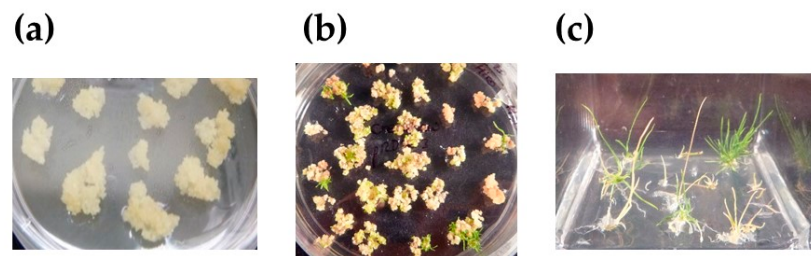


Figure 2. Production of *AsDREBL* mutants by CRISPR-editing vector pRD303. (a) Induction of embryogenic calli from *Crenshaw* seeds. Calli were transformed by pRD303 via gene gun bombardment and selected on medium with 200 mg/L hygromycin. (b) Regeneration of transgenic shoots after gene gun bombardment of the calli on medium containing 100 mg/L hygromycin. (c) Root induction on medium containing 50 mg/L hygromycin.

RFLP method was used to identify the putative *AsDREBL* mutants. Since the *EcoRV* site is part of the 20 nt target sequence and also part of the PAM, the 277 bp PCR product of the gDNA amplification from the WT plant would be digested into two fragments with the sizes of 203 bp and 74 bp. If the *EcoRV* site was mutated, the PCR products from the putative *AsDREBL* mutants would not be cut into two fragments. Since creeping bentgrass is tetraploid, and, in most cases, CRISPR-gene editing does not result in mutations in all alleles [12,13], the 277 bp WT PCR products produced from the WT alleles would be cut into the 203 bp and 74 bp fragments and the mutated 277 bp PCR products would be produced from the mutated alleles. Our results show that 6 out of the 21 plants transferred to soil, namely 303-3,4,6,7,15,21, produced an obvious undigested PCR band at the size of about 277 bp and smaller products similar to the ones by the WT plant at 203 bp and 74 bp, as shown in Figure 3. Nine other plants, 303-1,2,5,8,9,10,11,12,16, also produced a less obvious, seemingly undigested PCR product and two smaller digested products, while 6 plants (303-13,14,17,18,19,20) did not produce a clear undigested PCR product.

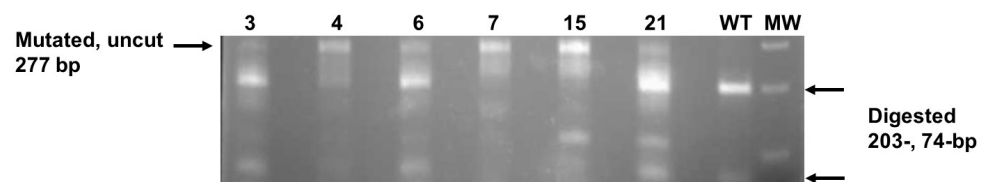


Figure 3. RFLP analysis of *AsDREBL* mutants. The 277 bp partial gDNA spanning the 20 nt target sequence was PCR-amplified from 303-3, 4, 6, 7, 15, 21 and WT plants. The PCR products were digested with *EcoRV* and electrophoresed on 1.2% agarose gel. The 100, 200, and 300 bp from the 1kb plus molecular weight (MW) marker (Thermo Fisher Life Technologies) are shown.

The seemingly undigested 277 bp bands from 15 plants were purified from the gel and were Sanger-sequenced by the forward primer. Figure 4a shows a variety of mutations that occurred in 303-1,2,3,4,5,6,7,8,9,10,12,15,16,19,21 around the target site. The PCR product of 303-15 was then cloned into pGEMT-easy and 10 individual clones were sequenced. The results show that one clone had the WT sequence, and there were six different mutation

types in total (Figure 4b), indicating the polyploidy nature of the genome [16] and that most of the alleles were mutated by our CRISPR-editing vector pRD303.

WT	CAAACGATGCCGATGCCGATGCCGATAT CGG CATGGTCAGAA
303-1	CAAA-----CGATGCCGATGCCGATATCGGCATGGTCAGAA
303-2	CAAAC-A-----ATGCCGATGCCGATATCGGCATGGTCAGAA
303-3	CAAACGATGCCGATGCC-A-----ATATCGGCATGGTCAGAA
303-4	CAAACGATGTTCGATGCCG-T-----TATCGGCATGGTCAGGA
303-5	CAAACGATGCCGATGCCGAT-----AT--CGGCATG----GAA
303-6	CAAACGATGCCGATGCCGAT-----AT-T-GGCATGGTCGGAA
303-7	CAAAATGATGCCGATGC---T---GATATCGGCATGGTCAGAA
303-8	CAAACGATGCCGATGCCGAT-----AT-T-GGCATGGTCAGAA
303-9	CAAA-----CGATGCCGATGCCGATATCGGCATGGTCAGAA
303-10	CAAACGATGCCGATGC---T---GATATCGGCATGGTCAGAA
303-12	CAAACGATGCCGAT-----T-CCGATATCGGCATGGTCAGAA
303-15	CAAA-----CGATGCCGATGCCGATATCGGCATGGTCAGAA
303-16	CAAACAATGCCGAT-----A--CCGATATCGGCATGGTCAGAG
303-19	CAAACGATG-C--T---GATGCCGATATCGGTATGGTCAGAA
303-21	CAAACGATGCCGAT-----AT-C-G-----G-CATG----GAA

(a)

WT	CAAACGATGCCGATGCCGATGCCGATAT CGG CATGGTCAGAA
8-1	CAAACGATGCCGATGCCGAT-----AT-T-GGCATGGTCAGAA
8-2	CAAACGATGCCGATGC---T---GATATCGGCATGGTCAGAA
8-3	CAAA-----CGATGCCGATGCCGATATCGGCATGGTCAGAA
8-4	CAAA---T---GATGCCGATGCCGATATCGGCATGGTCAGAA
8-5	CAAA-----CGATGCCGATGCCGATATCGGCATGGTCAGAA
8-6	CAAA-----CGATGCCGATGCCGATATCGGCATGATCAGAA
8-7	CAAACGATGCCGATGCCGATGCCGATATCGGCATGGTCAGAA
8-8	CAAA-----CGATGCCGATGCCGATATCGGCATGGTCAGAA
8-9	CAAACGATGCCGAT-----AT-C-GA-----CATGGTCAGAA
8-10	CAAACGATGC---T---GATGCCGATATCGGCATGGTCAGAA

(b)

Figure 4. Sequencing results of *AsDREBL* mutants. (a) Sanger sequencing results of PCR products amplified from WT and 303-1,2,3,4,5,6,7,8,9,10,12,15,16,19,21 gDNAs, spanning the 20 nt target site (underlined). The *EcoRV* site is italicized. The PAM site (CGG) is in bold. (b) The PCR product from 303-8 was cloned into pGEMT-easy and 10 individual clones were Sanger-sequenced.

3.3. *AsDREBL*-Edited Mutant Crenshaw Plants Were More Tolerant to Drought

Fifteen *AsDREBL* mutant and fifteen WT plants (with three replicates/each) were grown in the greenhouse and transferred to sand, then tested for drought tolerance by withholding water for five days. Our data show that the NDVI values of 15 mutant plants were not much different from the 15 WT plants from Day 0 to Day 5 of drought. The biomass of these 15 mutants also did not show a difference when compared to the 15 WT plants from Day 0 to Day 5 of drought. However, the greenness of 303-7 and 303-8 plants, as analyzed by ImageJ, was shown to consistently stand out compared to the other 13 mutant and all WT plants from Day 1 to Day 5 of drought (Figure 5a).

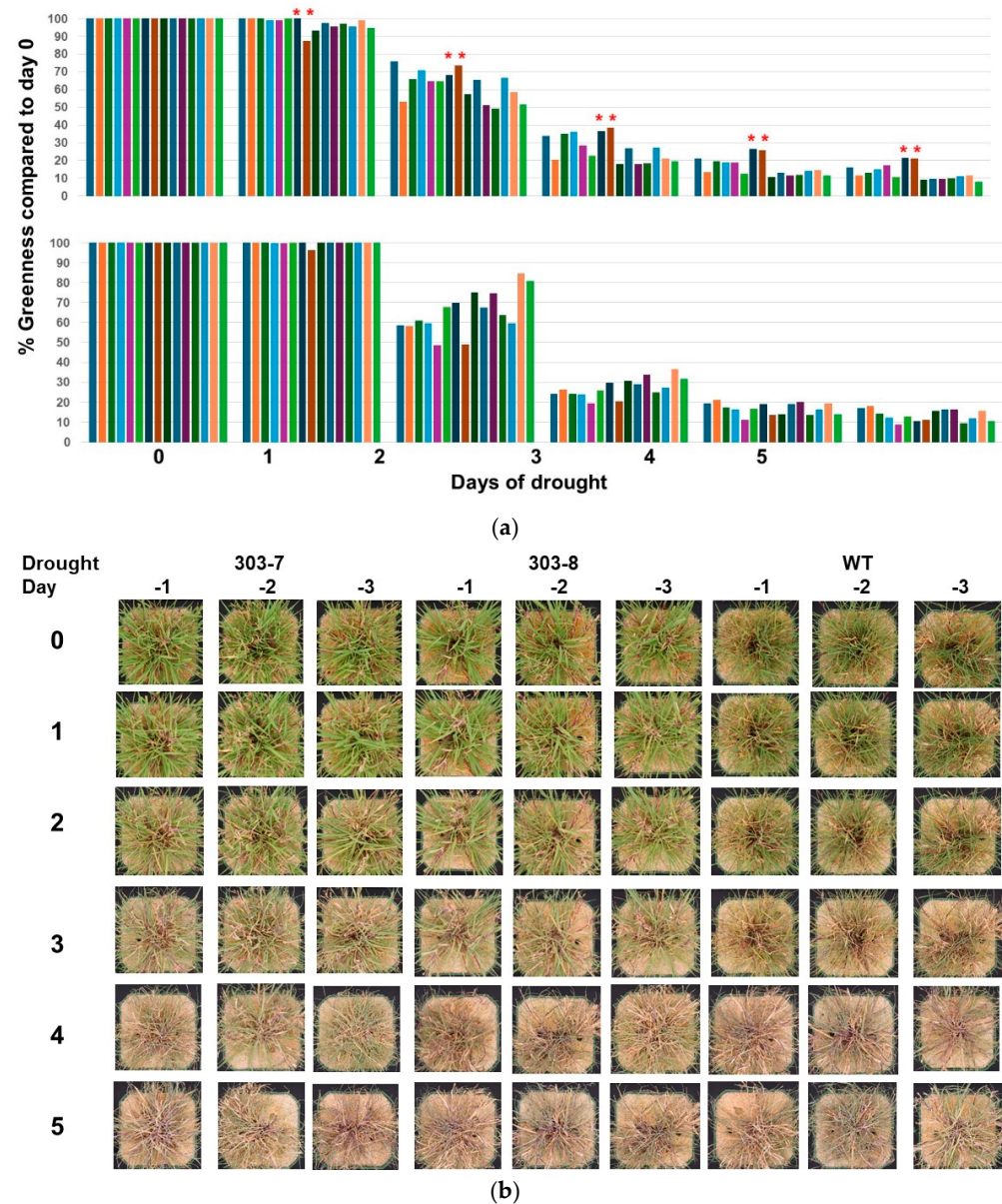


Figure 5. Drought testing. Three replicate plants from 15 *AsDREBL* mutant and 15 WT lines were withheld water for five days. Plants were photographed every day, and the greenness of individual plants was analyzed by ImageJ and compared to their values at Day 0 (a). The two * denote lines 303-7 and 303-8. The composite of these photos is shown (b).

3.4. *AsDREBL*-Edited Mutant Crenshaw Plants Were More Tolerant to Salinity

Three replicates from the 15 mutant and 15 WT lines were treated with daily spray of salt-containing 50% Hoagland solution for six weeks with increasing salt concentration from 1 DS to 6 DS per week. Plants were evaluated every week with the rating of “tip burn”, with 1 being total tip burn and 9 being no tip burn. Two to three weeks after the 2 DS and 3 DS treatment, some plants started to show signs of tip burn. The results of 5 DS salt treatment after week five and 6 DS salt treatment after week six were plotted. Figure 6a shows that after 5 DS salt treatment, most of the 15 mutant plants (three replicate plants/mutant line) had higher ratings compared to the 15 WT plant (three replicate plants/WT line). Figure 6b shows that after 6 DS salt treatment, 303-1,4,7,8,21, continued to display higher ratings compared to the WT plants.

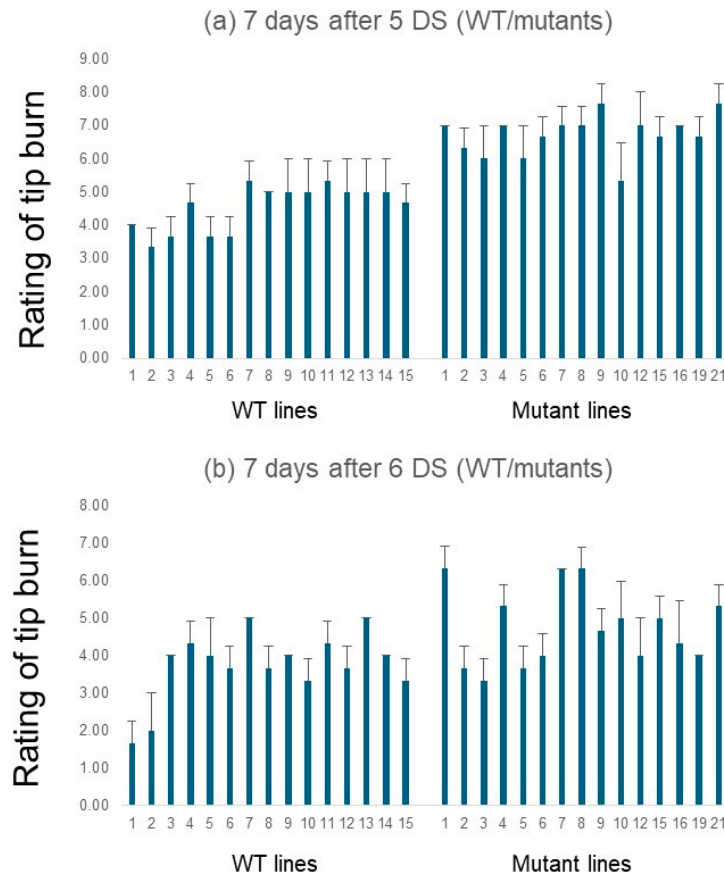


Figure 6. Salinity tolerance testing. Three replicate plants for each of the 15 WT and 15 *AsDREBL* mutant lines were sprayed every day in our salt tent with a weekly increasing concentration of salt from 1 DS to 6 DS. Plants were evaluated by the rating of “tip burn” as a result of salt spray, with 1 = total tip burn and 9 = no tip burn. The ratings from 5 DS (a) and 6 DS (b) treatments are plotted from the average ratings of three replicate plants with standard errors.

4. Discussion

As an extensively used cool-season creeping bentgrass on golf courses around the world, *A. stolonifera* L. is negatively affected by several diseases, including dollar spot caused by *Clarireedia jacksonii*, drought, and salinity conditions. In this project, we employed the powerful CRISPR-gene editing technology to mutate the *AsDREBL* in creeping bentgrass cv. Crenshaw and tested the mutant plants’ ability to tolerate drought and high salt conditions in greenhouse settings. The homolog of *AsDREBL* in Arabidopsis, *AtDREB1C* has been shown to be a negative regulator and plays a central role in stress tolerance in Arabidopsis [15]. Through bioinformatic analysis, we identified the *AsDREBL* cDNA sequence in the NCBI *A. stolonifera* EST library and subsequently cloned the partial gDNA of *AsDREBL*. We created our own sgRNA-based CRISPR-gene editing vector pRD303 containing wheat U6 promoter, driving the sgRNA, and the maize intron-ubiquitin promoter, driving the monocot codon-optimized Cas9, which was used to transform embryogenic calli generated from mature Crenshaw seeds. We optimized the transformation and regeneration tissue culture protocol and produced 21 transgenic Crenshaw plants (Figure 2).

Selecting the *EcoRV* containing the 20 nt target sequence enabled us to efficiently screen for *AsDREBL*-edited mutants by the RFLP method. By sequencing the *EcoRV* uncut PCR products of the gDNA spanning the 20 nt target site, we identified that 15 out of the 21 rooted plants carried various mutations (mostly deletions) around the 20 nt target

site (Figure 4a). It was possible that there were other mutants in which the mutations did not result in the disruption of the *EcoRV* site. By cloning and sequencing 10 clones from 303-8 mutant's *EcoRV*-uncut PCR product, we found six different mutation patterns, underlining the polyploidy nature of creeping bentgrass [16]. This result also indicates that our CRISPR-gene editing platform is efficient in knocking out genes in creeping bentgrass.

The well-grown 15 Crenshaw *AsDREBL* mutants and 15 WT plants were subjected to drought testing in the plant growth chamber by withholding water. Our results showed that 303-7 and 303-8 mutants performed better throughout the 5-day drought period compared to the other mutant plants. These two mutant plants also stayed greener and outperformed all WT plants at Day 3, 4, and 5 when all plants were under extreme drought (Figure 5). During the salinity testing in our salt tent, it was observed that for four weeks of treatment with increasing salt concentration from 1 DS to 4 DS, the difference in salt tolerance between *AsDREBL* mutant and WT plants was not obvious. However, after the fifth week with 5 DS daily salt spray, most of the *AsDREBL* mutants outperformed the 15 WT plants by the rating of “tip burn” (Figure 6a). Furthermore, five *AsDREBL* mutant plants, including 303-7,8, continued to do better after the sixth week with 6 DS daily salt spray.

To our knowledge, this is the first report of CRISPR-KO of *AsDREBL* in creeping bentgrass that improved its drought and salt tolerance. Compared to other crops and plants, there have been limited publications on using the CRISPR-gene editing technology to engineer turfgrass. An *Agrobacterium*-mediated transformation system has been reported to CRISPR-edit perennial ryegrass (*Lolium perenne* L.) with 29% efficiency [21]. Recently, *Zoysia matrella*, a C4 warm-season turfgrass, has also been edited with the CRISPR technology [22], with the 63.6% transformed showing mutations. Our results of 15 mutants carrying mutations at the target site out of a total of 26 regenerants (57.7% efficiency) seem comparable to these two reports. The mechanism of *DREB1C* negatively regulating plant stress response remains elusive since its discovery in *Arabidopsis* [15], even though its structural and functional roles in rice cold tolerance has been computationally characterized [23]. We took advantage of CRISPR-induced mutations and showed that our *AsDREBL*-KO mutant creeping bentgrass' tolerance to drought and salinity was enhanced. The mechanism of this induced stress tolerance needs to be further studied. This work highlights the feasibility and importance of applying CRISPR-gene editing technology to improve different turfgrass' agronomic traits.

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