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Stress-Inducible Overexpression of *SlDDF2* Gene Improves Tolerance against Multiple Abiotic Stresses in Tomato Plant

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Abstract: Dehydration-responsive element-binding protein 1 (DREB1)/C-repeat binding factor (CBF) family plays a key role in plant tolerance against different abiotic stresses. In this study, an orthologous gene of the *DWARF AND DELAYED FLOWERING* (*DDF*) members in Arabidopsis, *SlDDF2*, was identified in tomato plants. The *SlDDF2* gene expression was analyzed, and a clear induction in response to ABA treatment, cold, salinity, and drought stresses was observed. Furthermore, two transgenic lines (SlDDF2-IOE#6 and SlDDF2-IOE#9) with stress-inducible overexpression of *SlDDF2* under *Rd29a* promoter were generated. Under stress conditions, the gene expression of *SlDDF2* was significantly higher in both transgenic lines. The growth performance, as well as physiological parameters, were evaluated in wild-type and transgenic plants. The transgenic lines showed growth retardation phenotypes and had higher chlorophyll content under stress conditions in plants. However, the relative decrease in growth performance (plant height, leaf number, and leaf area) in stressed transgenic lines was lower than that in stressed wild-type plants, compared with nonstressed conditions. The reduction in the relative water content and water loss rate was also lower in the transgenic lines. Compared with wild-type plants, transgenic lines showed enhanced tolerance to different abiotic stresses including water deficit, salinity, and cold. In conclusion, stress-inducible expression of *SlDDF2* can be a useful tool to improve tolerance against multiple abiotic stresses in tomato plants.

Keywords: abiotic stresses; bioinformatics; DREB1; stress-inducible promoter; tomato; transcription factor

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1. Introduction

Abiotic stresses, such as cold, drought, high salinity, and extreme heat have adverse effects on plant growth and development. They are considered major constraints for plant production in many areas around the globe. Among them, drought is considered a major limiting factor for the productivity of any given crop. To minimize the negative impact of abiotic stresses on plants, it is necessary to develop new plants that utilize water more efficiently and tolerate such stresses [1]. In this perspective, a basic strategy is based on the cloning of key regulatory genes and the introduction of their active forms into plants so that they can acquire abiotic stress tolerance phenotypes [2].

Under stress, several morphological, physiological, and molecular processes are altered in different organs to improve plant tolerance [3]. Plants have developed different defense mechanisms against abiotic stresses that involve the interaction among a group

of transcription factors and the activation of key effector genes [4]. Transcription factors control plant responses to different environmental factors through sequence-specific interactions with *cis*-regulatory DNA elements, which are found in promoter and enhancer regions of their target genes [5]. Thus, the levels of expression of different abiotic stress-responsive genes are influenced by the manipulation of stress-responsive transcription factors [6]. Therefore, the manipulation of these transcription factors can improve the tolerance of different plants against different biotic and abiotic stress conditions [7]. In this perspective, members of dehydration-responsive element-binding protein 1 (DREB1)/C-repeat binding factor (CBF) family, encoding AP2 transcription factors, are known to regulate the expression of several stress-responsive genes by binding to *C-repeat/dehydration-responsive cis-element* in their promotors, thus enhancing cold, high salinity, and drought tolerance of plants [8]. In many plants, *DREB* genes work as the connecting points for multiple plant-response pathways to different stress factors, such as salinity, drought, ABA, and cold pathways [9–11]. For instance, the *DWARF AND DELAYED FLOWERING* (*DDF*) genes were upregulated in Arabidopsis plant under cold, drought, salinity stress conditions [12], while the overexpression of *CBF4* gene in Arabidopsis plants improved drought tolerance that was associated with upregulation of several stress-responsive genes and resulted in [13].

Tomato (*Solanum lycopersicon* L.) is one of the most grown vegetable crops all over the world, and it is mainly cultivated under irrigated conditions. Tomato yield is greatly affected in many growing areas by different abiotic stresses including drought, salinity, and low temperatures [14]. There is a growing need to improve stress tolerance in tomato plants to withstand such adverse conditions. In this study, a new member of DREB transcription factors in tomato plants (named *SlDDF2*) that is closely related to the *DDF* gene in Arabidopsis was isolated and characterized. The *SlDDF2* gene was expressed in response to a variety of abiotic stimuli, implying a potential role in tolerance against abiotic stresses. Transgenic tomato plants with different levels of inducible overexpression of the *SlDDF2* were generated and analyzed for their growth, physiological, biochemical, and gene expression responses to multiple abiotic stress factors. Transgenic tomato plants with inducible overexpression of *SlDDF2* showed reduced water loss and improved tolerance against multiple abiotic stresses.

2. Materials and Methods

2.1. Cloning of SlDDF2 Gene and Bioinformatics Analysis

To clone a *DDF* orthologous gene in tomato plants, bioinformatics and comparative genomics analyses were performed based on a previously published full-length sequence of Arabidopsis *DDF1* gene (GenBank accession number: NM_101131 [15]). A TBLASTN search was performed utilizing DDF1's amino acid sequence against the annotated ITAG2.3 predicted tomato cDNA sequences database [16]. The full-length coding sequences of novel and unstudied tomato DNA sequences encoding DDF transcription factors in tomato were retrieved and further analyzed. The predicted coding sequences of a selected tomato *DDF* gene (*SlDDF2*; *Solyc08g007820*) were used to design specific primers (*SlDDF2fwd*: 5′-ATGAATAACGACTCGAGTTTG-3′ and *SlDDF2Rev*: 5′-TCAAATACTATAACTCCACA-3′) using the NCBI Primer-BLAST tool to isolate its full-length CDS as described previously by Al-Abdallat et al. [16].

For *SlDDF2* cloning, leaves from two-week-old tomato cv. "Money Maker" plants were collected and used for total RNA extraction using the SV Total RNA Isolation System Kit (Promega, Madison, WI, USA), as directed by the manufacturer. Following the manufacturer's instructions, the extracted RNA was used to synthesize the first-strand cDNA library using the SuperScript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and the oligo T(18) primer. The full-length CDS of *SlDDF2* gene was then amplified from the synthesized cDNA using specific pair of primers (*SlDDF2Fwd* and *SlDDF2Rev*) in a PCR with a total volume of 25 µL containing 5 µL of cDNA as a template, 2.5 µL of dNTPs (100 µM), 5 µL of 5× PCR buffer, 0.5 µM of each primer and 0.25 µL of 5 U/µL GoTaq

DNA polymerase (Promega, Madison, WI, USA). The thermal reaction was conducted using GeneAmp® PCR system 9700 (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 30 s and 72 °C for 2 min and a final extension of 72 °C for 10 min. The amplified CDS fragments were resolved on a horizontal 1% agarose gels stained with ethidium bromide. The PCR products (estimated size 735 bp) were then eluted from the agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and then cloned into pGEM®-T Easy Vector System (Promega, Madison, WI, USA). DNA Plasmids containing PCR products were selected, and the DNA products were then sequenced using the M13 reverse and forward sequencing primers by ABI 3730XL machine by Macrogen (Seoul, Korea). The sequenced *SlDDF2* cDNA and its deduced amino acids sequences were analyzed by the Vector NTI software (https://www.thermofisher.com/jo/en/home/life-science/cloning/vector-nti-software.html, accessed on 1 July 2021; Invitrogen™, Carlsbad, CA, USA) and further confirmed by a BLAST search (https://solgenomics.net/tools/blast/, accessed on 1 February 2019) for DNA and amino acid sequence homology to further verify the identity of the cloned cDNA.

The Sol Genomics Network [17] was used to retrieve and analyze DNA and amino acid sequences, as well as chromosomal position and annotation prediction of several members of DREB-A1 and DREB-A2. Phylogenetic trees were constructed using MEGA version 10 software [18] and the amino acids sequences of SlDDf2 and selected ERF subfamily proteins from tomato and Arabidopsis from the DREB-A1 and DREB-A2 groups [19] that were retrieved from the Phytozome databases [20] were included. The retrieved sequences were aligned using the ClustalW algorithm and the alignment was used to calculate distance matrices for neighbor-joining analyses with the Kimura two-parameter model and Bootstrap analysis with 10,000 replicates was performed to test the robustness of the internal branches, as described previously by Alhindi and Al-Abdallat [21].

2.2. Plant Material and Stress Treatments

For gene expression analysis, tomato cv. "Moneymaker" (MM) plants were grown under growth conditions and were subjected to different treatments that included water deficit, salinity, cold, and ABA. Transgenic tomato lines with inducible overexpression of the *SlDDF2* gene, on the other hand, were utilized to compare abiotic stress tolerance in MM plants in response to drought, salinity, and cold. The transgenic plants were generated using the binary plasmid *pCABIMA1302* harboring the *SlDDF2* gene downstream of the stress-inducible *Rd29a* promoter. For this purpose, the *mgfp5* gene was replaced with *SlDDF2* CDS at the *Nco*I and *Bst*EII sites to generate *pCAMBIA1302/SlDDF2*. Thereafter, the *Rd29a* promoter (GenBank accession number: AY973635.1) was cloned into *pCAMBIA1302/SlDDF2* by replacing the *CaMV 35S* promoter using *Eco*RI and *Nco*I sites to produce *pCAMBIA1302/Rd29a::SlDDF2*. The constructs were then introduced into tomato cv. "MM" using *Agrobacterium*-mediated transformation, and two positive plants carrying a single copy of the transgene were identified and selected, as described previously by Al-Abdallat et al. [22]. Gene expression analysis levels in T2 homozygous selected transgenic lines were analyzed using the quantitative RT–PCR approach under water deficit and control conditions.

For stress treatments, tomato seeds of the selected genotypes were submerged in water for 24 h at 25 °C, and then, they were washed with sterilized water and sown into small pots (10 cm diameter × 10 cm depth) filled with acid-washed sand. The pots were incubated under controlled conditions (at constant temperature (25 °C) and photoperiod of 16 h light–8 h dark, with 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux density) till full germination. The tomato seedlings were then irrigated daily with a fixed amount of 1× Hoagland solution (Sigma-Aldrich, Gillingham, UK). For water deficit treatment, two-week-old tomato seedlings were exposed to water withholding for 0, 3, 5, and 7 days for gene expression analysis in MM plants and for 7 days for inducible expression analysis in transgenic plants. Furthermore, stress tolerance and wilting behavior in response to water withholding for 10 days were

investigated on transgenic and nontransgenic lines, in which 20 plants from each line were used per treatment and the survival percentage was calculated at the end of the dehydration treatment.

To investigate the effect of high salt treatment on *SlDDF2* gene expression, the roots of two-week-old MM seedlings were submerged in saline water (300 mM NaCl) for 0, 2, 4, 8, 12, and 24 h. For salinity stress tolerance in transgenic and nontransgenic lines, two-week-old seedlings (20 plants from each line were used per treatment) were irrigated every three days with a fixed volume of 1× Hoagland solution supplemented with 100 mM for 12 days and the wilting behavior was monitored, and survival percentage was calculated at the end of the high salt treatment. For the effect of cold treatment on *SlDDF2* gene expression, two-week-old MM seedlings were incubated at 4 °C for 0, 2, 4, 8, 12, and 24 h. For cold stress tolerance in transgenic lines and wild-type plants, two-week-old seedlings (20 plants from each line were used per treatment) were incubated for 24 h at 4 °C, and the wilting behavior was monitored, and the survival percentage was calculated. The gene expression of *SlDDF2* was analyzed in response to ABA treatment, two-week-old MM seedlings were sprayed with 100 µM ABA solution, and leaf tissues were collected after 0, 2, 4, 8, 12, and 24 h.

2.3. Growth and Physiological Measurements

Relative water content (RWC) was determined in well-watered and stressed plants (subjected to 10 days of water withholding period), as described in Al-Abdallat et al. [16], using fully expanded leaves, and the RWC was calculated according to Barrs and Weatherley [23]. The water loss rate was measured using fully expanded leaves excised from the well-watered and stressed transgenic lines (subjected to 10 days of water withholding period) that were placed on filter paper for 2 h, as described in Al-Abdallat et al. [16], and the water loss rate was measured according to Ristic and Jenks [24]. Stomatal resistance ($s \cdot cm^{-1}$) was measured using a fully expanded leaf from well-watered and stressed plants after 10 days of water withholding by using a Porometer device (AP4, Delta-T Devices, Cambridge, UK). The determination of chlorophyll content (Chla, Chlb, and total Chl) was carried out using excised leaves from stress-treated transgenic lines and MM plants following a modified protocol, as described previously by Al-Abdallat et al. [16]. Finally, growth-related measurements (leaf number, plant height (in cm), and leaf area (cm^2) were collected from stress-treated (subjected to 10 days of water withholding period) and well-watered transgenic lines and MM plant. Five replicates were used for all physiological and growth parameters, and the standard error of means was used to compare means.

2.4. Gene Expression Analysis

For quantitative real-time PCR (qRT–PCR) analysis, total RNA was extracted from leaf samples collected from treated plants at indicated time points using the SV Total RNA Isolation System Kit (Promega, Madison, WI, USA). The extracted RNA was used to synthesize the first-strand cDNA library as described above. Specific primers pairs for *SlDDF2* expression analysis (*SlDDF2Efwd*: 5′-ATGAATAACGACTCGAGTTTG-3′ and *SlDDF2ERev*: 5′-TCAAATACTATAACTCCACA-3′) were used. The qRT–PCR analysis was performed using the stress-inducible *Le16* gene (*Solyc10g075090*, a phospholipid transfer protein from tomato also known as Le16 (*Lycopersicon esculentum protein 16*) and *Solyc03g078400* (encoding actin, a house-keeping gene used as an internal reference control for relative gene expression analysis) as described in Al-Abdallat et al. [16]. All cDNA samples were analyzed in triplicate, and each replicate was derived from two biological replicates. The relative changes in gene expression were quantified as described in Vandesompele et al. [25].

2.5. Statistical Analysis

The data are presented as mean ± SD of three technical replicates from two biological replicates (n = 6) for gene expression analysis and five biological replicates for the growth performance and physiological parameters' measurements. Student's t-test was used

to determine significant group differences, and means were considered as statistically significant if $p < 0.05$.

3. Results and Discussion

3.1. Identification of DDF Orthologous Genes in Tomato

To identify *DDF* orthologous gene in tomato plants, a TBLASTN search was conducted against the annotated ITAG2.3 predicted tomato cDNA sequences database using the full-length amino acids sequence data of the *DDF1* gene from Arabidopsis (GenBank Accession: NM_101131). Using this approach, three tomato orthologous genes were identified: *Solyc12g056430*, *Solyc08g007820,* and *Solyc08g007830*, and the three genes were named *SlDDF1*, *SlDDF2*, and *SlDDF3*, respectively. Phylogenetic analysis using ERF subfamily proteins from tomato and Arabidopsis belonging to groups DREB-A1 and DREB-A2 revealed the identity of SlDDF proteins, which clustered with DDF1 and DDF2 proteins from Arabidopsis (Figure 1). The DDFs are members of the DREB-A1 subfamily of the ERF/AP2 transcription factor family in Arabidopsis, and they are implicated in stress responses and GA biosynthesis regulation [12], indicating that *SlDDF* may have a role in stress tolerance in tomato plants. To investigate this role in tomato plants, the *SlDDF2* gene was selected and cloned using specific pair of primers. The *SlDDF2* gene was found on the upper arm of chromosome 8, and it was annotated as *Solyc08g007820* that encodes ethylene-responsive transcription factor 10 with a single ORF (735 bp with a single exon) intron and a total length of 245 amino acids.

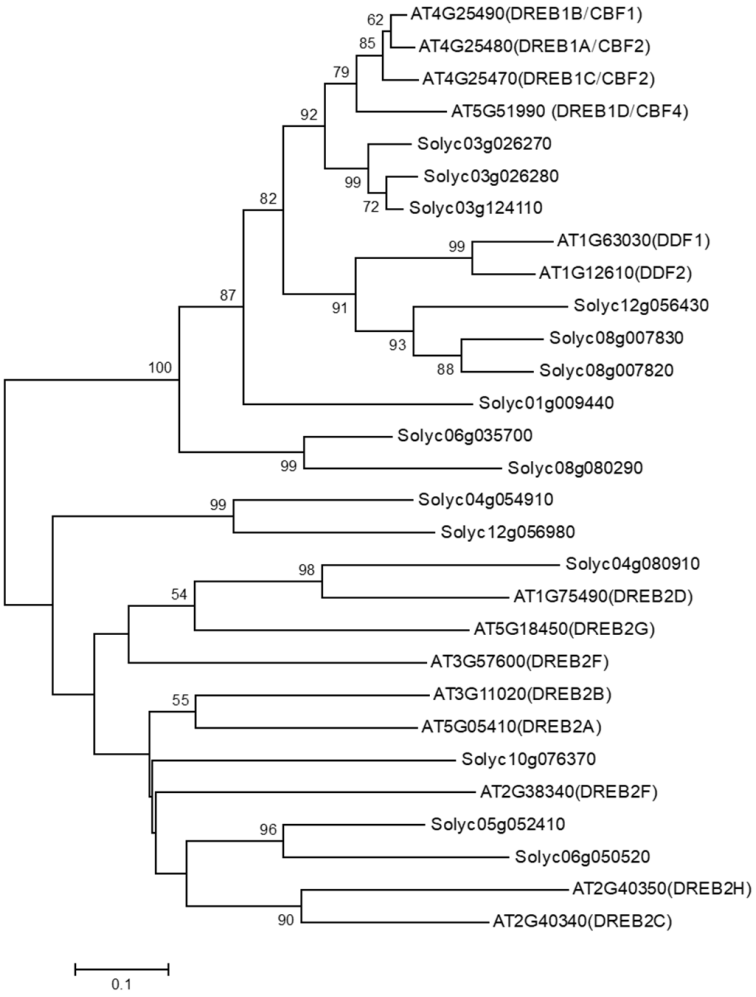

Figure 1. Phylogenetic analysis of *Arabidopsis* proteins belonging to the DREB-A1 and DREB-A2 groups and their closest orthologs in tomato plants.

3.2. Expression Analysis of SlDDF2

The expression behavior of *SlDDF2* in response to ABA and abiotic stresses was analyzed in MM plants using quantitative real-time PCR. The *SlDDF2* expression levels were compared with *Lycopersicon esculentum protein 16*; *Solyc10g075090* (*Le16*), a stress-inducible gene from tomato encoding a phospholipid transfer protein. The expression of the *SlDDF2* gene in MM plants was highly induced after two hours of ABA treatment before returning to the basal expression level of the control (zero time) after 4 h (Figure 2A). This is in general agreement with Li et al. [26], who reported that the ABA-induced expression of *SlDREB* in tomato plants started from 1 to 6 h after treatment before it returned to pre-treatment levels after 12 h. The expression of the *Le16* stress-responsive gene, on the other hand, was induced as expected in response to ABA treatment and peaked after 24 h, which is consistent with previous studies [27,28]. The expression patterns of *SlDDF2* and *Le16* genes in response to cold treatment (4 °C for 0, 2, 4, 8, 12, and 24 h) were also analyzed in tomato MM plants. The expression of *SlDDF2* was induced after 4 h, with a higher level of induction observed at 12 h of cold treatment, while *Le16* gene expression was at the highest level after 12 h of cold treatment (Figure 2B). These results are similar to the findings of Zhang et al. [29], who found that the expression of the *LeCBF1* gene was upregulated upon exposure to low temperature, reaching its highest level after 8 h before returning to its pretreatment levels after 24 h.

The expression of the *SlDDF2* gene in MM plants was induced after 2 h in response to 100 mM NaCl treatment, reaching its highest expression at 4 h of incubation (Figure 2D). *Le16* gene expression was induced in response to NaCl with time, which is in agreement with Al-Abdallat et al. [22], and the highest expression level was observed at 24 h. The upregulation of *SlDDF2* in response to high salt stress suggests a potential role in salinity tolerance, which was previously reported by Sakuma et al. [30] and Magome et al. [12], who found that *DDF1* gene expression was induced in Arabidopsis roots under high salinity conditions. These results are also in agreement with Hichri et al. [31], who reported the inducible expression of *SlDREB2* in tomato plants in response to NaCl treatments.

The expression patterns of the *SlDDF2* gene and *Le16* were analyzed in MM tomato plants in response to water deficit treatment by water withholding for 3, 5, and 7 days. The expression of the *Le16* gene in stressed MM tomato plants was induced under water deficit with time, reaching the highest expression level after seven days (Figure 2D), which is in general agreement with Al-Abdallat et al. [16]. Similarly, the expression of the *SlDDF2* gene was induced in response to water deficit after five days of stress, reaching its highest level after seven days (Figure 2D). Similar results were described by Hichri et al. [31], who reported drought-induced expression of *SlDREB2* in tomato plants.

3.3. Stress-Inducible Overexpression of SlDDF2 in Tomato

To check if *SlDDF2* inducible overexpression can enhance tolerance of tomato against various abiotic stresses, the coding sequence of *SlDDF2* was cloned into a binary plasmid under the control of the *rd29A*, a stress-inducible promoter from *Arabidopsis* plant, or under the control of the *CaMV 35S* constitutive promoter. Several independent transgenic tomato lines were generated with transgenic lines carrying the *CaMV 35S* constitutive promoter showed severe growth retardation phenotypes and did not produce any seeds and, therefore, were discarded from further analysis (data not shown). Severe pleotropic effects of constitutive overexpression of stress-related DREB transcription factors in tomato plants were reported previously [32]. Similarly, the overexpression of *DDF1* in Arabidopsis plants resulted in dwarfism and late-flowering phenotypes [12]. On the other hand, transgenic lines carrying the *rd29A* stress-inducible promoter were generated, and two transgenic lines carrying a single insertion event as revealed by real-time PCR analysis were selected for further analysis (SlDDF2-IOE#6 and SlDDF2-IOE#9). To validate the inducible expression of *SlDDF2* in response to stress, transgenic lines and MM plants were subjected to water withholding for seven days. The expression of the *SlDDF2* gene was significantly higher in both transgenic lines, compared with wild type; however, the expression level was much

higher in stressed plants than that in nonstressed plants (Figure 3A). Furthermore, the expression levels in SlDDF2-IOE#9 plants were significantly higher than that of SlDDF2-IOE#6 plants under both treatments. Stress-inducible expression of *DREB* genes the *rd29A* stress-inducible promoter was reported previously in different plants species including tomato [33,34].

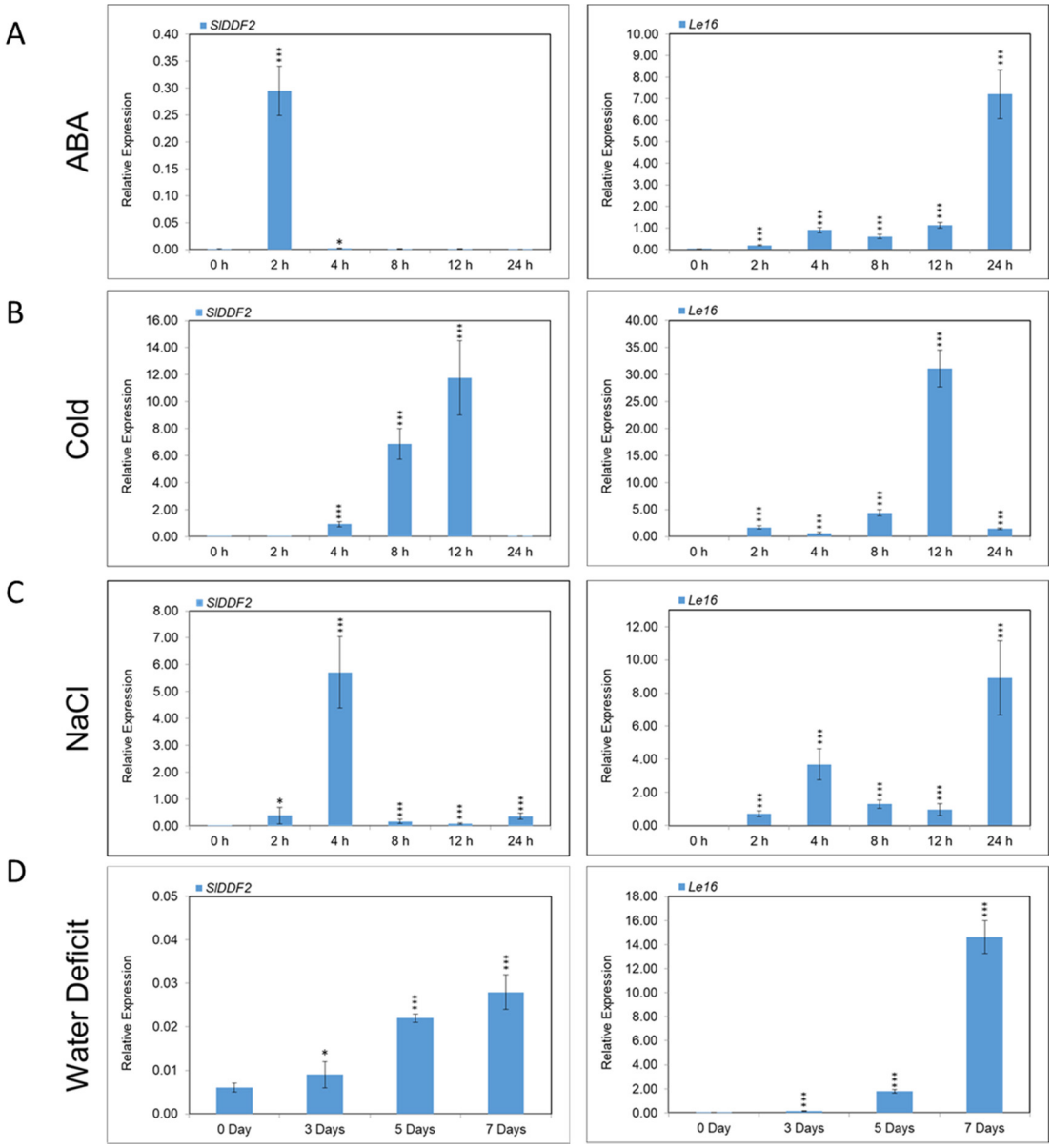

Figure 2. Relative gene expression analysis of *SlDDF2* and *Le16* in response to (**A**) ABA, (**B**) cold, (**C**) NaCl, and (**D**) water deficit treatments. MM plants were compared to untreated plants (control). The stress-responsive *Le16* (*Solyc10g075090*) gene was included as a control. Values are the means ± SD. of six replicates. Relative expressions are significantly different from those at zero time at different levels: * $p < 0.05$, *** $p < 0.001$.

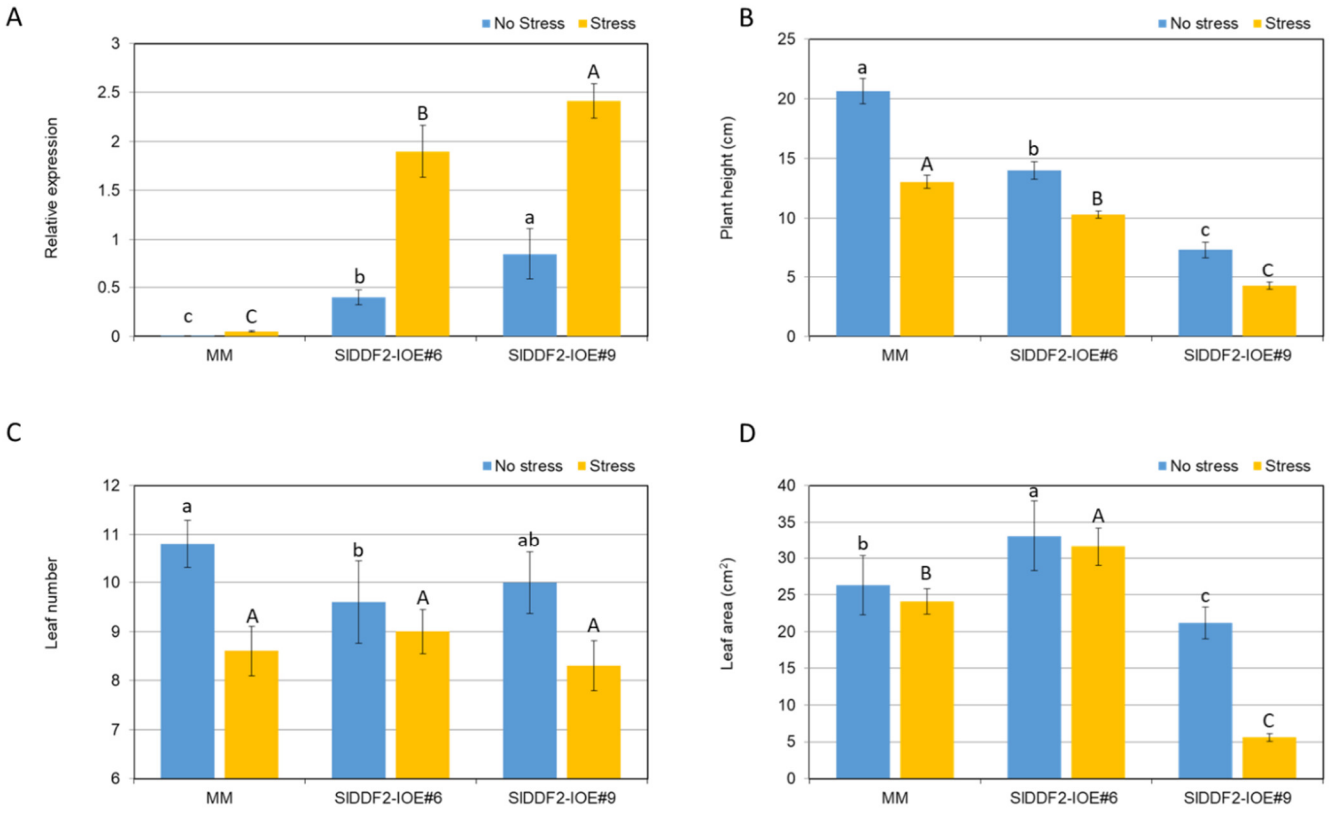

Figure 3. (**A**) Relative gene expression analysis of *SlDDF2* in MM and SlDDF2-IOE#6 and SlDDF2-IOE#9 transgenic plants in response to water withholding for seven days; values are the means ± SE of six replicates; (**B**) plant height, (**C**) leaf number, and (**D**) leaf area of two-week-old seedlings of SlDDF2-IOE#6 and SlDDF2-IOE#9 transgenic lines and MM plants under normal and water withholding for seven days (stress). Values are the means ± SD. Different lower-case letters indicate a significant difference between transgenic and wild-type plants under nonstressed conditions, and different capital letters indicate a significant difference between transgenic and wild-type plants under stress conditions ($p < 0.05$).

When compared with the wild-type plants, the two selected transgenic lines (SlDDF2-IOE#6 and SlDDF2-IOE#9) showed growth retardation phenotypes under normal and water deficit conditions, with clear shorter plants and shorter internodes phenotypes (Figure 3B). The wild-type and SlDDF2-IOE#9 plants showed a significant reduction in leaf number mean values under stress conditions when compared with nonstressed plants (Figure 3C). In addition, the SlDDF2-IOE#9 plants showed a significant reduction in leaf area means values under stress conditions when compared with well-watered plants and SlDDF2-IOE#6 plants (Figure 3D). On the contrary to the findings of this study, the use of the stress-inducible *rd29A* promoter for the overexpression of *AtDREB1A* in transgenic tomato did not show negative effects on plant growth and development [33]. However, the observed growth retardation phenotypes in the SlDDF2-IOE lines are similar to previous phenotypes reported in *rd29A*:DREB1A transgenic tobacco [35,36] and *rd29A*:*AtCBF3* potato plants, in which growth retardation phenotypes were observed. The differences in growth retardation phenotypes between the two transgenic lines can be explained by the higher expression levels in SlDDF2-IOE#9, as reported previously by Pino et al. [36]. Additionally, the observed behaviors were comparable to those found in transgenic tomato plants with constitutive overexpression of SlDREB, which has been linked to reduced internode elongation due to lower gibberellin levels [26].

Under water deficit conditions, the transgenic plants were found to have darker green leaf color, which was associated with increased chlorophyll a, chlorophyll b, and total chlorophyll pigments concentrations in comparison with the wild-type plants (Figure 4).

The levels of Chla, Chlb, and total chlorophyll were increased only under water deficit stress conditions compared with the nonstressed groups. These findings are in general agreement with Li et al. [26] and Al-Abdallat et al. [16], who observed increased total chlorophyll pigments in transgenic tomato plants overexpressing *DREB* genes, which was attributed previously to reduced GA levels in transgenic lines [37].

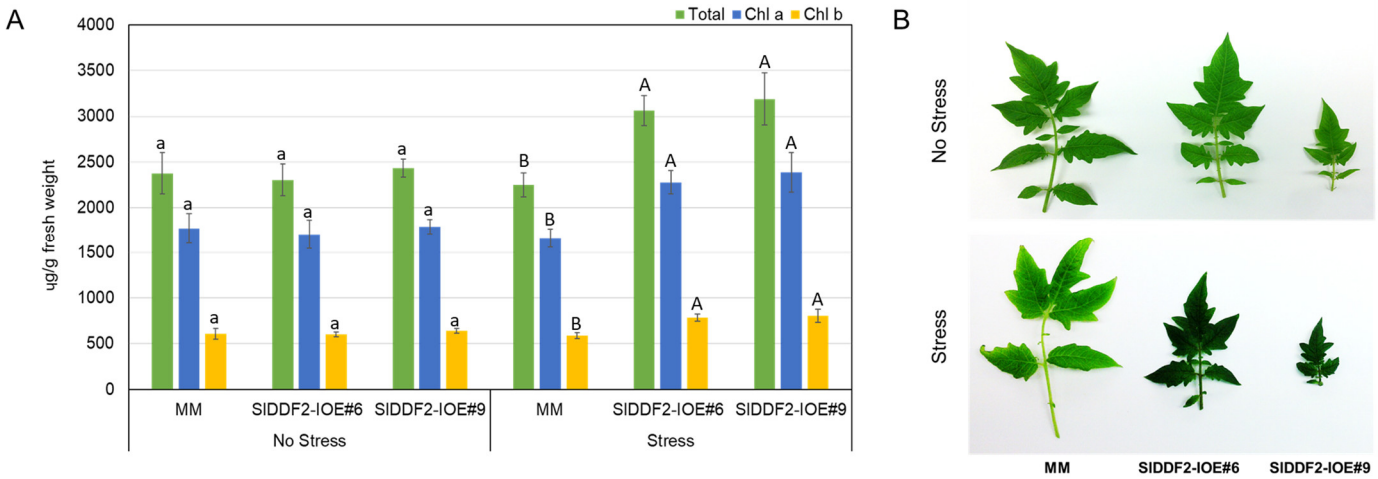

Figure 4. (**A**) Total chlorophyll (green), Chlorophyll a (blue), and Chlorophyll b (yellow) contents in MM and SlDDF2-IOE#6 and SlDDF2-IOE#9 transgenic plants in response to water withholding for seven days or no stress conditions; (**B**) detached leaves from stressed and nonstressed wild-type plants and the two transgenic lines plants. Values are the means ± SD. Different lower-case letters indicate a significant difference between transgenic and wild-type plants under nonstressed conditions, and different capital letters indicate a significant difference between transgenic and wild-type plants under stress conditions ($p < 0.05$).

The physiological behavior of the transgenic plants under water deficit stress was compared with MM plants. Initially, the relative water content was measured immediately after detaching leaves from each plant. As shown in Figure 5, MM plants exposed to stress conditions showed lower RWC than the transgenic lines, which indicates a better water status in cells of transgenic lines. To investigate the effect of water deficit on water loss rate ($g \cdot h^{-1} \cdot g^{-1}$ DW), fully expanded wild-type and transgenic-line leaves from both treatments were detached and subjected to dehydration for 2 h. The results showed that the water loss rate from the stressed transgenic lines was lower than that in control and stressed wild-type plants (Figure 5). These results suggest that the drought resistance of the transgenic plants overexpressing the *SlDDF2* gene was improved, compared with MM plants. It has been reported that transgenic tomato plants overexpressing *DREB* genes showed enhanced drought tolerance by maintaining higher water content and reduced water loss rate [38].

To analyze the impact of water deficit on physiological responses of *SlDDF2* transgenic lines, two-weeks old transgenic and MM (included as control) seedlings were grown under stress conditions for 10 days by water withholding and observed for their growth and wilting behaviors at the end of treatment. Under drought stress conditions, the majority of MM plants were welted (60% survival rate), and an obvious, adverse effect was observed, while transgenic tomato lines showed enhanced tolerance to water deficit stress and showed a delayed wilting behavior and higher survival rate when compared with MM plants (Figure 6B). For salinity stress tolerance, SlDDF2-IOE#9 transgenic plants displayed improved tolerance to high salt stress (survival rate 50%), followed by SlDDF2-IOE#6 plants (survival rate 50%), while MM wild-type plants suffered severely from salinity stress (Figure 6C). For cold stress tolerance, the survival rate of the wild-type plants was 10%, whereas the SlDDF2-IOE transgenic plants showed enhanced tolerance to cold stress and higher survival rate when compared with wild-type plants, with 45% and 75% for SlDDF2-IOE#6 and the SlDDF2-IOE#9, respectively (Figure 6D). These results

suggested that the overexpression of the *SlDDF2* gene improved drought, cold, and salt stresses tolerance in tomato plants. In line with our results, the overexpression of *SlDREB2* enhanced Arabidopsis and tomato tolerance to salinity stress (125 mm NaCl) [31], while the stress-inducible overexpression of Arabidopsis *CBF1* in transgenic tomato plants improved tolerance against low temperatures, water-deficit, and high salt treatments [34].

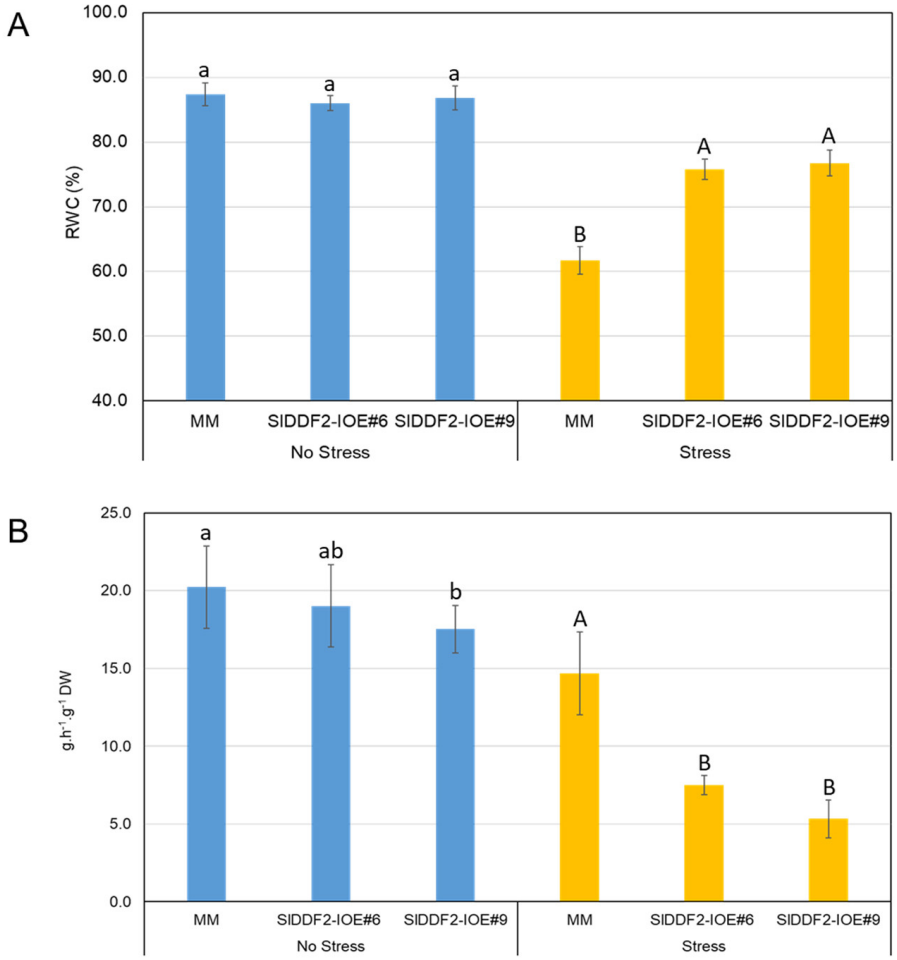

Figure 5. Relative water content of leaves of MM and SlDDF2-IOE#6 and SlDDF2-IOE#9 transgenic lines after 10 days of (**A**) water withholding and (**B**) water loss rate, as measured by decrease in fresh weight after two hours in detached leaves from MM and SlDDF2-IOE#6 and SlDDF2-IOE#9 transgenic plants. Values are the means ± SD. Different lower-case letters indicate a significant difference between transgenic and wild-type plants under nonstressed conditions, and different capital letters indicate a significant difference between transgenic and wild-type plants under stress conditions ($p < 0.05$).

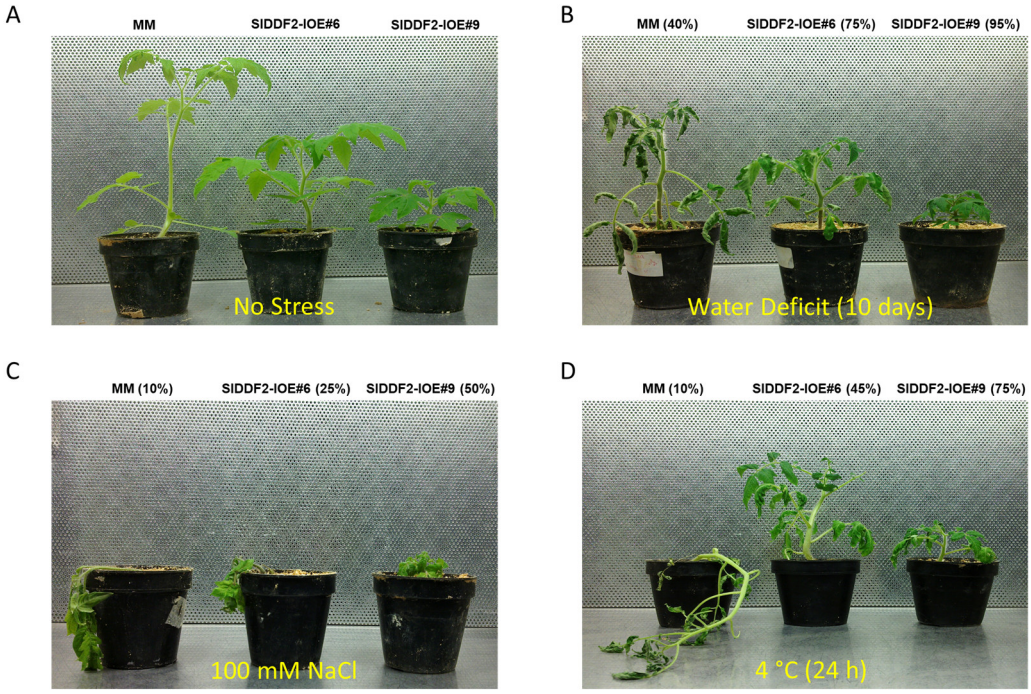

Figure 6. Representative MM and SIDDF2-IOE#6 and SIDDF2-IOE#9 transgenic lines grown under (**A**) normal conditions, (**B**) water deficit, (**C**) salinity, and (**D**) cold stresses (percentages are describing survival rate out of 20 plants per treatment).

4. Conclusions

The *SlDDF2* gene was identified in tomato plants, and the phylogenetic analysis clustered it with the DREB1 family, indicating a potential role in abiotic stress tolerance. Furthermore, gene expression analysis of *SlDDF2* showed inducible expression patterns in response to multiple abiotic stresses including cold, salinity, and drought. Stress-inducible overexpression of the *SlDDF2* gene in tomato plants enhanced tolerance against different abiotic stresses when compared with MM plants, with clear pleotropic effects observed on them. The identified stress-related *SlDDF2* gene could be a useful tool for tomato improvement and tolerance under abiotic stress conditions.

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