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Identification of *PtGai* (a DELLA protein) in trifoliate orange and expression patterns in response to drought stress

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Abstract: Gibberellins (GAs) are an important hormone in regulating plant growth and development, and DELLA protein is an essential negative regulator of GA signal transduction. The aim of the study was to clone a GA-inhibiting protein DELLA from trifoliate orange (*Poncirus trifoliata* L. Raf.) and to analyze the bioinformations and expression patterns of the protein gene in tissues and in response to drought stress. A DELLA protein was isolated from trifoliate orange and named as *PtGai* (Genebank number: MZ170959). The PtGai protein had 1731 bp open reading frames, along with 576 amino acid codes, and also grouped with sweet orange (XM_006430552.4). The PtGai protein sequence was 65% homology with the sequences of DELLA proteins in other plant families. PtGai protein existed in the nucleus based on the prediction of subcellular localization. *PtGai* protein could be expressed in roots, stems, and leaves, along with the highest expression in stems. *PtGai* was upregulated by drought stress in leaves and roots, along with the decrease of root total GA concentration and the inhibition of shoot and root biomass production. It indicated the characteristics of PtGai protein and the roles of *PtGai* in GA synthesis and plant growth.

Introduction

Due to the abnormal global climate change, various abiotic stresses have caused tremendous damage to ecosystems and crop production, whilst drought stress as a kind of abiotic stress has become a major factor for limiting crop growth (Cheng *et al.*, 2021; Zou *et al.*, 2021a). To mitigate the damaging effects of drought and to enhance plant vigor in the face of abiotic stress, plants adopt various strategies to cope with soil water deficit, including osmoregulator accumulation, stomatal closure, and accumulation of stress proteins, in order to maintain normal plant growth and development (Natanella *et al.*, 2020). As an important endogenous hormone, the hormonal network of gibberellins (GAs) regulates plant growth processes and responses to abiotic stress (Stirk *et al.*, 2019). GAs regulate the plant growth cycle and developmental processes through a variety of mechanisms (Theo *et al.*, 2020). It has been found that under certain drought levels, GAs enhanced plant growth and mitigated the negative effects of drought stress (Shan *et al.*, 2019).

DELLA proteins are a group of independent transcription regulators of GRAS, mainly present in plant cell nuclei, and are also key inhibitory proteins for the signal transduction of GAs, inhibition of GA responses, and plant growth (Floss *et al.*, 2013; Bai *et al.*, 2019). The result of Zhou and Underhill (2020) showed that the GA signaling pathway could relieve the inhibition of DELLA protein during plant growth and development, and the decrease of GA biosynthetic gene expression was accompanied by the increase of DELLA protein expression. Bilova *et al.* (2016) also observed that under the condition of low GA concentration, DELLA protein was closely linked with specific transcription factors, complementing each other and blocking plant DNA activity, thus inhibiting plant growth. Therefore, the removal of DELLA proteins can effectively eliminate the inhibitory function on GA activity and promote plant growth (Harberd *et al.*, 2009). On the contrary, more stable proteins may be the result of mutations in DELLA proteins or an important functional domain of GA signal transduction (Bilova *et al.*, 2016),

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TABLE 1

Gene-specific primer sequences used in the experiment

Primer names	Primer sequence (5'→3')	Purposes
PtGai	F: ATGAAGAGAGATCACCAAC	Subcellular vector construction
	R: GTCCACTCTCGTGAGTTGA	
PtGai	F: GTCAACGGGAAATGCAAATAAGGC	qRT-PCR and qPCR
	R: CCATCACCATCTCCAGCTGTTCAA	
β-Actin	F: CCGACCGTATGAGCAAGGAAA	Reference gene in qRT-PCR
	R: TTCCTGTGGACAATGGATGGA	

which reflects the malleability of the GA signal. During plant growth and development, DELLA forms a huge regulatory network pathway, thus, suggesting an interaction between GA signals and DELLA (Arro *et al.*, 2019).

DELLA proteins interact with numerous transcription factors in different signaling pathways and emit functions associated with plant regulation of drought (Park *et al.*, 2013; Bai *et al.*, 2019). The results from Nir *et al.* (2017) showed that the tomato (*Solanum lycopersicum*) DELLA protein *PROCERA* (*PRO*) regulated plant stomata and reduced water loss by increasing abscisic acid (ABA) sensitivity. In *Oryza sativa*, the DELLA protein Slender Rice1 (*SLR1*) had an important role on four pathogens (Vleesschauwer *et al.*, 2016). Jusovic *et al.* (2018) reported that the altered structure of the wheat DELLA mutant contributed to the mitigation of photosynthetic tissue damage under salt stress. The yield increase in wheat under drought stress was closely linked to the DELLA protein gene encoding a reduced *Rht* gene (Kocheva *et al.*, 2014). These results indicate that DELLA proteins are linked to the stress responses of plants.

Citrus is one of the most widely cultivated fruit crops in the world (Wu *et al.*, 2019), and citrus yield and fruit quality are seriously impacted by drought stress (He *et al.*, 2020; Zou *et al.*, 2021b). Trifoliate orange (*Poncirus trifoliata* L. Raf.) is the principal rootstock used in Southeast Asia and is also drought-sensitive (Zhang *et al.*, 2020). Studies on DELLA proteins mainly focused on model plants such as *Arabidopsis thaliana*, rice, and wheat (Jusovic *et al.*, 2018; Noel *et al.*, 2020), whereas the information regarding DELLA proteins in woody plants such as citrus is scarce. The aim of this study was to clone DELLA protein from trifoliate orange and to analyze the characteristics and basic functions of the protein gene in response to drought stress and the relationship with plant GAs concentrations and plant growth.

Materials and Methods

Cloning and sequence analysis of PtGai protein

The roots of trifoliate orange were pre-treated with liquid nitrogen to avoid DNA degradation. The PrimeScriptTM RT Master Mix (Baori Medical Biotechnology Beijing Co., Ltd., Beijing, China) was used to extract total RNA. The PrimeScriptM RT Reagent Kit with gDNA Eraser (Baori Medical Biotechnology Beijing Co., Ltd., Beijing, China) was used to reverse the RNA into cDNA. On the basis of sweet orange database (http://citrus.hzau.edu.cn/orange/index.php) and our transcriptome data (SRR10413223, SRR10413224, SRR10413225, and SRR10413226), the specificity primer of DELLA protein was designed (Tab. 1). The PCR product (cDNA) was purified and ligated into the 1300-35S-YFP vector. The PCR product (cDNA) was purified and ligated into the 1300-35S-YFP vector, and the ligated product was transferred into *Escherichia coli* for cloning and sequencing to obtain the cloned full-length sequence. Sequence alignment was carried out using NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in combination with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to download the sequences of different plant DELLA proteins. The amino acid sequence evolutionary tree of plant DELLA protein was constructed using the MEGA-X software. Gene structures were predicted using the online SWISS-MODEL tool (https://swissmodel.expasy.org/). Evolutionary tree construction was obtained using the Neighbor-joining (NJ) method (Saitou and Nei, 1987), and the bootstrap method with a repetition number of 500 was used to validate the evolutionary tree (Felsenstein, 1985). Evolutionary tree distances were achieved using the

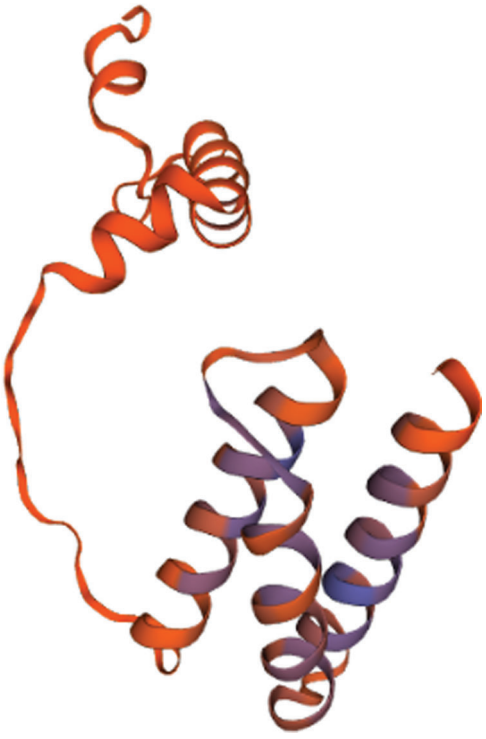


FIGURE 1. The three-dimensional structure of the PtGai protein.

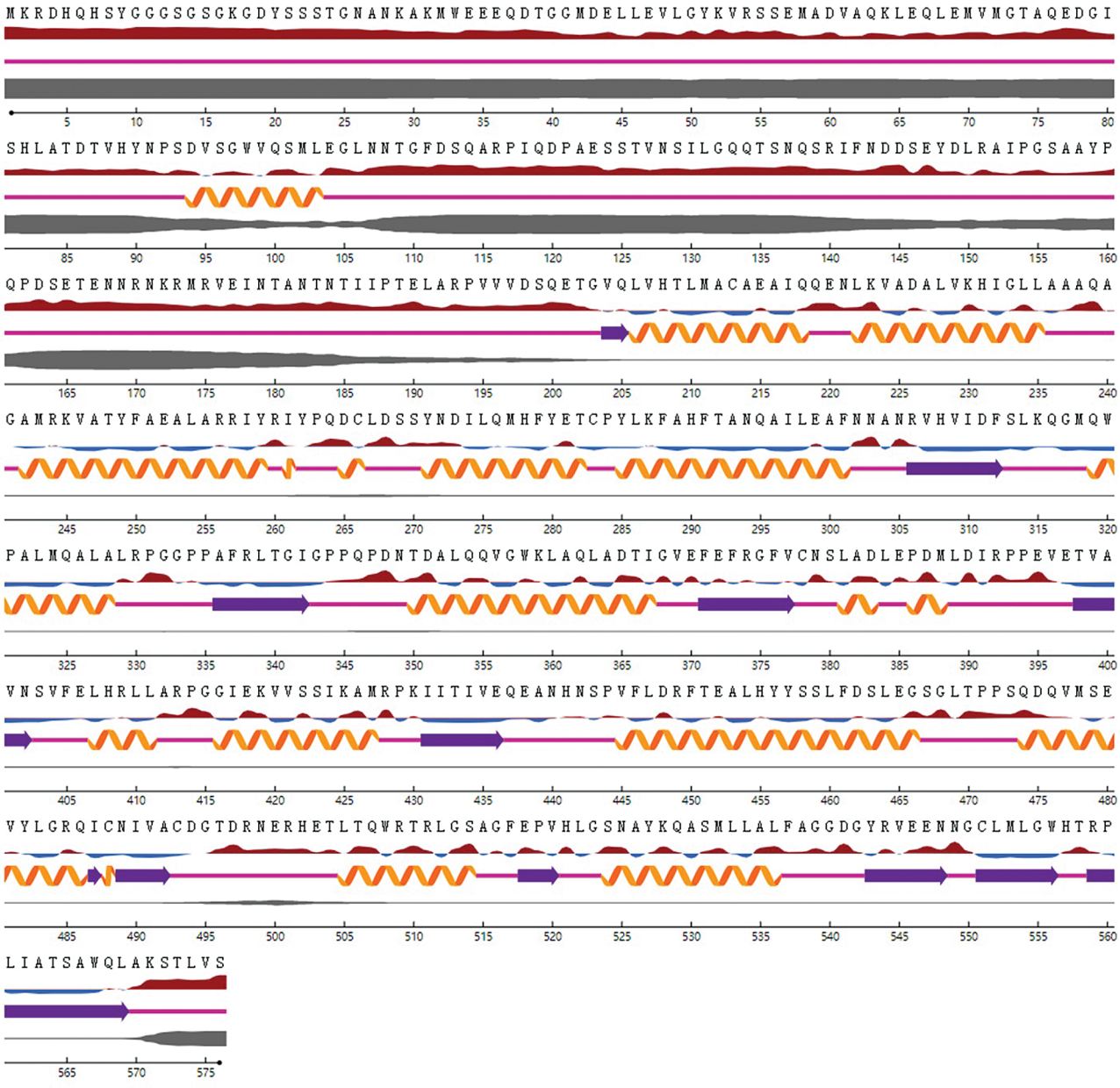
p-distance method (Nei and Kumar, 2000), with the number of amino acid differences per locus as a unit. Subcellular structural localization analyses were performed online using the WoLF-PSORT (https://wolfpsort.hgc.jp/).

Plant culture and drought treatment

Seeds of trifoliate orange were soaked in 10% NaOH for 10 min, washed with distilled water for three times, disinfected with 75% alcohol for 15 min, and then placed in sterilized sands in 28/20°C (day/night temperature), 1200 Lux light intensity, and 80% relative air humidity. Five-leaf-old seedlings with consistent growth were transplanted into a plastic pot with autoclaved (0.11 MPa, 121°C, 2 h) mixture of soil and sand (5:2). Three seedlings were planted in each pot, with a total of 24 pots. After transplanting, the plants were grown in 75% of the maximum field capacity (WW) for 8 weeks. Subsequently, half of the plants were exposed to 55% of the maximum field capacity (DS) for 10 weeks, and the other plants were kept in WW status for another 10 weeks. The soil moisture was controlled by weighting. After the drought treatment lasted for 10 weeks, the experiment was ended, and the seedlings were harvested. Plant samples were frozen in liquid nitrogen and stored at −80°C for further study on the response of DELLA protein to drought stress.

qRT-PCR analysis

The obtained cDNA products were used to design primers for the clone of the DELLA sequence in combination with Olige-7 software (Tab. 1). After the successful primer was obtained, qRT-PCR analysis was performed using the Chamq^{TM}



Relative Surface Accessibility: Red is exposed and blue is buried, theresholded at 25%.
Secondary Structure: Helix, Strand, Coil.
Disorder: Thickness of line equaals probability of disordered residue

FIGURE 2. PtGai protein secondary structure and relative solvent accessibility.

Universal SYBR qPCR Master Mix kit (Vazyme Biotech Co., Ltd., Nanjing, China). The CFX96 Real-time PCR Analyzer (BIO-RAD, Hercules, California, USA) was used to analyze the quantitative results. Three biological replicates were performed on each sample, and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative quantification (Livak and Schmittgen, 2001). The *β-actin* was used as a reference gene.

Statistical analysis

The experimental data such as the relative expression of *PtGai*, root total GA concentrations, and shoot and root biomass were statistically analyzed by the analysis of variance (ANOVA) based on the SAS software. We used Duncan's Multiple Range test to compare the significant ($P < 0.05$) difference between treatments.

Results

Full length and bioinformatics analysis of PtGai protein

Based on the Transcription Factor Database (http://planttfdb.gao-lab.org/), phylogenetic relationships between DELLA proteins of the GRAS family and their homologous proteins indicate that all proteins have different families. PtGai protein (registration number: MZ170959) has a 1731 bp open reading frame, 576 amino acid coding, oligo-state monomer structure and its molecular weight is 63.49 kDa. The amino acid contents analyzed by WoLF-PSORT were alanine 70, cysteine 45, glutamine 94, histidine 78, isoleucine 32, leucine 63, serine 46, and valine 30. Within iPSORT range, the first 30 has N-terminal residues, and under the condition of 12 of the maximum hydrophilic value, the protein value of PtGai is 1; the first 20 n has N-terminal residues, and the protein value of PtGai is 45 when the maximum total negative charge is 12 (Fig. 1). Secondary structure predictions indicated that approximately 46.88% of the PtGai protein sequence was α-helix, and 9.72% of the extended chain, and 43.40% of the coil, with a threshold of 25% (Fig. 2). The average hydrophobicity of the PtGai protein was −0.49. Phylogenetic tree analysis of DELLA proteins showed that PtGai protein grouped with *Citrus sinensis* and also closely related to *Pistacia vera* L. (Fig. 3). The WoLF-PSORT predicted that PtGai protein was localized in the nucleus.

Sequences of PtGai proteins blast

The published amino acid sequences of DELLA proteins from other seven plants were compared with the amino acid sequences of PtGai proteins by MEGA-X software. The results showed that the PtGai protein sequence was 65% homology with the sequences of DELLA proteins of different plant families (Fig. 4).

Expression of PtGai protein in different tissues and in response to drought stress

Tissue-specific quantitative expression of *PtGai* protein was detected by qRT-PCR. *PtGai* was expressed in roots, stems, and leaves, and the highest expression of *PtGai* was observed in stems, followed by the roots and leaves in the decreasing order (Fig. 5a). *PtGai* protein in roots and stems was 4.36 and 4.79 times higher than that in leaves (Fig. 5a). Leaf and root *PtGai* protein could be induced by drought stress, by 2.98 times and 1.26 times, respectively (Fig. 5b).

Changes in root total GA concentrations and biomass production

The drought treatment significantly reduced the total GA content of roots in trifoliate orange by 22.06%, compared to the well-watered treatment (Fig. 6a). On the other hand, the drought treatment also strongly suppressed shoot and root biomass production of trifoliate orange by 3.28% and 6.24%, respectively (Fig. 6b).

Discussion

The present study cloned a DELLA protein from *P. trifoliata*, having a registration number MZ170959. Phylogenetic tree structure showed that the PtGai protein was close to that of sweet orange (*C. sinensis*), consistent with the species evolution. The results also indicated that the DELLA protein of *P. trifoliata* had highly homologous (65%) with other plants. Li *et al.* (2018a) found that the RcDELLA protein of *Ricinus communis* had a typical conserved DELLA domain, which was most similar to the plants with a genetic relationship. DELLA protein sequences of different plants showed that DELLA proteins mainly existed in the nucleus. The results of subcellular localization of PtGai in this study showed that PtGai protein was mainly localized in the nucleus, which was also consistent with the previous results (Li *et al.*, 2018b).

PtGai protein gene had tissue-specific expression characteristics, and the high expression of *PtGai* was found in stems. Jia *et al.* (2019) also found that whole-genome DELLA proteins (BNA A06G34810D, BNA A09G18700D, and BNA C09G52270D) of *Brassica napus* showed the highest expression in stem tissue. It may be that DELLA proteins controlled the establishment of shoot rip tissue (Benavent, 2017). DELLA proteins might regulate the expression of the *SPL* gene in stem tissues and thus delay

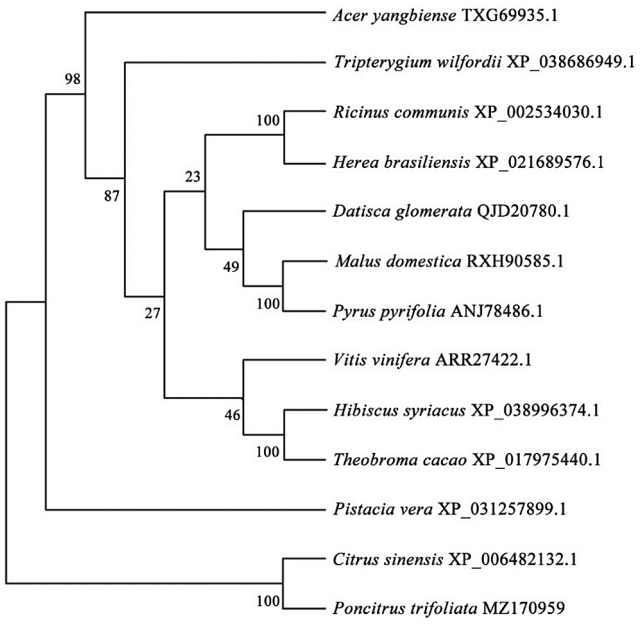


FIGURE 3. Phylogenetic trees of PtGai and other DELLA proteins.

FIGURE 4. Multiple sequence alignments between PtGai protein and other plant DELLA proteins. The conserved amino acids in all proteins are identified in purple.

the flowering of stem tissues (Galvao *et al.*, 2012). However, Song *et al.* (2016) reported the highest expression of *JrGAI* gene in flower buds at the dormant stage. A higher expression of *PaGAI* gene was found in flowers and phloem of cherry plum than in fruits and leaves (Zhang *et al.*, 2012). Such tissue-specific expression of *PtGai* showed the regulatory dependence and tissue-specific function of *PtGai* protein (Grimplet *et al.*, 2016; Arro *et al.*, 2019).

In our study, *PtGai* protein gene was induced in leaves and roots of trifoliate orange in response to drought stress, showing a stressed responsive pattern, which was consistent with the results of Arro *et al.* (2019) and An *et al.* (2015). *DELLA* protein genes negatively regulated plant growth (Liu *et al.*, 2021). Our study further showed that drought stress markedly inhibited root total GA content and shoot and root biomass production, which was contrary to the induced expression of *PtGai* under drought stress. Therefore, it was concluded that the overexpression of *PtGai* under drought conditions inhibited plant GA content, which thus inhibited plant growth performance. Karel *et al.* (2017) also found that the decrease of GA synthesis led to the up-regulation of *DELLA* protein genes. Guo *et al.* (2014) revealed that *DELLA*

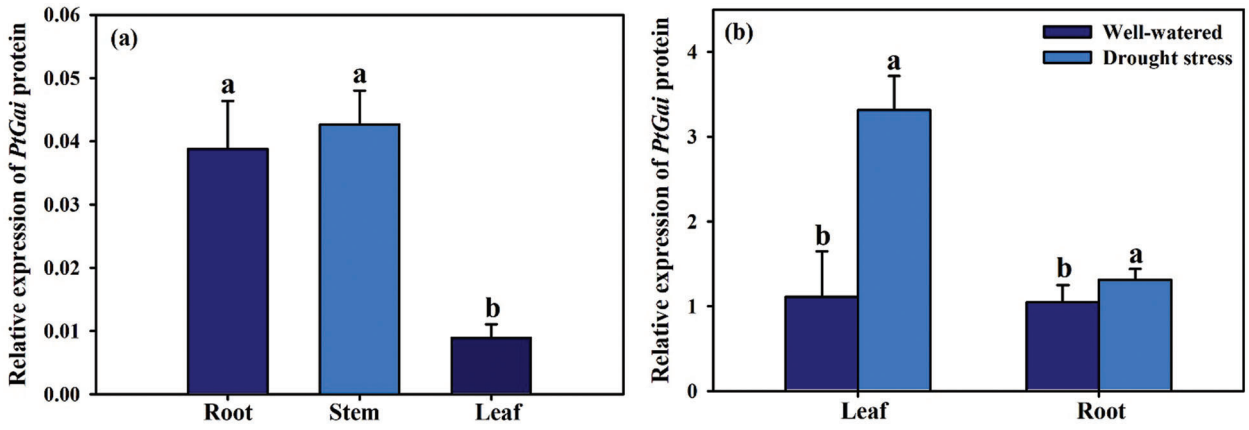


FIGURE 5. Tissue-specific expressions (a) and drought stress response (b) of *PtGai* protein gene. Data (mean ± SD, $n = 3$) with different letter at the bar indicated the significant difference among treatments at 0.05 levels.

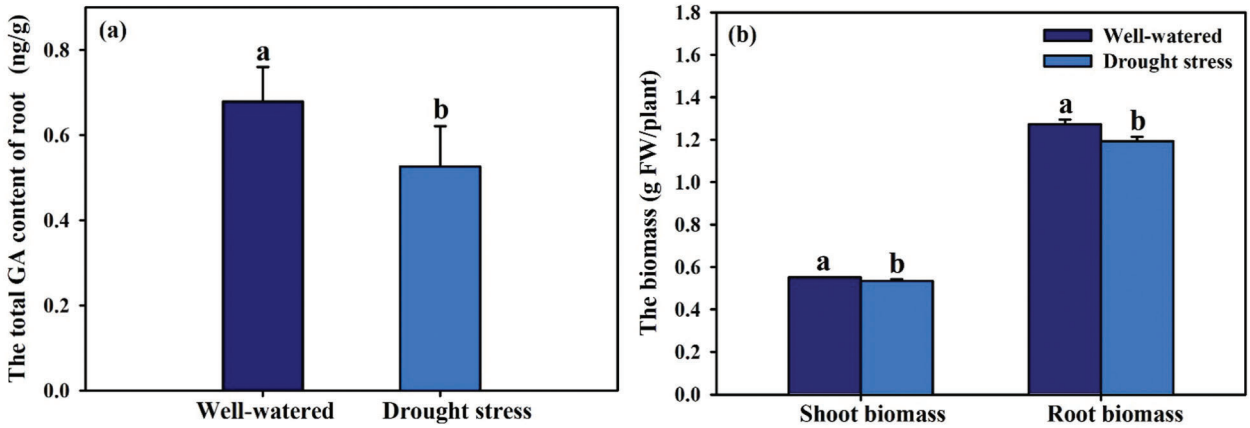


FIGURE 6. Root total GA concentrations (a) and shoot and root biomass production (b) of trifoliate orange under well-watered and drought stress. Data (mean ± SD, $n = 12$) with different letter at the bar indicated the significant difference among treatments at 0.05 levels.

protein genes regulated the remodeling of plant development and improved the degradation of harmful substances under long-term Mg stress. However, Joanna *et al.* (2020) observed the increase of the active GA20 under drought conditions. As a result, more work needs to be done around the types of GAs, along with the expression of *DELLA* genes.

Conclusions

In this study, a DELLA protein PtGai was cloned from trifoliate orange. Bioinformatics analysis revealed that PtGai proteins were grouped with sweet orange, highly homologous (65%) with DELLA proteins from other plants, and localized in the nucleus, with tissue-specific expression and drought-induced expression. Coupled with the changes of biomass and total GAs content, *PtGai* protein as the GA inhibitor modulated GA biosynthesis to regulate plant growth. These results provide a basis for further analysis of the function of *PtGai* protein gene and its regulatory network in response to drought stress. More work is needed to further uncover the upstream transcription factors.

Availability of Data and Materials: All data generated or analyzed in this study are included in this published article.

Author Contribution: The authors confirm the contribution of the paper as follows: study conception and design: Q-SW and KK; data collection: X-FC and Q-SW; analysis and interpretation of results: X-FC, AH, EFAA, and Q-SW; draft manuscript preparation: X-FC and Q-SW. All authors reviewed the results and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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