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Strigolactones in Sugarcane Growth and Development

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Abstract: Sugarcane is a complex polyploid aneuploid cash crop, and transgenic varieties are important for molecular genetic and traditional breeding approaches. Herein, the sugarcane variety ROC22 served as the receptor, the *Bar* gene served as a screening marker, and positive and negative fragments of the *ScD27.2* gene, upstream of strigolactones (SLs) biosynthesis genes driven by the *35S* promoter, were introduced by *Agrobacterium tumefaciens*-mediated transformation. Regenerated plants were obtained by co-culture, screening culture, and differentiation induction, and 27 sense and antisense *ScD27.2* transgenic seedlings were obtained by herbicide screening. PCR detection and 1% Basta (Glufosinate) application on leaves revealed *Bar* in all lines, with all testing positive for herbicide application and 23 containing the target gene (positive resistance screening rate = 87.5%). q-PCR and phenotypic analyses showed that *ScD27.2* expression, plant height, tiller number, root length, stem diameter, and fresh weight were decreased in transgenic (ScD27.2R-9) compared with non-transgenic (NT and ScD27.2F-2) lines. *ScD27.2* expression was downregulated, and growth potential was inhibited. Under 20% PEG treatment, malondialdehyde (MDA) content in ScD27.2R-9 was higher than in NT, while proline content was lower. Under drought stress, *ScD27.2* expression, MDA levels, and proline content in ScD27.2F-2 and NT were higher than in non-treated controls, *ScD27.2* expression increased with time, and MDA and proline levels also increased. *ScD27.2* expression in ScD27.2R-9 decreased under 20% PEG treatment, MDA and proline increased (but not to NT levels), and growth was lower than NT. The 20% PEG treatment also increased the levels of (±)-2′-epi-5-deoxystrigol and (+)-abscisic acid in the rooting culture media of ScD27.2F-2, ScD27.2R-9, and NT lines, but the levels of (+)-abscisic acid content in ScD27.2R-9 was lower than in NT. Thus, interfering with *ScD27.2* expression decreased resistance to 20% PEG treatment. *ScD27.2* encodes a β-carotene isomerase involved in SLs biosynthesis that might function in sugarcane resistance to drought stress. It explains the role of SLs in sugarcane growth and development and responses to drought stress.

Keywords: DWARF27; tilling; sugarcane; strigolactones biosynthesis; *ScD27.2*

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1. Introduction

Sugarcane (*Saccharum* spp. *hybrids*) is a critical sugar crop and bioenergy plant. It is also the most crucial sugar and cash crop for national food security. In 2021, China's sugarcane planting area totaled 1316 thousand hectares, with a sugarcane output of approximately 107 million tons. Sugarcane yield is affected by many intrinsic and extrinsic factors, including growth conditions, genotype, soil conditions, biological and abiotic stresses [1], etc. It is strategically vital to improve sugarcane yield and drought resistance through transgenic research [2]. With the development of dense genome sequencing for sugarcane [3], regulating plant hormones may become an essential genetic engineering target to obtain new, stable crop germplasms that are resistant to abiotic stress [4,5].

Strigolactones (SLs) are plant hormones that regulate tillering characteristics of crops [6]. These plant hormones that are derived from carotenoids [7] regulate branching [8,9], secondary growth [10,11], root development [12,13], abiotic stress [14–16], yield [17,18], and responses to soil nutrient elements [19–21]. The mining of genes related to the SLs biosynthesis could improve sugarcane yield.

The β-carotene isomerase encoded by the DWARF27 gene is a plastid localized protein and is the first catalytic enzyme in SL biosynthesis [22]. Reversible isomerization catalyzed by β-carotene isomerase may play a key role in regulating SL biosynthesis [23].

Since both SLs and abscisic acid (ABA) are involved in the cleavage of carotenoids, and since both are derived from carotenoids, a connection between SLs and ABA metabolism is inevitable [24]. The interactions between SLs and the ABA pathway may involve the D27 protein, which is vital in communicating ABA and SL levels in rice [24,25]. The interactions between SLs and ABA may be the key to abiotic stress tolerance, and it is possible to improve crop viability under natural stress conditions using plant hormone signaling and metabolic regulation [26,27].

The carotene isomerase OsDWARF27 has stereoscopic, double-bond specificity [28] and strict substrate and region specificities, and it can specifically isomerize the all-trans-β-carotene substrate into 9-cis-β-carotene, using iron as a cofactor [24].

Arabidopsis thaliana AtD27 is widely expressed, with the highest transcript levels in immature flowers, and it can be induced by phosphorus deficiency, auxin [24], and ABA treatment [24]. ABA levels in AtD27 mutants are low, and the ABA pathway is often disturbed [24]. There are five members of the D27 gene family in Sugarcane (*Saccharum spontaneum*), all of which contain the typical domain of β-carotene isomerase DUF4033.

A rice *d27* mutant could not produce SLs. The tiller number was increased, plant height was decreased, and the normal phenotype could be restored by exogenous SLs [23]. The GenBank accession number of the *ScD27.2* gene is KP987221.1. The gene is located at SSPon.06G0016140-2C on chromosome 6C of *S. spontaneum* L., and shares only 31.40% homology with the other four family genes. The gene includes a complete open reading frame (ORF) of 867 bp encoding a protein of 288 amino acid residues. The conserved region of DWARF27 may contain two zinc finger protein domains (ZnF_TAZ and ZnF_A20) that respond to drought stress-induced high expression, but the enzyme activity of DWARF27 in sugarcane is unknown.

We used PCAMBIA3301 as a vector, and *ScD27.2* gene sense and antisense expression are driven by the Cauliflower Mosaic Virus (*CaMV) 35S* promoter and a plant expression vector harboring the *Bar* gene as the screening marker gene was constructed. The vector was introduced into sugarcane cultivar ROC22 (a *Saccharum* spp. *hybrid* from ROC5 × 69-463) to obtain the *ScD27.2* gene sense and antisense transgenic lines with significantly different transcript levels. Polyethylene glycol (PEG) was used to simulate drought stress treatment, *ScD27.2* gene transcript levels were measured, changes in MDA and proline were compared, and the effects of drought stress on sugarcane were analyzed. The responses of the *ScD27.2* gene to sugarcane drought stress were clarified, and its role in regulating sugarcane growth was preliminarily explored. This study will provide a theoretical basis for improving sugarcane yield and drought resistance through the manipulation of SLs.

2. Materials and Methods

2.1. Materials

The *A. tumefaciens* strain EHA105 and plant expression vector PCAMBIA3301 (containing the *CaMV 35S* promoter) were provided by Shuzhen Zhang, Institute of Tropical Biotechnology, Chinese Academy of Tropical Agricultural Sciences. ROC22, a sugarcane cultivar used for genetic transformation, was planted in the Sugarcane Research Institute of Yunnan Academy of Agricultural Sciences. The basic medium for sugarcane tissue culture was the Murashige and Skoog (MS) medium [29]. The *Escherichia coli* strain DH5α and carrier PMT18-T were purchased from TAKARA (Dalian, China).

2.2. Construction of Plant Expression Vectors

The forward target fragment was amplified using primers D27FNcoI-F and D27FBglII-R, and the reverse target fragment was amplified with primers D27RNcoI-F and D27RBglII-R, containing *Nco*I and *Bgl*II double restriction sites (see Table A1, Appendix A). NcoI and BglII digested the recovered fragments, and the 867 bp fragments were recovered.

Meanwhile, plasmid PCAMBIA3301 was digested with *Nco*I and *Bgl*II to recover large fragments. The small fragments were mixed with the recovered large fragments at a DNA molar ratio of 3:1, and T_4 DNA ligase (1 μL) and 2 × T_4 DNA ligase (1 μL) were added. Then, 10 μL of sterile water was added. Samples were mixed and centrifuged, and incubated overnight at 16 °C. A 5 μL volume of the combined product was transferred into *Escherichia coli* DH5α competent cells, and cells were plated on a kanamycin plate for positive screening. PCAMBIA3301-ScD27.2F and PCAMBIA3301-ScD27.2R vectors were identified via enzyme digestion and DNA sequencing, and the two plasmids were transformed into *Agrobacterium tumefaciens* EHA105 receptive cells via the freeze–thaw method.

2.3. A Tumefaciens-Mediated Genetic Transformation of ROC22

Sugarcane transformation was carried out using an *A. tumefaciens*-mediated method, as described previously [30,31], with modifications for the ROC22 variety. Whorled leaves were cut from the tips of ROC22 and used as materials for embryogenic callus induction.

Transverse sections approximately 1 mm thick were cut directly into the meristem and placed on a callus induction medium (MS + 30 g/L sucrose + 2 mg/L 2,4-D and 8 mg/L Agar) [32]. Transverse sections approximately 1 mm thick were cut directly into the meristem and placed on a callus induction medium; callus cultures were kept in the dark for 28 days, and the medium was replaced with a fresh medium every two weeks for 45 days to induce callus formation. Calluses were then selected and chopped.

The in vitro-cultured Agrobacterium bacilli were collected by centrifugation, suspended in a medium (1/5 MS medium + 35 g/L sucrose + 35 g/L glucose + 150 mM acetosyringone), and diluted to OD_{600} 0.6–1.5. Calluses were collected, placed in an agrobacterium infection solution containing expression vectors, and gently shaken in the dark for 30 min. The Agrobacterium suspension was removed, calluses were transferred to a Petri dish, and excess Agrobacterium suspension was absorbed with filter paper. Calluses were transferred to a new medium and incubated at 22 °C in darkness for 3 days. Infected calluses were transferred to a co-culture medium (MS + 1.0 mg/L 2,4-D + 35 g/L sucrose + 8 g/L agar + 200 mg/L timentin) and cultured at 28 °C in the dark for 7 days.

Infected calluses were then transferred to a screening medium (MS + 2.0 mg/L 2,4-D + 8 g/L agar + 200 mg/L timentin + 35 g sucrose + 0.05% Basta) and incubated at 28 °C for 35 days under dark conditions. Resistant calluses were transferred to a medium (MS + 1.0 mg/L 6-BA + 8 g/L agar + 200 mg/L timentin + 35 g sucrose + 0.05% Basta) and cultured in a light incubator for 2 weeks. The differentiated green buds were transplanted into a rooting medium (MS + 2 mg/L NAA + 8 g/L agar + 300 mg/L timentin + 35 g sucrose + 0.05% Basta) and cultured in a light incubator for 3 weeks. Each main bud was transplanted into a fresh rooting medium and cultured for 3 weeks. The leaves of seedlings were taken for molecular detection and transplantation.

2.4. PCR Detection of Bar and ScD27.2 Genes in Transgenic Lines

The DNA of transformed sugarcane lines was extracted for PCR detection, and untransformed NT lines served as negative controls. According to the *Bar* gene within the PCAMBIA3301 sequence used to design primers, upstream and downstream primers (5′-CATCGTCAACCACTACATCGAGACAAGC-3′ and 5′-CAGAAACCCACGTCATGCCAG TTCC-3′) were designed to amplify a 397 bp fragment. Primers for ScD27.2 gene amplification (ScD27.2F, ATGCTCCTTGCTCACGCTC; ScD27.2R, TCAAATAGAGCAATTCACTTG) were designed to amplify the 860 bp fragment.

The reaction system consisted of 20 ng of template DNA, 0.5 pM of forward and reversed primers, 1 mM of dNTPs, 2.5 U of Taq DNA polymerase, 10× buffer (1 μL), and 10 μL of sterile water. Thermal cycling included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, and extension at 72 °C for 60 s, then extension at 72 °C for 10 min. Products were assessed by 1.5% agarose gel electrophoresis.

Leaves of transformed sugarcane were smeared with 0.1% Basta herbicide, and untransformed NT lines served as negative controls. The experiment was repeated three times, leaves were photographed, and the results were statistically analyzed.

2.5. qRT-PCR

Total RNA was extracted from sugarcane leaves using an RNA Plant Kit (TaKaRa, Dalian, China). RNA was dissolved in RNase-free water, the concentration was determined using a NanoDrop Instrument (Thermo Fisher, Waltham, MA, USA), and RNA quality was evaluated according to OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios. RNA integrity was determined by 1% agarose gel electrophoresis, and RNA was stored at −70 °C for later use. A 1 μg sample of total stem tip RNA was extracted to synthesize cDNA according to the TaKaRa RNA PCR Kit (TaKaRa, Dalian, China).

Real-time PCR primers were designed according to the ScD27 gene sequence. *GAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) was the reference gene [33]. A TaKaRa Prime Script RT Reagent Kit with gDNA Eraser and SYBR Premix Ex Taq (TaKaRa, China, Dalian) was used with specific primers designed for *ScD27.2*. The amplification reactions were present at 20 μL, and *ScD27* gene expression was measured by an ABI Vii7 real-time PCR System (Applied Biosystems, USA) [34,35]. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative transcript levels of genes [36]. Three replicates were included, and the average value was calculated. The expression of the *ScD27.2* gene was measured for whole transgenic sugarcane lines and under different drought treatment conditions.

2.6. Phenotypic Analysis of Transgenic Sugarcane Lines

Lines with significantly different gene transcript levels (sense ScD27.2F-2, antisense ScD27.2R-9, and NT) were transplanted into sand beds and planting cups after survival experiments. The morphological characteristics of five sugarcane lines were observed and compared after 60 days of open-air culture. Tiller number, stem diameter, plant height, and fresh weight were measured for cultured transgenic and wild-type sugarcane lines. Three lines with consistent growth were selected from each strain, experiments were performed in triplicate, lines were photographed, and average values were calculated.

2.7. Simulated Drought Stress Treatment

ScD27.2F-2, ScD27.2R-9, and NT were inoculated into an MS medium containing 20% PEG-8000 [37]. The MS medium without PEG was used as a control, and 20% PEG treatment was performed at 0, 2, 4, and 6 days. *ScD27.2* gene transcript levels were measured by qRT-PCR. Treatment started at day 0 (0-day material refers to samples taken within half an hour of processing).

ScD27.2F-2, ScD27.2R-9, and NT were treated with 20% PEG-8000 MS medium for 6 days, and some lines were used for phenotypic analysis to assess morphological indices, including tiller number, plant height, stem diameter, root length, and fresh weight. The other lines were used for the determination of physiological indicators. All leaves of lines were used to determine proline (Pro) and malondialdehyde (MDA) levels by colorimetric methods [38,39]. Leaves of four lines were mixed for each treatment, all treatments included three replicates, and average values were calculated.

2.8. Hydroponic Culture

Sugarcane tube seedlings were grown sterile for 3 weeks until 8–10 cm tall. After 3 days of opening the bottle, roots were rinsed to remove the MS AGAR medium, and

plants were transferred to a hydroponic solution containing 2 mM NH_4NO_3, 0.65 mM $MgSO_4$, 0.25 mM KH_2PO_4, 0.75 mM K_2SO_4, 2 mM $CaCl_2$, 0.1 mM KCl, 1×10^{-3} mM $ZnSO_4$, 1×10^{-3} mM $MnSO_4$, 1×10^{-4} mM $CuSO_4$, 0.2 mM Fe-EDTA, 1×10^{-3} mM H_3BO_3, 5×10^{-6} mM, and $(NH_4)_6Mo_7O_4$. The pH was adjusted to 6.0 using 3 M KOH. Culturing was conducted in an incubator with a photoperiod of 14/10 h and a light intensity of 100–150 μmol m^{-2} S^{-1} at 28/25 °C. The culture was performed in 1/3 nutrient solution for 60 days, and 20% PEG-8000 was added and cultured for another 30 days. The root culture medium was collected, each sample was repeated three times, and six lines were included in each repetition. Samples were stored at −80 °C.

2.9. Analysis of SLs Content in Sugarcane Root Tissue

Appropriate modifications were made as described by Haider [25] and López-Ráez [40]. The content of SLs in root secretions of sugarcane plants was determined, and root secretions were collected and extracted after 30 days. A 50 mL fresh root hydroponic solution was taken, and 1 ng (±)-2′-epi-5-deoxystrigol was added as an internal standard. After filtration through filter paper, the solution was loaded onto a Solid Phase Extraction column (Oasis HLB cartridges and 96-well plates, USA) for vacuum filtration. The column was cleaned with 5% methanol and eluted with 2 mL acetone. The eluted solvent was dried to the size of a tiny droplet under nitrogen, redissolved in 200 μL acetonitrile, and UPLC-MS/MS analysis was performed. Data were collected and analyzed using MassLynx 4.1 (TargetLynx) software (Waters, MA, USA). Concentrations of (±)-5-deoxystrigol and (+)-ABA were calculated based on standard calibration curves and corrected by IS. All the experiments were repeated three times, and the results are expressed as mean ± standard deviation (SD).

2.10. Data Analysis

Microsoft Excel 365 was used for sorting and statistically analyzing data, SPSS 17.0 software was used for variance analysis, and Duncan's method was used for significant difference analysis ($p < 0.05$).

3. Results

3.1. Agrobacterium-Mediated Genetic Transformation of Sugarcane and Detection of Transformed Lines

In order to investigate the role of the *ScD27.2* gene in sugarcane, transgenic lines were generated. Using the plasmid PMT18-T-ScD27.2 preserved in our laboratory as a template, ROC22 as the experimental material, forward and reverse target fragments were ligated into the pCAMBIA3301 vector by PCR amplification and double enzyme digestion. Hence, the 860 bp sequence of the *ScD27.2* cDNA forward and reverse fragments was introduced into the pCAMBIA3301 vector under the control of a *CaMV 35S* promoter. DNA sequencing verified that *Agrobacterium* engineering strains containing pCAMBIA3301-ScD27.2F and pCAMBIA3301-ScD27.2R vectors were successfully obtained.

The resulting *ScD27* construct was transformed into sugarcane cv ROC22 by *Agrobacterium tumefaciens*-mediated transformation, resulting in 12 independent antisense lines and 15 independent sense lines. The *A. tumefaciens*-mediated method induced embryonic calluses in ROC22 sugarcane lines (Figure 1A,B). After adding 0.05% Basta to the culture medium for resistance screening, small resistant buds were differentiated (Figure 1C–F) and then cultured until rooting and transplanted to the greenhouse for barrel cultivation.

PCR detection and screening results showed that all 15 PCAMBIA3301-SCD27 sense lines and 12 PCAMBIA3301-ScD27 antisense lines were positive for the *BAR* gene, and 13 and 10 lines were positive for *ScD27.2* genes, respectively (Figures 2 and 3, Table 1). DNA sequencing confirmed the PCR product to be the desired fragment of the *Bar* and *ScD27.2* gene.

Leaves of 27 transformed sugarcanes were coated with 0.1% Basta herbicide, all showed herbicide resistance, and the untransformed NT lines showed herbicide sensitivity (Figure 4).

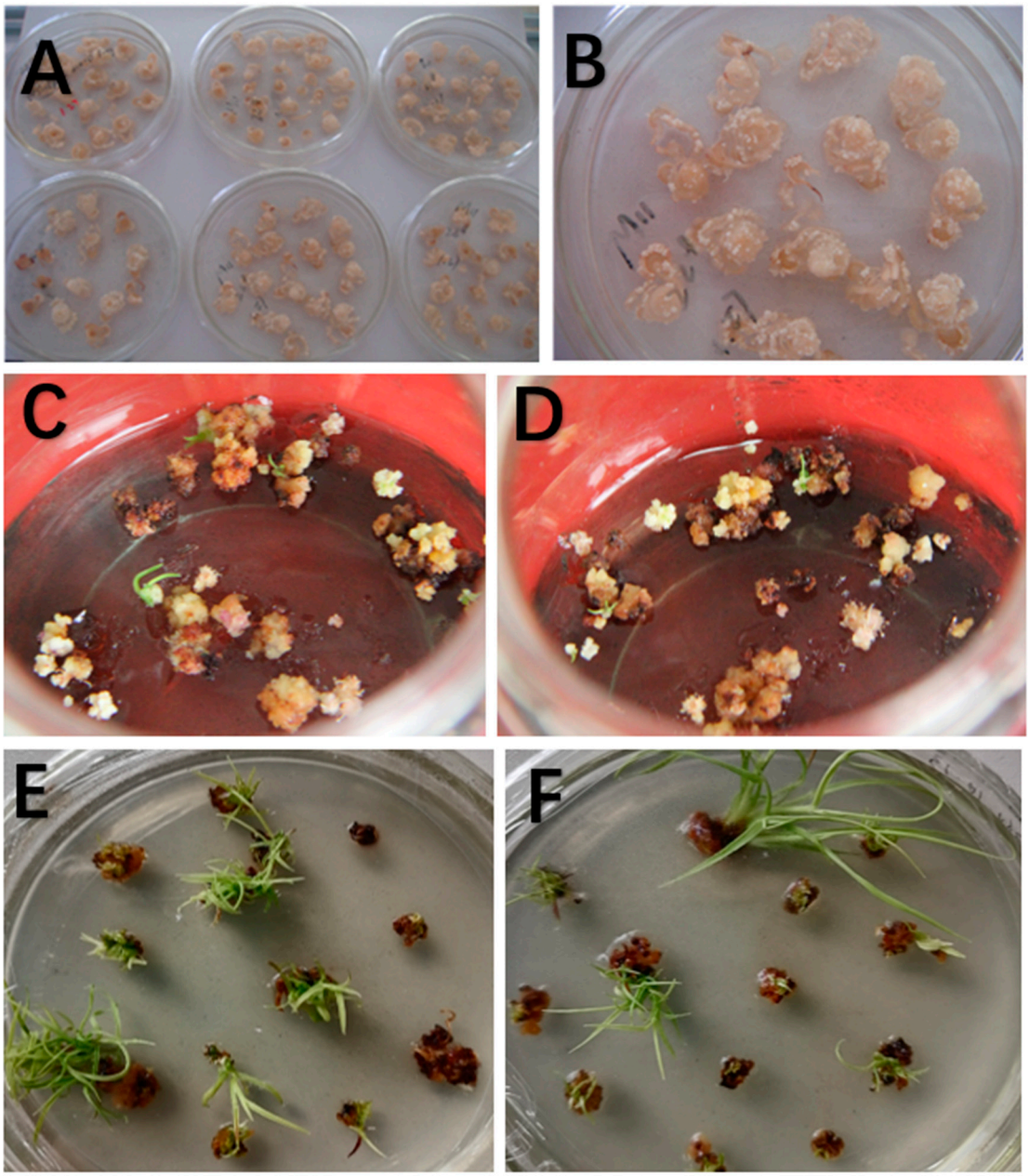


Figure 1. Agrobacterium-mediated genetic transformation of sugarcane. (**A**,**B**) Induction and culture of embryogenic callus from sugarcane; (**C**–**F**) differentiation of herbicide-resistant embryogenic callus and herbicide-resistant seedlings.

Table 1. Statistics for PCR detection of *Bar* and *ScD27.2* genes in transformed lines.

Bar	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
PCAMBIA3301-ScD27 sense lines	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PCAMBIA3301-ScD27 antisense lines	+	+	+	+	+	+	+	+	+	+	+	+			
ScD27.2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
PCAMBIA3301-ScD27 sense lines	+	+	−	+	+	+	+	+	+	+	+	+	−	+	+
PCAMBIA3301-ScD27 antisense lines	+	+	+	−	+	+	+	+	+	−	+	+			

+, positive for the target fragment; −, negative for the target fragment.

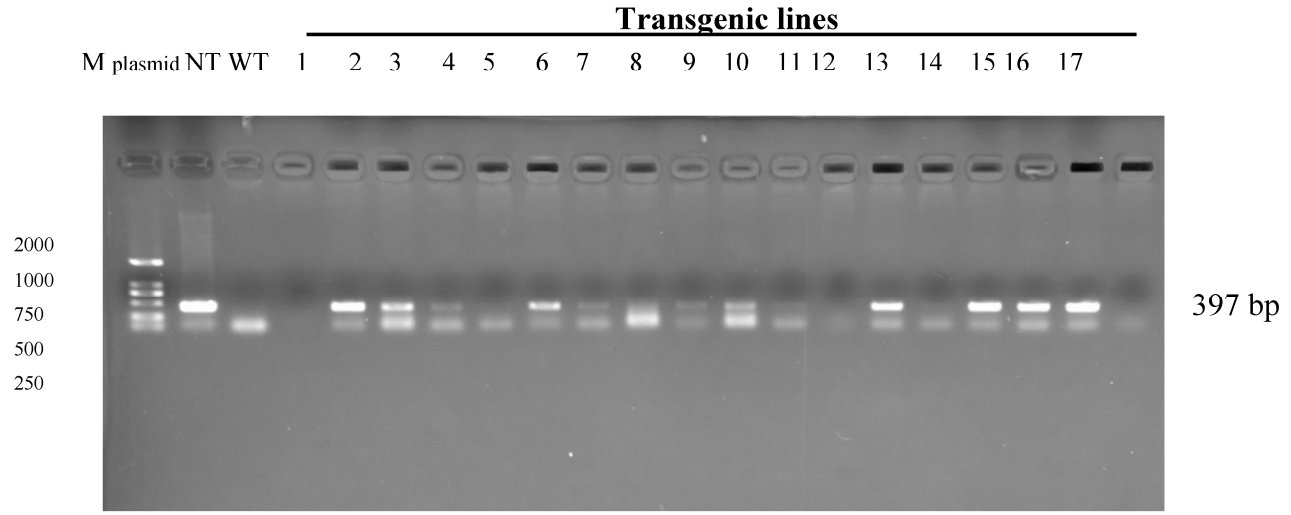


Figure 2. PCR detection of the *Bar* gene in transformed seedlings. M, Marker; NT, Non-transgenetic lines; WT, Wild type.

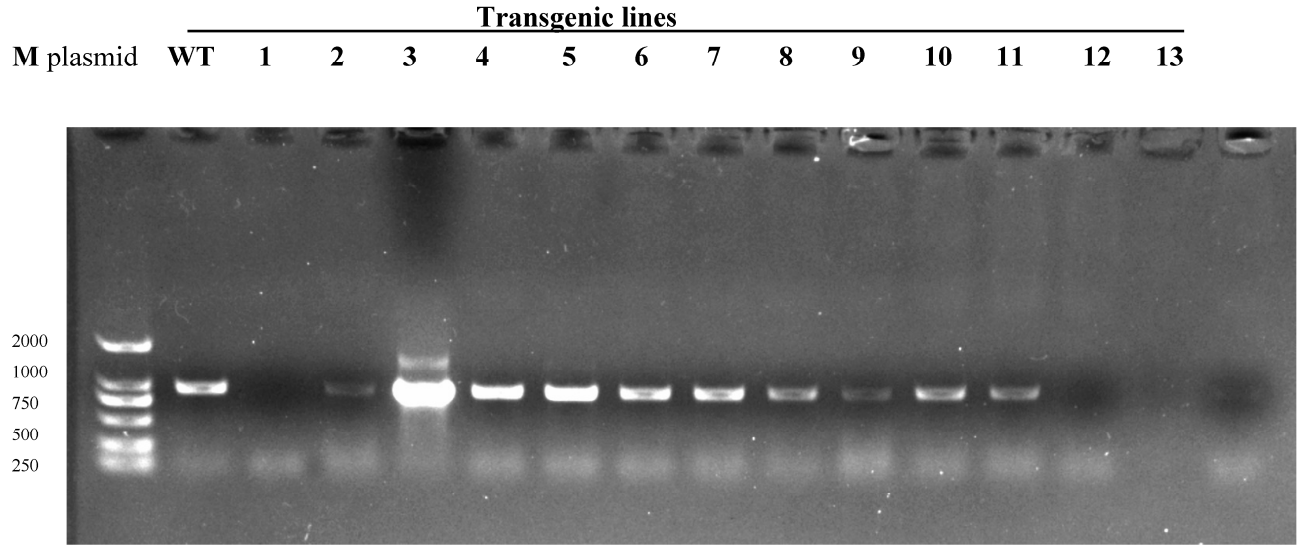


Figure 3. PCR detection of the *ScD27.2* gene in transformed seedlings. Numbers 1 to 13 are the transformed plant sample. M, Marker; WT, Wild type.

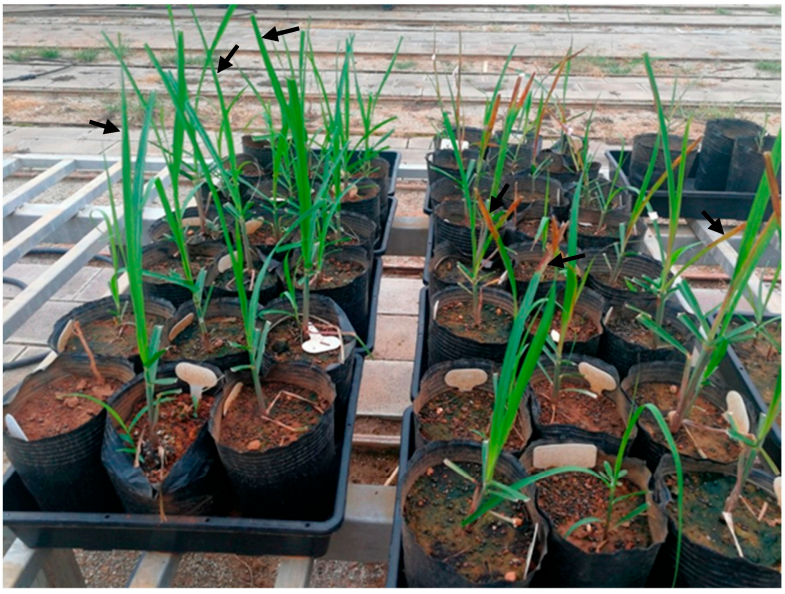


Figure 4. Leaves coated with 0.1% Basta after transplanting. Transformed sugarcane lines are on the left, and NT lines are on the right. The black arrows indicate differences between transformed and NT lines.

3.2. Down-Regulation of ScD27.2

The *ScD27.2* transcript level was assayed by qRT-PCR in transgenic sugarcane and cv ROC22. The accumulation of the ScD27.2R-9 specific transcript examined was up to two-fold less than in the wild-type controls. The relative gene expression of antisense ScD27.2R-9 was 0.442 (Figure 5).

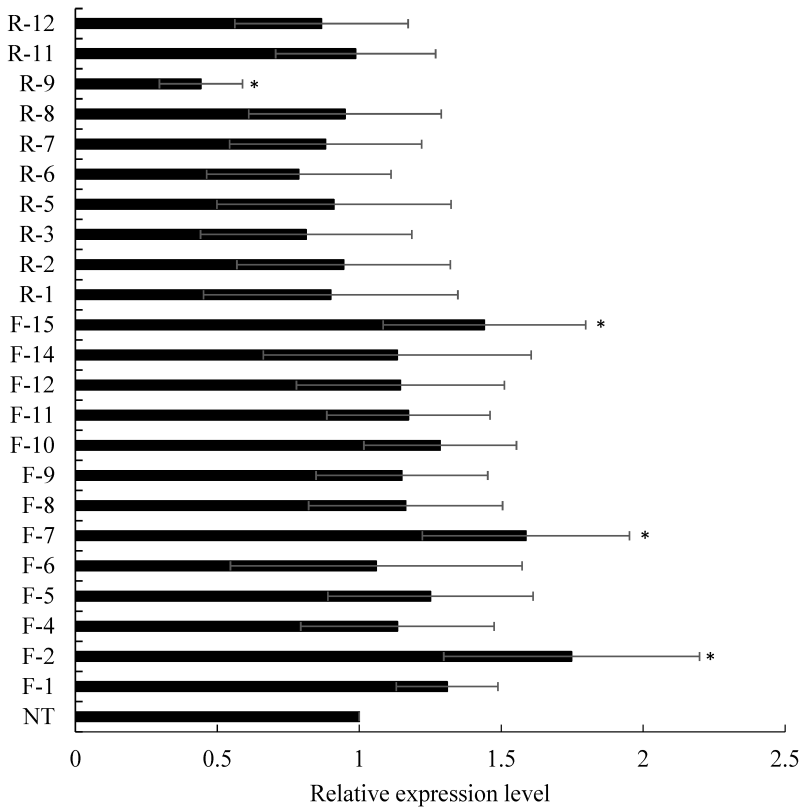


Figure 5. qPCR analysis of *ScD27.2* gene expression in transgenic lines. NT, non-transgenic lines; ScD27.2F-2, sense overexpression lines; ScD27.2R-9, antisense interference lines. R-1 to 12 are PCAMBIA3301-ScD27

antisense lines; F-1 to F-15 are PCAMBIA3301-ScD27 sense lines. * indicate significant differences at the 0.05 level. The error line represents the standard error (n = 9).

The *ScD27.2* gene transcript levels were highest in ScD27.2F-2 lines (Figure 5). The relative gene transcript levels of both ScD27.2R-9 and ScD27.2F-2 genes significantly differed from those of NT and were significantly different from those of other lines (Figure 5).

ScD27.2F-2 and ScD27.2R-9 also present severe phenotypes when compared with wild-type sugarcane. Therefore, in subsequent experiments, they will be used as experimental materials (Figure 6).



Figure 6. Images of plant phenotypes. ScD27.2F-2 sense overexpression lines, ScD27.2R-9 antisense interference lines, and non-transgenic control lines (NT) are shown left to right.

3.3. Increased Shoot Branching in ScD27.2R-9

In order to investigate the gene function of *ScD27.2* in sugarcane, agronomic characters of ScD27.2F-2 and ScD27.2R-9 were analyzed (Table 2). Figure 6 shows the phenotypes of transgenic lines ScD27.2F-2, ScD27.2R-9, and NT cultured in the open air for 60 days. Table 2 shows phenotypic data, including stem diameter, plant height, tillering number, and fresh weight. The results revealed no significant differences in stem diameter and tiller number compared with NT controls, but plant height and fresh weight of ScD27.2R-9 were lower than NT. These results indicate that following *ScD27.2* gene interference, plant height was decreased, tiller number was increased, plant height was inhibited to a certain extent, and fresh weight was reduced.

Table 2. Phenotypic analysis of ScD27.2 sense overexpression and ScD27.2R-9 antisense interference transgenic lines.

Plant Line	Tillering Number	Plant Height (mm)	Stem Thickness (cm)	Fresh Weight (g)
ScD27.2F-2	1.33 ± 0.47 a	47.99 ± 3.69 a	15.65 ± 0.50 a	239.06 ± 26.01 a
ScD27.2F-3	2.1 ± 0.58 b	51.23 ± 3.56 a	15.42 ± 0.60 a	245.36 ± 28.36 a
ScD27.2R-5	2.5 ± 0.21 b	48.68 ± 3.25 a	16.35 ± 0.85 a	262.53 ± 27.51 a
ScD27.2R-7	3.0 ± 0.24 b	45.36 ± 2.36 a	16.53 ± 0.75 a	255.59 ± 12.36 a
ScD27.2R-9	4.67 ± 1.25 c	18.01 ± 2.06 b	14.53 ± 0.88 a	198.08 ± 9.40 b
NT	2.00 ± 0.82 b	50.98 ± 2.51 a	15.07 ± 0.89 a	244.94 ± 6.18 a

Note: NT, non-transgenic lines; ScD27.2F-2, sense overexpression lines; ScD27.2R-9, antisense interference lines. Duncan's new multiple range method was used for significance analysis, and different lowercase letters in the same column indicate the significance of differences at the 0.05 level, $n = 3$.

3.4. Transcript Level of the ScD27.2 Gene in ScD27.2R-9

The transcript level of the *ScD27.2* gene in ScD27.2F-2 and ScD27.2R-9 transgenic lines was measured by qPCR following 20% PEG treatment. The results showed that the transcript level of the *ScD27.2* gene in ScD27.2R-9 changed significantly (Figure 7). The 20% PEG treatment could induce significant expression of the ScD27.2 gene in ScD27.2F-2 and NT lines, but not in ScD27.2R-9.

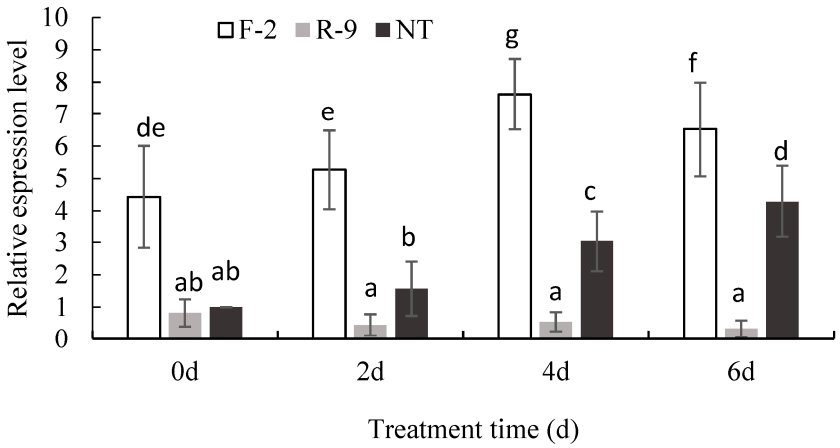


Figure 7. Effects of drought stress on gene expression in ScD27.2 F-2, ScD27.2R-9, and NT lines. Duncan's new multiple range methods were used to analyze significant differences in gene expression at different time points after 6 days of stress treatment in 20% PEG-8000 MS medium. Different lowercase letters indicate significant differences at the 0.05 level. The error line represents the standard error ($n = 9$).

3.5. Increase MDA Content in ScD27.2R-9

Under the 20% PEG treatment, the MDA content of ScD27.2F-2 was significantly lower than that of NT, while the MDA content of ScD27.2R-9 was higher than that of NT. The MDA content in the three lines increased gradually with 20% PEG treatment duration. The general trend was ScD27.2R-9 > NT > ScD27.2F-2 (Figure 8).

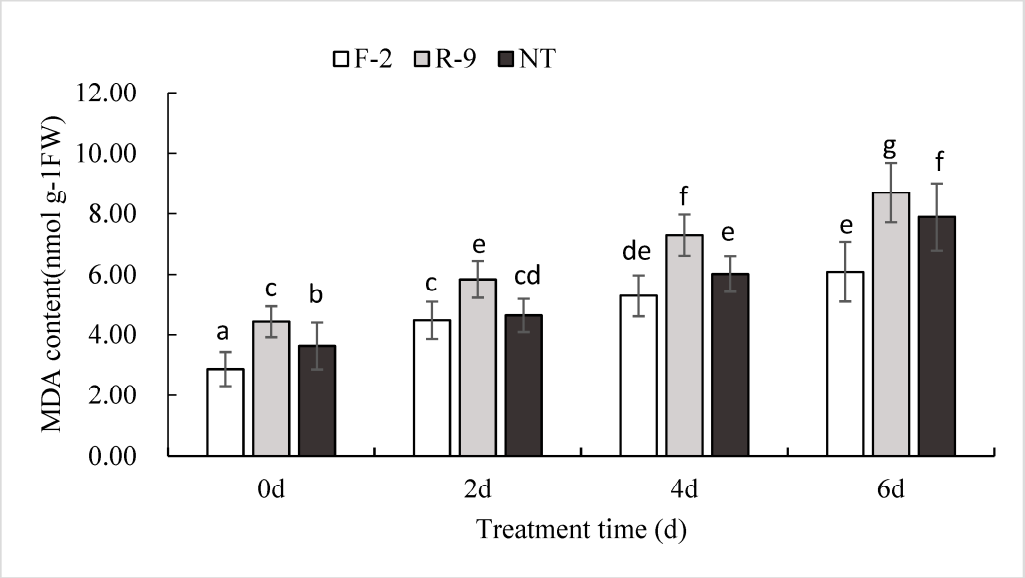


Figure 8. Malondialdehyde content of ScD27.2 F-2 and ScD27.2R-9 transgenic lines and NT non-transgenic controls. Duncan's new multiple range methods were used to analyze significant differences in gene expression at different time points after 6 days of stress treatment in 20% PEG-8000 MS medium. Different lowercase letters indicate significant differences at the 0.05 level. The error line represents the standard error (n = 9).

3.6. Lower Free Proline Content in ScD27.2R-9

Under 20% PEG treatment, the free proline content in ScD27.2F-2 transgenic lines was significantly higher than in control NT sugarcane (Figure 9). The free proline content in ScD27.2R-9 was lower than that in NT. The content of the free proline increased gradually. The general trend was ScD27.2F-2 > NT > ScD27.2R-9.

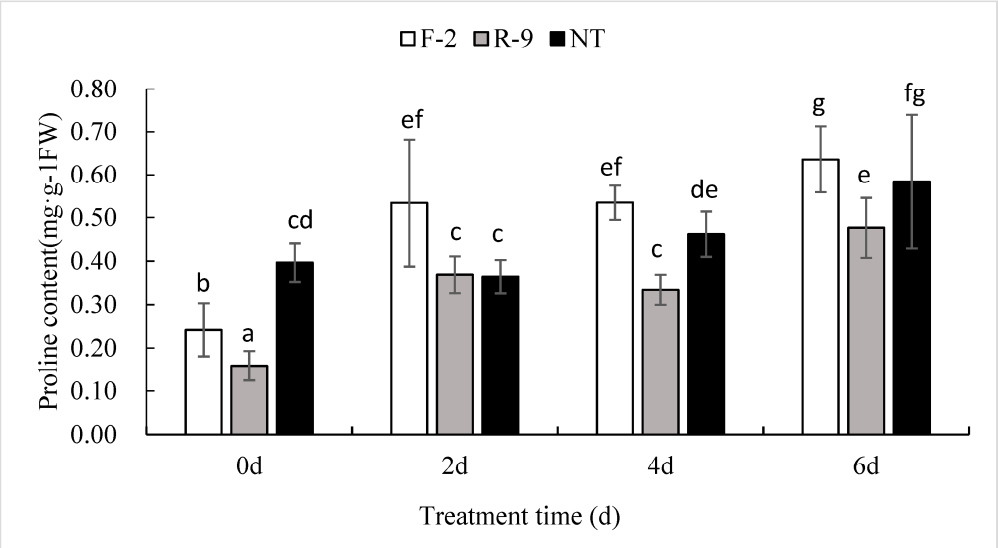


Figure 9. Proline content of ScD27.2 F-2 and ScD27.2R-9 transgenic lines and NT non-transgenic controls. Duncan's new multiple range methods were used to analyze significant differences in gene expression at different time points after 6 days of stress treatment in 20% PEG-8000 MS medium. Different lowercase letters indicate significant differences at the 0.05 level. The error line represents the standard error (n = 9).

3.7. The Tiller Number of ScD27.2R-9 after 15 Days of 20% PEG

After 15 days of stress treatment in the 20% PEG-8000 MS medium, agronomic traits, including tiller number, plant height, stem diameter, root length, and fresh weight of the transgenic lines, were analyzed. The results showed that the tiller number of ScD27.2R-9 was significantly higher than that of ScD27.2F-2 and NT. However, plant height, stem diameter, root length, and fresh weight of ScD27.2R-9 were lower than NT lines but slightly higher than NT for scD27.2F-2 lines (Table 3 and Figure 10). Results might indicate that the growth of ScD27.2R-9 was significantly inhibited, and resistance to drought stress was decreased, while the growth of ScD27.2F-2 was similar to controls.

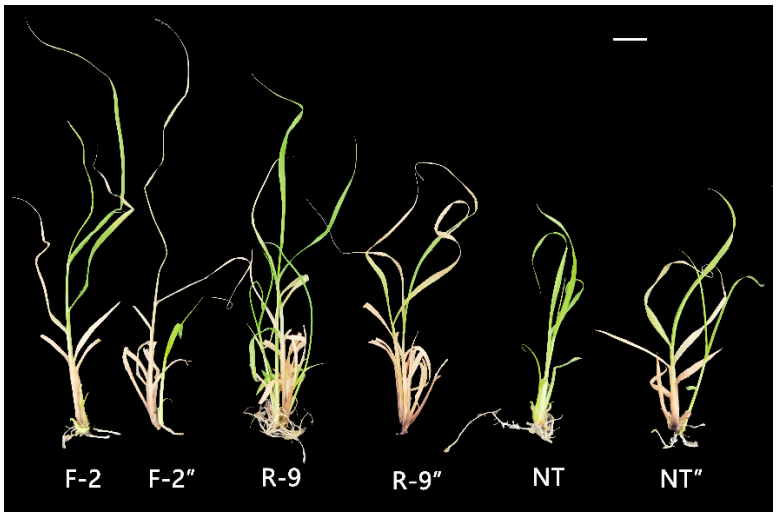


Figure 10. Phenotypes of ScD27.2F-2 and ScD27.2R-9 transgenic and NT non-transgenic lines subjected to drought stress. F-2, R-9, and NT are F-2 and R-9 drought-stressed lines and normal lines. Scale bar = 1 cm.

Table 3. Effects of drought stress on the phenotypes of ScD27.2F-2 and ScD27.2R-9 transgenic and NT non-transgenic lines.

Plant Line	Tillering Number	Plant Height (cm)	Stem Thickness (cm)	Root Length (cm)	Fresh Weight (g)
ScD27.2F-2	1.20 ± 0.40 a	10.77 ± 1.35 b	0.30 ± 0.05 b	2.24 ± 0.67 b	4.17 ± 0.53 b
ScD27.2R-9	5.30 ± 1.10 b	6.12 ± 0.74 a	0.25 ± 0.03 a	1.36 ± 0.25 a	2.12 ± 0.41 a
NT	1.40 ± 0.49 a	11.80 ± 0.91 c	0.31 ± 0.05 b	2.22 ± 0.20 b	4.24 ± 0.87 b

Note: After 15 days of stress treatment in 20% PEG-8000 MS medium, the phenotypes of the three lines were analyzed for significant differences. Duncan's new multiple range methods were used for analysis, significant differences at the 0.05 level are indicated by different lowercase letters, and the standard error line is shown (n = 10).

3.8. PEG Increase in the SL Content in Sugarcane Lines

To explore the effect of 20% PEG treatment on SL content, we measured the content of (±)-2′-epi-5-deoxystrigol in the rooting culture media ScD27.2F-2 and ScD27.2R-9 transgenic lines and NT lines [25]. The results showed that the content of (±)-2′-epi-5-deoxystrigol was 122.43 ng/g DW in ScD27.2F-2, 137.36 ng/g DW in ScD27.2R-9, and 119.23 ng/g DW in NT (Figure 11). After 30 days of 20% PEG treatment, SL content in ScD27.2F-2, ScD27.2R-9 and NT lines were 134.13 ng/g DW, 146.65 ng/g DW, 139.33 ng/g DW, respectively. The content of (±)-2′-epi-5-deoxystrigol increased significantly in all three strains under 20% PEG treatment.

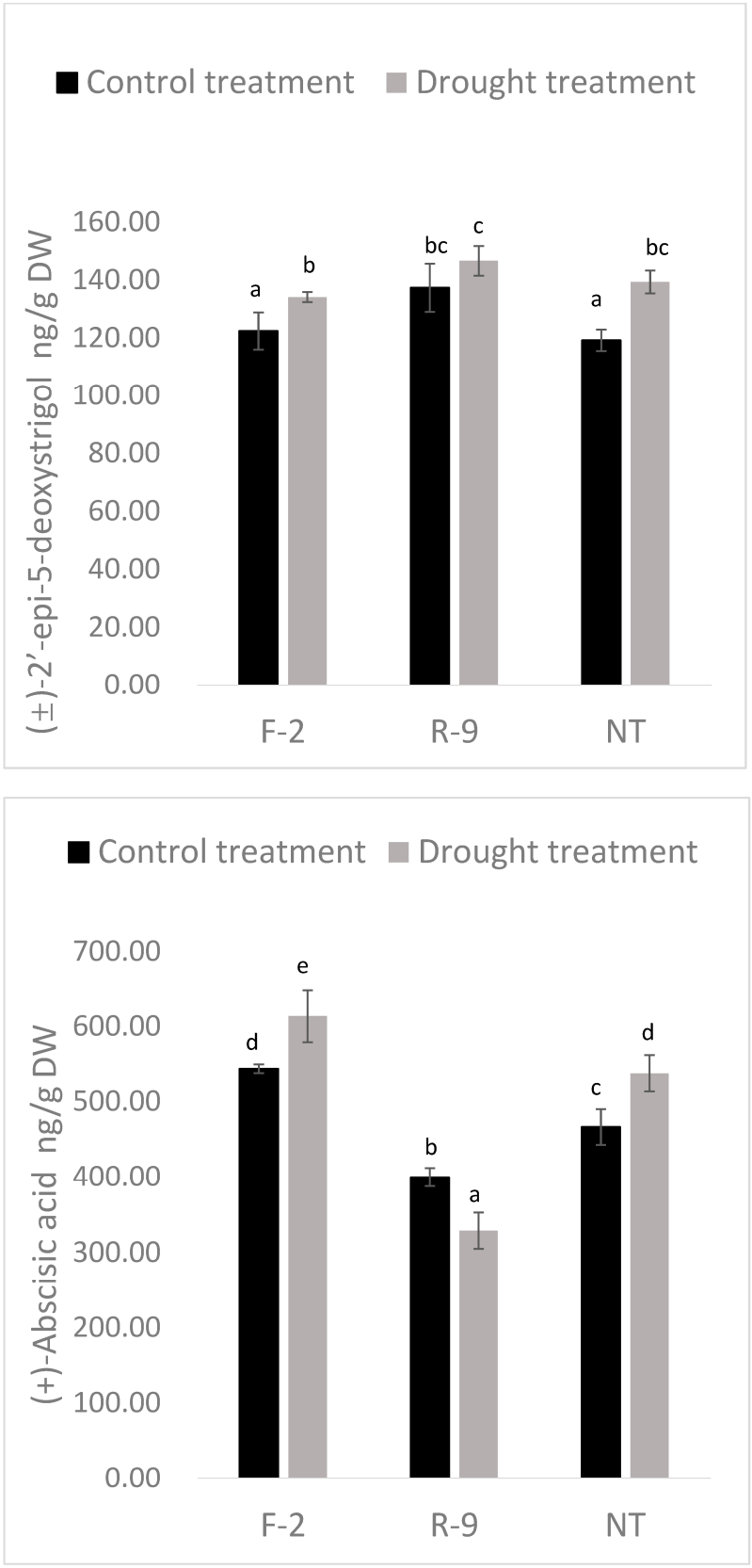


Figure 11. Content of (±)-2′-epi-5-deoxystrigol and (+)-abscisic acid in the rooting culture medium of ScD27.2F-2 and ScD27.2R-9 transgenic lines and NT non-transgenic lines after 30 days of drought stress treatment. Different lowercase letters indicate significant differences at the 0.05 level. The error line represents the standard error (n = 9).

3.9. Lower ABA Levels in ScD27.2R-9

To explore the effect of 20% PEG treatment on ABA content, we measured the content of (+)-ABA in the rooting culture medium ScD27.2F-2 and ScD27.2R-9 transgenic lines and NT lines. The average content of (+)-ABA in transgenic ScD27.2F-2, ScD27.2R-9, and NT lines was 544.29 ng/g DW, 399.62 ng/g DW, and 466.35 ng/g DW, respectively. In ScD27.2R-9 lines, (+)-ABA levels were significantly lower than in the other two strains. Following 20% PEG treatment, (+)-ABA levels in all three lines were significantly increased, but levels were lower than in non-drought stress controls.

4. Discussion

Recent research has demonstrated that DWARF27 is an SL biosynthesis gene that encodes an iron-containing carotenoid isomerase. Using trans-β-carotene as a substrate, the catalytic production of 9-cis-β-carotene is the first step in SL biosynthesis, and the catalytic reaction is reversible. It is speculated that *DWARF27* may be a key regulator of SL levels and is highly sensitive to drought induction [24].

4.1. ScD27.2R-9 Antisense Line with a Highly Branched Dwarf Phenotype

Some physiological characteristics observed in the ScD27.2R-9 antisense line in sugarcane were similar to those observed in other species that reduced DWARF27 gene expression [22,23]. TaD27-B is vital in regulating the wheat tiller number by participating in SLs biosynthesis [9]. Since mutations in all SL biosynthetic genes result in a highly branched dwarf phenotype, which is characteristic of plants with impaired SL biosynthesis [8], a similar phenotype in sugarcane may be caused by downregulation of DWARF27, a sugarcane homologous gene. ScD27.2R-9 showed dwarf and multiple tillering phenotypes and decreased fresh weight, reduced levels of *ScD27.2* gene expression. The transcript level of the *ScD27.2* gene was increased in ScD27.2F-2; ScD27.2F-2 had a similar phenotype to NT.

4.2. Lower ABA Levels Lead to ScD27.2R-9 Drought Sensitivity

Increasing ABA content under drought stress might be a protective mechanism for plants. It might help plants cope with adversity and ensure survival by driving stomatal closure, reducing water loss, controlling transpiration, and reducing plant growth [41,42]. Low levels of ABA were produced by ScD27.2R-9 lines under drought stress, while MDA content increased and proline content decreased compared with NT lines. The results show that ScD27.2R-9 was more sensitive to drought stress in sugarcane than NT, and similar results were found in other crops [43,44].

Drought stress is one of the most frequently encountered restrictive environmental conditions during plant development and growth. Under water shortage conditions, SLs and ABA interact to help plants achieve the optimal allocation of resources to survive [45,46]. The results showed that drought stress could enhance the expression of the *ScD27.2* gene in sugarcane and increase the accumulation of SLs and ABA. Drought can increase the transcription of SLs biosynthesis genes in Arabidopsis leaves [46], and the expression of the SLs biosynthesis gene, Carotenoid Cleavage Dioxygenases 8(CCD8), is also increased under drought stress [43]. Both the D27 gene and *CCD8* were induced by drought stress, which may indicate the existence of drought-related transcriptional regulatory factors during SLs biosynthesis. The *ScD27.2* gene could be induced by drought stress in ScD27.2F-2 and NT lines. Strigolactones might play an important role in plant responses to drought stress [46,47], so ScD27.2R-9 is very sensitive to 20% PEG treatment [43,48].

4.3. The Fresh Weight of ScD27.2R-9 Lines Was Significantly Lower

The analysis of MDA and free proline content levels showed that ScD27.2R-9 might decrease drought resistance. These results are consistent with previous reports showing that drought reduces leaf yield and leaf area and inhibits tillering [49,50]. Furthermore, the dwarf and multiple tillering phenotypes and decreased drought resistance of ScD27.2R-9 appear to be correlated with decreased expression of the *ScD27.2* gene. These results might

indicate a decreased expression of the ScD27.2 gene in ScD27.2R-9, increased peroxidation of PEG, and similar membrane lipids, resulting in damage to the ScD27.2R-9 cell membrane system and diminished drought stress resistance in ScD27.2R-9 lines. Hence, the expression of the *ScD27.2* gene was decreased in ScD27.2R-9 lines under drought stress, and phenotypic analysis also showed that the growth of ScD27.2R-9 lines was inhibited by drought stress. Moreover, the fresh weight of ScD27.2R-9 lines was significantly lower than that of control lines.

4.4. Lower ABA Levels in ScD27.2R-9 Lines Indicated an Interference with the ABA Pathway

ScD27.2R-9 exhibited dwarf and multiple tillering phenotypes, expression of the *ScD27.2* gene was decreased, and the ABA content was significantly lower than in NT controls under 20% PEG treatment. Biosynthetic precursors of both SLs and ABA are carotenoid-derived terpenoids [51]. The decreased SL biosynthesis in roots under drought conditions may be one of the reasons for increased ABA levels. Plants rely on ABA as an early warning chemical signal of drought [52]. After 30 days of 20% PEG treatment, (±)-2′-epi-5-deoxystrigol was increased in all three lines, but the content of (+)-ABA in ScD27.2F-2 was increased significantly compared with the controls. Meanwhile, the content of (+)-ABA in ScD27.2R-9 decreased significantly compared with controls. In ScD27.2R-9 lines, the expression of the *ScD27.2* gene was decreased, and the ABA content was significantly decreased, resulting in the decreased drought resistance of ScD27.2R-9.

SL content analysis proves that PEG increases the SL content in sugarcane lines. In this study of sugarcane, the phenotype shown by the ScD27.2R-9 antisense line is complex and provides new insights into the role of SLs, particularly in the theory of sugarcane drought resistance. The function of *ScD27.2* should be further analyzed through protein interactions with transcriptional regulators and promoters to improve our understanding of the effects of SLs [53,54].

5. Conclusions

The agrobacterium-mediated method obtained the ScD27.2 transgenic sugarcane seedlings with herbicide resistance sense and antisense. Driven by the 35S promoter, the ScD27.2F-2 and ScD27.2R-9 transgenic lines with significantly up-regulated and down-regulated expression were obtained. The transcript level of the *ScD27.2* gene in ScD27.2R-9 was significantly lower than in NT lines, which resulted in a dwarf and multi-branched phenotype where growth was inhibited and drought resistance was decreased under the 20% PEG treatment. The expression of *ScD27.2* gene in ScD27.2F-2 was significantly higher than in NT lines, and the phenotype and drought resistance of *ScD27.2* were similar to those of controls. The expression of the *ScD27.2* gene in ScD27.2R-9 lines decreased under 20% PEG treatment, which affected the growth and development of sugarcane and reduced drought resistance. The 20% PEG treatment increased (±)-2′-epi-5-deoxystrigol and (+)-abscisic acid in the rooting culture media of ScD27.2F-2, ScD27.2R-9, and NT lines, but (+)-abscisic acid content in ScD27.2R-9 was lower than in NT. ScD27.2R-9 decreased resistance to 20% PEG treatment. These results might indicate that the ScD27.2 gene may be related to drought resistance responses of plants and further isolation of drought-related transcriptional regulatory factors can further study the relationship between SLs and sugarcane growth and drought stress.

Author Contributions: Z.W., F.Z. and J.G. planned and designed the research. F.Z., Z.W., W.W. and S.Z. completed the genetic transformation of sugarcane. F.Z. and Z.W. performed experiments, analyzed the data, and wrote the manuscript. C.W., X.L., J.L., L.Z., X.H. and L.F. helped the experiment. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The datasets supporting the conclusions of this article and materials used in this study are available by contacting with the corresponding author (judith1123@126.com).

Conflicts of Interest: The authors declare that they have no competing interests.

Appendix A

Table A1. Primer Sequence.

Primer Name.	Accession	Sequence (5′–3′)
D27FNcoIF		CCCATGGATGGAGGTCGCCGCCACTTGCATGC
D27FBglIIR		GAAGATCTTCAAATAGAGCAATTCACTTGACGG
D27RNcoIF		CCCATGGTCAAATAGAGCAATTCACTTGACG
D27RBglIIR		GAAGATCTGTCGCCGCCACTTGCATGC
QScD27F		GGATGAAGAACGGAAAGGAC
QScD27R		ACGAGCCAAGGGAAGAATAT
GAPDHF		CACGGCCACTGGAAGCA
GAPDHR		TCCTCAGGGTTCCTGATGCC

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