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ARTICLE

Genome-Wide Identification and Functional Characterization of *TIFY* Gene Family in *Verbena bonariensis* with Insights into *VbTIFY16*'s Role in Petal

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ABSTRACT: The TIFY transcription factor family plays a major role in plant growth and development. Petal size is a very important agronomic characteristic in the ornamental species of *Verbena bonariensis*. This study identifies 16 *TIFY* genes (*VbTIFYs*) in the *V. bonariensis* genome. Phylogenetic reconstruction divided these genes into six distinct subclades, indicating a high degree of homology between Verbena bonariensis and Arabidopsis thaliana. Promoter sequence analysis illustrated that the promoters of TIFY genes harbor not only cis-acting elements related to hormone regulation, but also functional motifs involved in light responses and low-temperature adaptation. Chromosomal localization results shows that *VbTIFY* genes are distributed across six chromosomes of *V. bonariensis*. Synteny analysis revealed four significant pairs of homologous genes within the *VbTIFY* gene family. Tissue expression profiling based on RNA-seq data showed that all 16 *VbTIFY* genes exhibited tissue-specific expression patterns across different developmental stages. Subsequently, six highly expressed genes were selected for qRT-PCR validation. The results indicated that both *VbTIFY12* and *VbTIFY16* were highly expressed in flowers, with the expression level of *VbTIFY16* being 12.7-fold higher than that of *VbTIFY12*. Subsequently, we focused subcellular localization assay confirmed that VbTIFY16 protein was localized in the nucleus. Overexpression of *VbTIFY16* in transgenic tobacco significantly reduced petal size, and further cytological analysis revealed that this phenotypic change was caused by the inhibition of cell expansion. Collectively, this study provides a comprehensive transcriptional framework of the *TIFY* gene family in *V. bonariensis*, lays a theoretical foundation for elucidating the molecular mechanism of petal size regulation, and offers valuable candidate genes for molecular breeding of ornamental traits in *V. bonariensis*.

KEYWORDS: *TIFY* gene family; *Verbena bonariensis*; flower; growth and development

1 Introduction

As a perennial herbaceous species of the genus *Verbena* in the family Verbenaceae, *Verbena bonariensis* is recognized as one of the typical and representative perennial herbaceous germplasm resources within this genus [1], has a typical quadrangular stem and grows to a height of 1–1.5 m, belonging to the medium to tall ornamental herb category. The flowers are terminal or axillary, displaying purple-red or blue coloration. Native to South America, it is now widely cultivated in many provinces across China, including the east, south, northwest and southwest. *Verbena bonariensis* prefers full sunlight and has weak cold resistance. It thrives best at temperatures between 20–30°C, with a flowering period that extends from spring to autumn, offering a long ornamental period [2]. As a flowering plant, it also contributes to insect pollination and is considered a valuable nectar source. In addition to its ornamental value, *V. bonariensis* is recognized for its

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medicinal properties, exhibiting efficacy in detoxification, reducing swelling, and relieving spasms. It may serve as an effective hepatoprotective agent and has potential for alleviating liver diseases [3]. Given the escalating demand for *V. bonariensis*, investigations into its growth, development and stress regulation are of paramount importance.

A transcription factor gene family unique to plants, the TIFY family is distinguished by the presence of a conserved TIFY domain that acts as its representative structural characteristic [4]. According to their conserved sequence characteristics, the TIFY transcription factor family can be divided into four distinct subfamilies, namely TIFY, JAZ, ZML and PPD [5]. All members of this family possess a signature TIFY domain, which harbors the highly conserved core motif TIF [F/Y]XG [6]. The TIFY subfamily members contain only the TIFY domain. The JAZ subfamily possesses a Jas domain that exhibits high sequence homology with the N-terminal sequence of the CCT domain [6,7]. For the ZML subfamily, members carry both a CCT domain and a GATA-type zinc finger domain. In contrast, the PPD subfamily is distinguished by the presence of a PPD domain and a truncated Jas domain [4–6].

To date, *TIFY* genes have been identified in multiple plant species, such as maize with 47 members [8] and *Camellia sinensis* with 16 [9]. As an important regulatory gene family in plants, members of the TIFY family are involved in the regulation of plant developmental processes, enhancement of stress resistance, and responses to abiotic stress through multiple pathways, and simultaneously perform core regulatory functions in the transduction pathways of plant hormone signals [10].

The TIFY family members are broadly engaged in regulating the development of diverse organs and tissues throughout plant growth. For example, *AtTIFY1* (ZML), the first TIFY member identified in *A. thaliana*, is not only associated with inflorescence development and flowering but promotes petiole and hypocotyl elongation by mediating cell extension as well [11]. Hakata et al. found that *OsTIFY11* and *OsTIFY10b* can increase crop yield by enhancing grain size and grain number, respectively; overexpression of *OsTIFY11* also reduces spikelet fertility through JA-mediated regulation, thereby increasing rice yield [12]. Yu et al. demonstrated that *SlTIFY2* in tomato promotes lateral bud germination and early flowering, accelerating the transition from vegetative to reproductive growth [13]. In *chrysanthemum*, In chrysanthemum plants, the regulation of petal size by *CmJAZ1* is achieved through protein-protein interaction with bHLH transcription factor CmBPE2. This binding process impedes the transcriptional activation of expansin gene *CmEXPA7* by *CmBPE2*, and since *CmEXPA7* acts as a critical gene governing cell expansion, petal growth is consequently restrained [14].

TIFY proteins play a crucial role in response to abiotic stresses. For example, *TdTIFY11a* enhances the germination rate of wheat under salt stress [9]. *ZmTIFY16* promotes root growth and development in both *Arabidopsis* and maize, improving drought and salt resistance [15]. *AtJAZ10* in *Arabidopsis* [16] and *OsJAZ8* in rice [17] contribute to enhanced disease resistance in plants. *TaJAZ1* increases resistance to biotic stress in wheat [18]. And overexpression of *CaTIFY7* and *CaTIFY10b* confers enhanced cold tolerance in pepper [19].

While TIFY transcription factors act as key regulators governing plant growth and development, research on the identification and expression characteristics of the *TIFY* gene family in *V. bonariensis* remains extremely limited in the academic community. Within this research, we performed genome-wide identification of *TIFY* gene family members in *V. bonariensis* using genomic data obtained by our research team. Physicochemical properties, gene structures, domains, conserved motifs, phylogenetic relationships, chromosomal distribution and cis-acting elements of these genes were analyzed, and their expression patterns in various tissues were further examined via a combination of transcriptome sequencing and qRT-PCR. Furthermore, subcellular localization analysis of the *VbTIFY16* gene belonging to the TIFY

family verified its nuclear localization. An overexpression vector of this gene was constructed and stably transformed into tobacco, demonstrating that *VbTIFY16* negatively regulates petal size in transgenic plants by inhibiting cell expansion. This study lay a theoretical foundation for elucidating the regulatory mechanism of petal size in *V. bonariensis* and provide additional candidate genes for molecular breeding.

2 Materials and Methods

2.1 Plant Materials

Verbena bonariensis seeds were obtained from Benary Seed Company (Hann. Münden, Germany). Seedling maintenance and multiplication were carried out in the Germplasm Resource Nursery at the Key Laboratory of Mountain Plant Resources Protection and Germplasm Innovation (Ministry of Education), Guizhou University. Wild-type tobacco seeds were provided by our laboratory's nursery, and all plant materials were identified and authenticated by Dr. Yan Li and Dr. Sumeera Asghar.

2.2 Identification of TIFY Gene Family Members in V. bonariensis

The *V. bonariensis* genome annotation (PRJCA027875) was downloaded from the National Genomics Data Cen ter (NGDC, https://ngdc.cncb.ac.cn) [20], downloaded the TIFY conserved domain (PF06200) from Pfam database (http://pfam.xfam.org/). Candidate members containing the conserved structural domain were obtained by aligning the conserved structural domain to the genomic data of *V. bonariensis* using HMMER 3.1 (http://hmmer.org/download.html). In addition, 18 *Arabidopsis* TIFY members were compared to the Verbena willow genome using BLASTP (E-value < 1×10^{-5}) to obtain candidate members. All the candidate members obtained were submitted to: SMART (http://smart.embl-heidelberg.de/) and NCBI-CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to determine the conserved structural domains of TIFY, and finally obtain the TIFY family members.

2.3 Physico-Chemical Properties of TIFY Gene Family in V. bonariensis

The physicochemical parameters of *V. bonariensis* TIFY family proteins, including protein length, molecular weight, theoretical isoelectric point (pI), instability index, and grand average of hydropathicity (GRAVY), were analyzed using the ProtParam (https://web.expasy.org/compute_pi/) [21]. The secondary structure composition was analyzed using the SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). Transmembrane helices were predicted via the TMHMM (https://services.healthtech.dtu.dk/services/TMHMM-2.0/), subcellular localization was determined by WoLF PSORT (https://wolfpsort.hgc.jp/) [22]. And the precise positions of conserved structural domains were mapped using NCBI's Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

2.4 Visualization of Gene Structures, Domains, and Conserved Motifs in the TIFY Gene Family of V. bonariensis

The intron-exon organization profiles of all identified *VbTIFY* genes were retrieved from the official genome annotation dataset of *V. bonariensis*. Conserved domain information was acquired by searching against the NCBI CDD database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). A total of 10 evolutionarily conserved motifs were identified using the MEME (http://meme-suite.org/tools/meme) [23] with default parameter configurations. All the above structural features were ultimately visualized using TBtools software (v2.042) [24].

2.5 Analysis of Phylogenetic Evolution and Chromosomal Distribution Characteristics of TIFY Genes in V. bonariensis.

Using the MUSCLE program within MEGA software, multiple sequence alignment was performed between the identified *TIFY* family genes from *V. bonariensis* and from *A. thaliana.* A phylogenetic tree was generated using MEGA version7.0 Bootstrap with parameter 1000 [25]; Subsequently the phylogenetic tree of *VbTIFY* genes was then reconstructed via the neighbour-joining method. Finally, the constructed phylogenetic tree was annotated and visualized using the Evolview v2 (https://evolgenius.info/evolview-v2/) [26]. Based on the genomic annotation data of *V. bonariensis*, the chromosomal localization of members of the *VbTIFY* gene family was visualized using TBtools v2.042 [24].

2.6 Cis-Acting Regulatory Element Analysis of TIFY Gene Family Members in V. bonariensis

The 2000 bp genomic sequences upstream of the translation initiation codon (ATG) of each identified *VbTIFY* gene were extracted from the *V. bonariensis* genome file using TBtools v2.042 [24]. Putative cis-acting regulatory elements embedded in these promoter sequences were annotated via the PlantCARE online database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [27]. The distribution patterns and functional classification of all identified cis-elements were subsequently visualized and plotted using the same TBtools software [24].

2.7 Characterization of TIFY Gene Family Collinearity in V. bonariensis

Genome-wide datasets and annotation files were acquired on the basis of *V. bonariensis* genome annotation. Collinear relationships among the gene family members were analyzed using the MCScanX algorithm [28], and TBtools v2.042 was subsequently applied for figure visualization [24].

2.8 Characterization of Expression Discrepancies in TIFY Gene Family

Using the transcriptome dataset generated from *V. bonariensis*, we retrieved the expression level (FPKM) values of all identified *VbTIFY* genes across three distinct tissues (flowers, stems, and leaves) and in samples collected pre- and post-12-hour 4℃ cold stress. To eliminate the influence of extreme values, we normalized the raw expression data via $\log_2(\text{FPKM} + 1)$ conversion, and subsequently generated heatmaps to visualize the expression profiles using TBtools 2.042 [24].

2.9 qRT-PCR Verification

Two-month-old healthy *Verbena bonariensis* plants were selected as experimental materials. Different organs (leaf, flower, and stem) were sequentially collected, placed into sterile, nuclease-free cryotubes, and immediately frozen in liquid nitrogen. These samples were then stored at −80°C for subsequent RNA extraction. Total RNA was isolated using the OMEGA Extraction Kit (OMEGA, Norwalk, CT, USA), and first-strand cDNA was synthesized via reverse transcription using the StarScript III RT Kit. Quantitative real-time PCR (qRT-PCR) reactions were performed on a qTOWER^{3}G Real-Time PCR System (Analytik Jena, Beijing, China) using 2× RealStar Fast SYBR qPCR Mix (GENStar, Beijing, China), with the *VbACTIN* gene serving as the internal reference. The 10 μL reaction system consisted of 1 μL of cDNA template, 0.5 μL of reverse primer, 0.5 μL of forward primer, 5 μL of 2× RealStar Fast SYBR qPCR Mix, and DEPC-treated water to a final volume of 10 μL. Three biological replicates and three technical replicates were set up for each sample. Additionally, primers were designed using NCBI Primers—blast and Primer Premier 5 software. The relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method [29], and results are expressed as mean ± standard deviation.

2.10 Subcellular Localization of VbTIFY16

The pCAMBIA1300-*VbTIFY16*-EGFP fusion expression vector was constructed using the seamless cloning method, and transformed into *Nicotiana benthamiana* leaves via Agrobacterium tumefaciens strain GV1301-mediated transient transformation. After 2 d of dark incubation, the subcellular localization of VbTIFY16 protein in tobacco leaf epidermal cells was observed using a laser scanning confocal microscope.

2.11 Plasmid Construction and Plant Transformation

The complete coding sequence of *VbTIFY16* was amplified via PCR and cloned into the pCAMBIA1301-35S vector using double restriction enzyme digestion, generating the overexpression construct pCAMBIA1301-35S-*VbTIFY16*. The constructed vector was transferred into *Agrobacterium tumefaciens* strain EHA105, followed by *Agrobacterium-mediated* transformation of *Nicotiana benthamiana* referring to the protocol described by Ning et al. [30]. As reported by Jefferson et al. [31], histochemical staining of β-glucuronidase (GUS) was employed to validate the identity of transgenic plants.

2.12 Phenotypic Analysis of Transgenic N. benthamiana under Normal Conditions

Both transgenic overexpression (OE) plants and wild-type (WT) control plants were maintained in a greenhouse culture system for comprehensive phenotypic characterization. At 65 days following transplantation, multiple growth-related phenotypic indices (plant height, leaf length, leaf width and leaf area) were collected and statistically analyzed; the floral traits (flower diameter and petal area) were assayed at the full blooming (FB) developmental stage. To characterize the cellular features of petals, petal tissue samples were harvested from OE and WT for sectioning, which was followed by morphological observation and size measurement of petal cells. The morphology of petal epidermal cells was observed and photographed using a microscope (Olympus, BX43, Tokyo, Japan) [32]. Cell length and area were determined using ImageJ software (NIH, Bethesda, MD, USA), and the total number of epidermal cells in petals was calculated by multiplying the average number of cells per 1 mm^2 by the total petal area.

3 Results

3.1 Identification and Physicochemical Property Analysis of VbTIFY

After comprehensive screening and identification, 16 *VbTIFY* genes were identified as from *VbTIFY1* to *VbTIFY16* (Table 1). The encoded proteins had amino acid lengths of 136–372 molecular weights of 15,075.11–40,696.34 Da, and theoretical pI values from 4.74 to 9.75. Thirteen out of 16 VbTIFYs had values higher than 7.0, indicating that most of the *V. bonariensis* TIFYs were alkaline proteins. Protein instability coefficients of VbTIFYs proteins ranged from 41.55 (*VbTIFY15*) to 78.85 (*VbTIFY3*), and the instability index was noticed higher than 40, which was a variable protein. The aliphatic index varied from 53.7 (*VbTIFY6*) to 82.76 (*VbTIFY13*). The overall mean of hydrophobicity ranged from −0.842 (*VbTIFY2*) to −0.007 (*VbTIFY9*), The average value of grand average of hydropathicity. Additionally, subcellular localization analysis suggested that all VbTIFY proteins are localized to the nucleus (Table 1). Among these, the nuclear localization of VbTIFY16 was further validated experimentally (see Section 3.8, Fig. 1).

Transmembrane domain analysis confirmed that among the 16 members of the *VbTIFY* family only *VbTIFY9* and *VbTIFY12* possessed a single transmembrane domain, while the remaining 15 members exhibited no transmembrane domain characteristics. Secondary structure predictions showed that all VbTIFY members had no β bridges, among which random coil was the most common secondary structure

in the amino acid sequence of *V. bonariensis* TIFY, accounting for 70.81% (*VbTIFY13*) to 85.95% (*VbTIFY9*), followed by α helix 8.75% to 21.08%, and extended chain 4.21% to 10.29% (Table 2).

Table 1: Physicochemical characteristics of TIFY proteins in *V. bonariensis*.

Gene Name	Gene ID	Amino Acids Number (aa)	Molecular Weight (Da)	Theoretical Pi	Instability Index	Aliphatic Index	GRAVY	Subcellular Localization (Predicted)
VbTIFY01	Vb2C062900.1	357	37,958.08	9.4	52.53	75.57	−0.264	Nucleus
VbTIFY02	Vb2C137200.1	261	28,552.85	9.59	70.91	64.21	−0.842	Nucleus
VbTIFY03	Vb2C166500.1	136	15,075.11	9.48	73.85	71.69	−0.581	Nucleus
VbTIFY04	Vb2C389800.1	357	39,227.4	8.37	61.24	69.16	−0.641	Nucleus
VbTIFY05	Vb3C120500.1	229	24,769.09	9.19	48.55	69.08	−0.464	Nucleus
VbTIFY06	Vb3C349600.1	362	38,426.21	9.03	53.7	53.7	−0.738	Nucleus
VbTIFY07	Vb4C322400.1	302	31,964.17	8.96	48.23	73.01	−0.268	Nucleus
VbTIFY08	Vb5C015400.1	229	24,735.21	9.52	52.16	74.24	−0.403	Nucleus
VbTIFY09	Vb5C074900.1	299	32,010.94	9.1	45.76	80.67	−0.007	Nucleus
VbTIFY10	Vb5C365300.1	372	40,696.34	4.94	45.56	69.97	−0.559	Nucleus
VbTIFY11	Vb6C003500.1	299	32,542.02	6.26	43.79	58.06	−0.763	Nucleus
VbTIFY12	Vb6C009600.1	285	31,101.09	9.33	47.5	73.61	−0.515	Nucleus
VbTIFY13	Vb6C097300.1	185	20,838.9	8.91	65.02	82.76	−0.43	Nucleus
VbTIFY14	Vb6C200900.1	271	29,171.24	9.75	58.56	71.96	−0.409	Nucleus
VbTIFY15	Vb7C301500.1	320	34,616.45	5.91	41.55	63.12	−0.681	Nucleus
VbTIFY16	Vb7C319400.1	166	18,489.27	9.75	56.82	67.71	−0.569	Nucleus

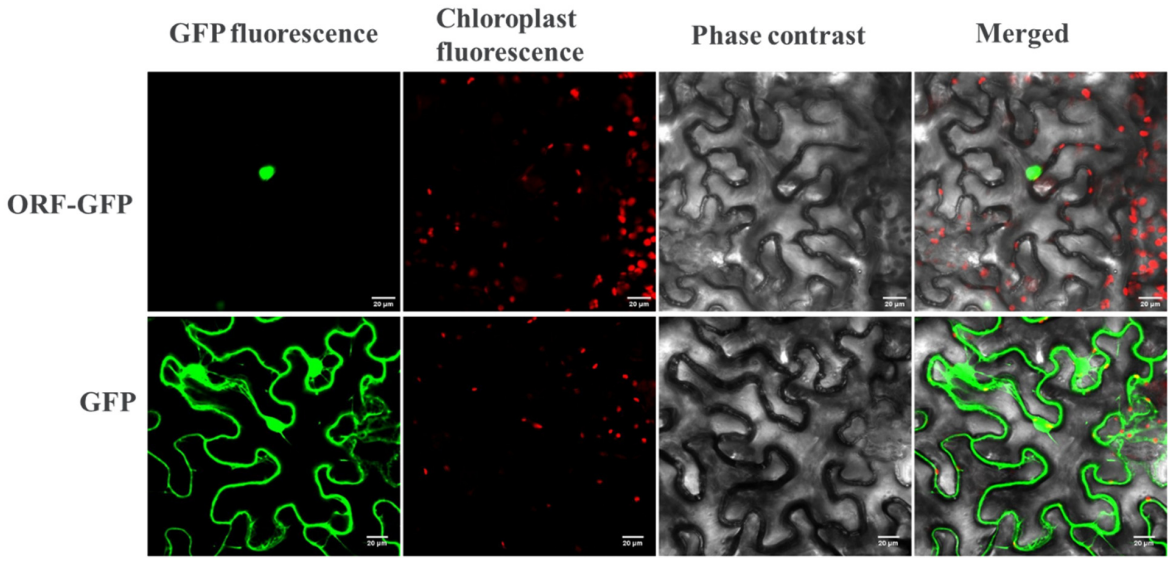

Figure 1: Subcellular localization of VbTIFY16.

Table 2: Secondary structure and *Subcellular Localization* of *TIFY* gene family proteins in *V.bonariensis*.

Protein	Alpha Helix (aa) (%)	Extended Strand (aa) (%)	Beta Bridge (aa) (%)	Random Coil (aa) (%)	Number of Transmembrane Domains
VbTIFY01	32 (8.96)	25 (7.00)	0 (0)	300 (84.03)	0
VbTIFY02	42 (16.09)	14 (5.36)	0 (0)	205 (78.54)	0
VbTIFY03	23 (16.91)	14 (10.29)	0 (0)	99 (72.79)	0
VbTIFY04	71 (19.89)	19 (5.32)	0 (0)	267 (74.79)	0
VbTIFY05	38 (16.59)	15 (6.55)	0 (0)	176 (76.86)	0

Table 2: *Cont.*

Protein	Alpha Helix (aa) (%)	Extended Strand (aa) (%)	Beta Bridge (aa) (%)	Random Coil (aa) (%)	Number of Transmembrane Domains
VbTIFY06	32 (8.84)	23 (6.35)	0 (0)	307 (84.81)	0
VbTIFY07	30 (9.93)	15 (4.97)	0 (0)	257 (85.10)	0
VbTIFY08	40 (17.47)	15 (6.55)	0 (0)	174 (75.98)	0
VbTIFY09	27 (9.03)	15 (5.02)	0 (0)	257 (85.95)	1
VbTIFY10	43 (11.56)	19 (5.11)	0 (0)	310 (83.33)	0
VbTIFY11	43 (14.38)	20 (6.69)	0 (0)	236 (78.93)	0
VbTIFY12	40 (14.04)	12 (4.21)	0 (0)	233 (81.75)	1
VbTIFY13	39 (21.08)	15 (8.11)	0 (0)	131 (70.81)	0
VbTIFY14	24 (8.86)	26 (9.59)	0 (0)	221 (81.55)	0
VbTIFY15	28 (8.75)	25 (7.81)	0 (0)	267 (83.44)	0
VbTIFY16	25 (15.06)	11 (6.63)	0 (0)	130 (78.31)	0

3.2 Structural Composition and Conservative Motif Analysis of TIFY Family Proteins in V. bonariensis

The results revealed the presence of four putative conserved domains within the VbTIFY family proteins: the TIFY domain, JAS motif domain, CCT domain, and GATA domain. All 16 VbTIFY proteins contain the TIFY domain, confirming their classification as members of the TIFY family. The CCT and GATA domains were exclusively identified in *VbTIFY10/11/15*, indicating that these three proteins are classified into the ZML subfamily. *VbTIFY06* contains only the TIFY domain, suggesting possible structural loss during evolution. The remaining 12 members all possess two conserved domains: TIFY and JAS (Fig. 2). A total of 10 conserved motifs were identified among the *TIFY* family members in *V. bonariensis* (Fig. 3). The number of conserved motifs varied among different VbTIFY proteins, ranging from 1 to 6. All *VbTIFY* members shared Motif 1, which corresponds to the TIFY domain (Fig. 2). The findings confirmed that the *VbTIFY* gene family has retained high evolutionary conservation. Further phylogenetic analysis indicated that proteins within a given clade displayed analogous domain organizations and conserved motif patterns, which suggests that genes with comparable motif compositions are likely to have related biological functions. Furthermore, Exon-intron structures and 5′/3′ UTR sequences of the 16 identified *VbTIFY* genes in *V. bonariensis* were systematically analyzed, which provides essential support for clarifying the structural heterogeneity of the *VbTIFY* gene family. The findings indicated that the number of exons varied significantly among different members, ranging from 2 to 10. Except for *VbTIFY06*, which contained 3 non-coding regions, the number of non-coding regions in other *VbTIFY* members ranged from 0 to 2, indicating that the *VbTIFY* family genes are relatively conserved during evolution.

3.3 Phylogenetic Tree and Chromosome Mapping of VbTIFY

To elucidate the evolutionary divergence of TIFY proteins in *V. bonariensis*, multiple sequence alignment was performed on 34 TIFY protein sequences, (16 from *V. bonariensis* and 18 from *A. thaliana*), followed by the construction of a phylogenetic tree using the neighbor-joining (NJ) method. (Fig. 4). Phylogenetic analysis results showed that the 34 TIFYs were divided into six independent groups, which were sequentially named Group 1 to Group 6. Members of the VbTIFYs family were mainly concentrated in Groups 1, 3, 5 and 6, while only one VbTIFY family member was contained in each of Group 2 and Group 4. Based on the structural characteristics of the TIFY domain, this gene family can be divided into four major subfamilies: JAZ, ZML, TIFY and PPD. Group 1 belongs to the ZML subfamily, containing three members including *VbTIFY10/11/15*, as well as three members derived from *A. thaliana*. Group 2 falls into the TIFY sub-family

and contains one member, *VbTIFY6*. Group 4 is assigned to the PPD sub-family and includes one member, *VbTIFY4*. The remaining three groups Group 3/5/6 belong to the JAZ subfamily and contain a total of 11 members. Furthermore, the phylogenetic tree analysis demonstrated tight clustering of TIFY proteins within the same group, a pattern consistent with the tree constructed using VbTIFY proteins in *V. bonariensis*. (Figs. 2 and 4).

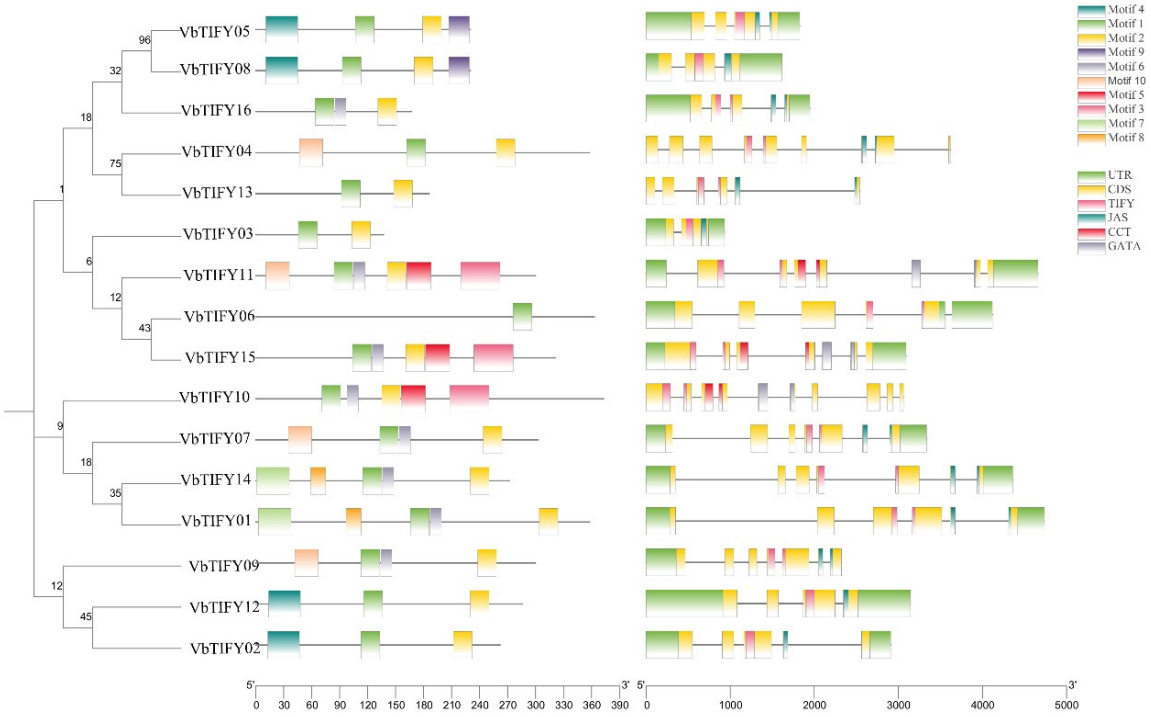

Figure 2: Conserved motif profiles, gene structural characteristics and evolutionary relationships of the *TIFY* gene family in *V. bonariensis*.

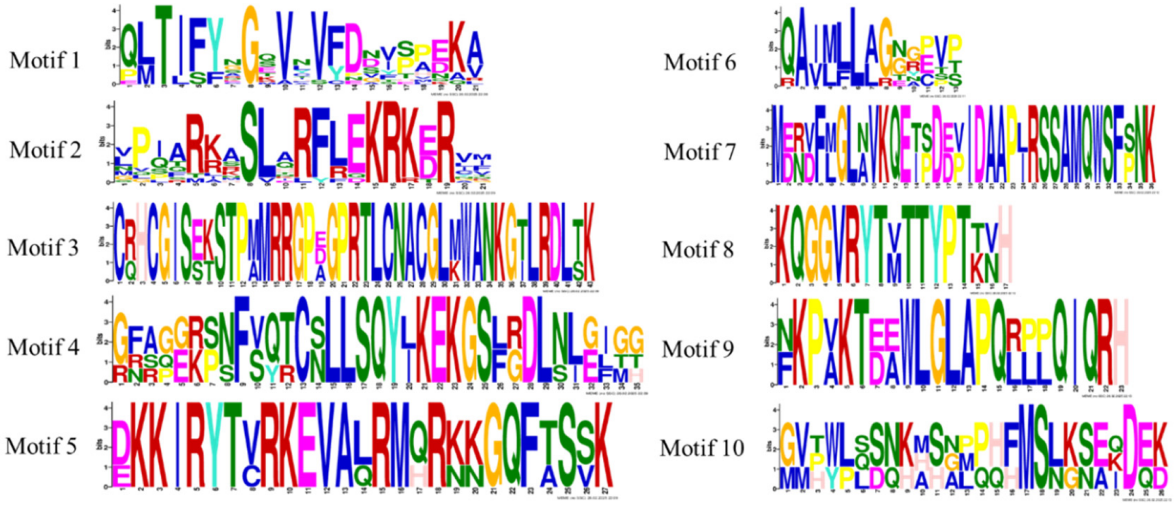

Figure 3: Conservative motif of *TIFY* gene family members in *V. bonariensis*.

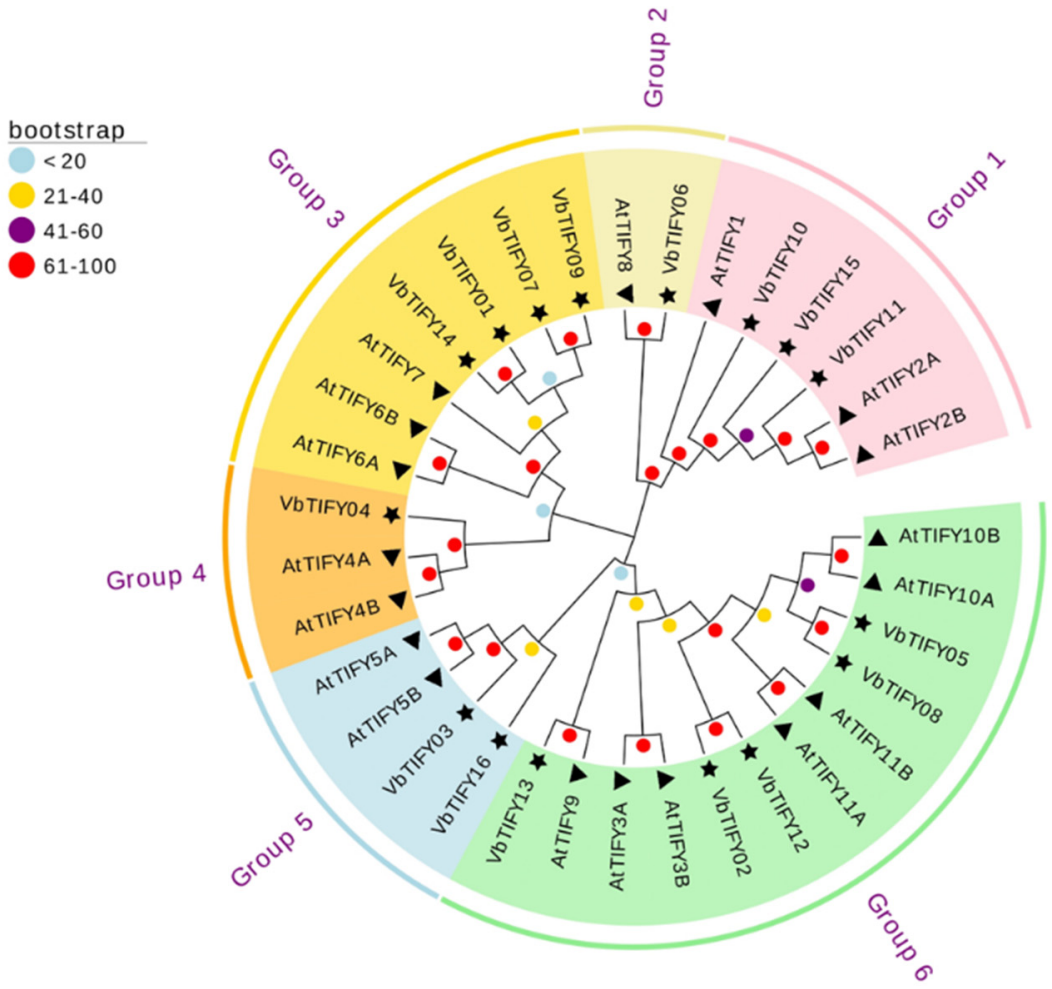

Figure 4: Phylogenetic tree of *TIFY* gene family members from *V. bonariensis* and *A. thaliana*.

The tree includes 16 VbTIFY proteins (red ★) and 18 AtTIFY proteins (black ●). The proteins are divided into six subgroups (Groups 1–6), corresponding to four subfamilies: ZML (Group 1), TIFY (Group 2), JAZ (Groups 1). Actually (Groups 3,5,6), and PPD (Group 4). The NJ tree was generated with 1000 bootstrap replicates.

16 *VbTIFY* genes were mapped to the chromosomes of *V. bonariensis* (Fig. 5). It is important to note that *V. bonariensis* is an autotetraploid species (4n), and the genome assembly used in this study represents a single subgenome (n). Therefore, the 16 VbTIFY genes identified here are expected to represent only a subset of the total TIFY complement, with additional copies potentially present in the three remaining subgenomes. All members were successfully located, with the distribution across chromosomes as follows: Chr.02 and Chr.06 contained the highest number of *VbTIFY* genes, each harboring four members, followed by Chr.05 with three members. Chr.03 and Chr.07 each carried two *VbTIFY* genes, while Chr.04 contained only one.

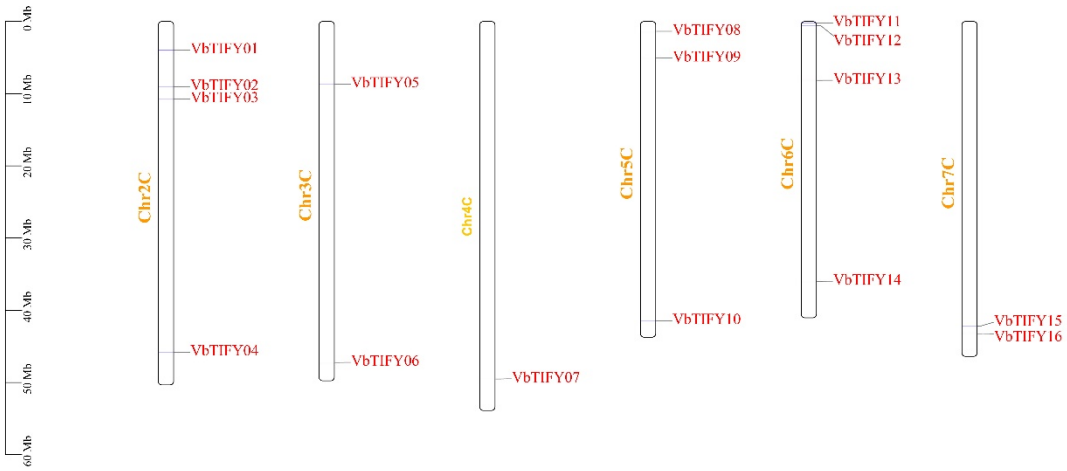

Figure 5: Chromosomal location of *TIFY* gene family members in *V. bonariensis*.

3.4 Colinearity Analysis of TIFY Gene Family Members in V. bonariensis

To investigate the evolutionary conservation of the TIFY gene family, collinearity analysis detected four homologous gene pairs (Red line in Fig. 6). As tabulated in Table 3, Ka/Ks ratios for these 4 pairs were all <1, demonstrating that the homologous genes evolved under negative selection (purifying selection pressure).

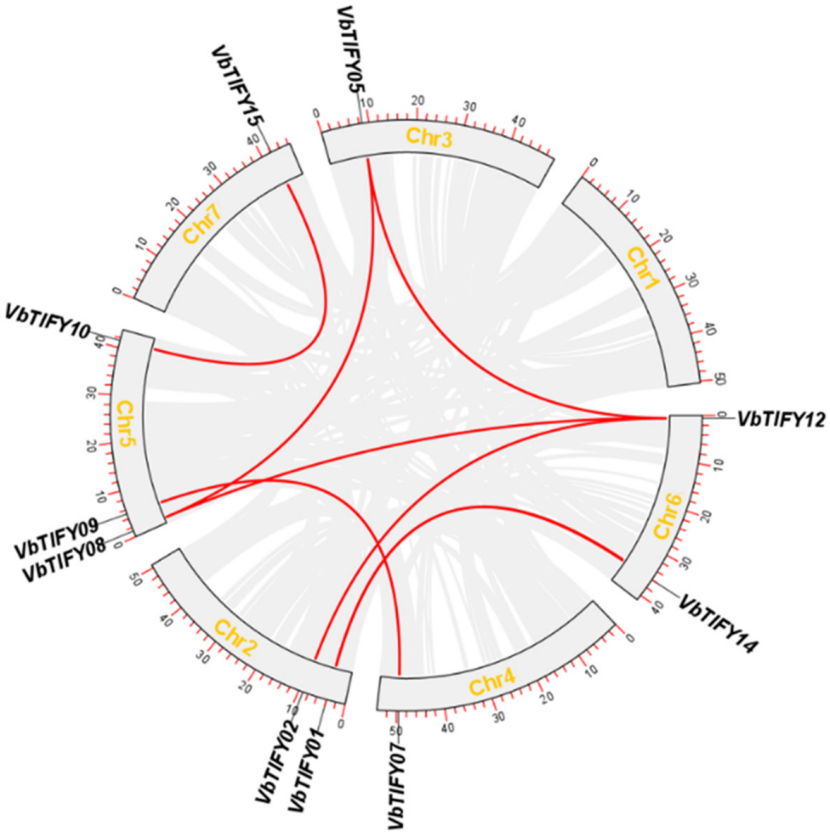

Figure 6: Colinearity relationship of *TIFY* gene family members in *V. bonariensis*.

Table 3: Ka and Ks values of highlight homologous gene pairs of *TIFY* gene family in *V. bonariensis*.

Gene ID		Ka	Ks	Ka/Ks
VbTIFY1	*VbTIFY14*	0.260123	0.832926	0.3123
VbTIFY2	*VbTIFY12*	0.281919	0.679076	0.415151
VbTIFY5	*VbTIFY8*	0.280921	1.267748	0.22159
VbTIFY7	*VbTIFY9*	0.349633	0.926227	0.377481

3.5 Analysis of Cis-Elements in the Promoter Regions of VbTIFY Genes

To elucidate the putative regulatory roles of the *TIFY* gene family in *V. bonariensis*, we screened the 2000 bp promoter regions upstream of the start codon for cis-acting regulatory elements (Fig. 7). The results revealed that *VbTIFY* genes harbor a diverse array of cis-elements, with light-responsive elements being the most prevalent (93), abscisic acid responsiveness (64), MeJA-responsiveness (28), anaerobic induction (35), low-temperature-responsive elements (10), auxin responsiveness (7), gibberellin responsiveness (8), among others. Specifically, 14 members contained abscisic acid-responsive cis-elements, apart from *VbTIFY6* and *VbTIFY13*. MeJA-responsive elements were identified in eight *VbTIFY* genes (*VbTIFY2/3/4/8/10/11/15/16*). Regarding other phytohormone-related cis-elements, for instance, *VbTIFY8* contained five types of auxin, gibberellin, salicylic acid, abscisic acid, and MeJA responsiveness. The presence of these cis-regulatory elements indicates that the *TIFY* gene may play a role in the cross-regulation of multiple hormones.

Regarding environmental response elements, all members carried multiple light-responsive cis-elements. Thirteen genes contained cis-elements essential for anaerobic induction, excluding *VbTIFY6*, *VbTIFY11*, and *VbTIFY13*. Additionally, six genes (*VbTIFY3/4/5/6/12/13*) contained defense and stress responsiveness elements; seven (*VbTIFY2/3/5/9/10/13/15*) contained low-temperature responsiveness elements; and nine (*VbTIFY2/3/4/6/7/11/13/14/15*) contained drought-inducibility elements. Furthermore, several genes carried tissue-specific or functionally specific cis-elements. For example, *VbTIFY4/6/10/15* contained elements associated with endosperm expression; *VbTIFY4/6/7/10/11* possessed elements related to meristem expression; and *VbTIFY8* contained a cis-acting element involved in seed-specific regulation (Fig. 7).

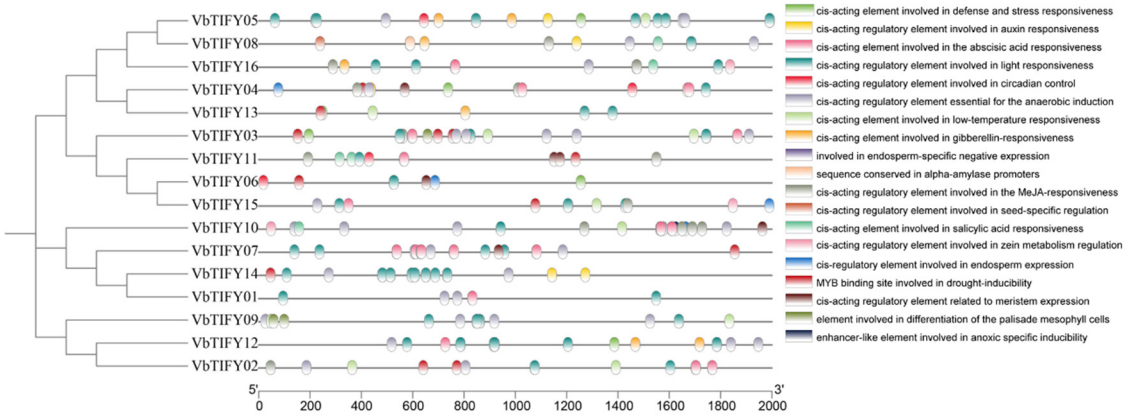

Figure 7: Cis-elements analysis in the promoters of *TIFY* genes in *V. bonariensis*. The 2000 bp promoter sequences of *VbTIFY* genes were used to analyze cis-elements.

3.6 Differences in the Expression of TIFY Gene Family Members in V. bonariensis

To further investigate the regulatory roles of *TIFY* gene family members in the growth and development of *V. bonariensis*, we calculated and visualized the transcriptomic FPKM values of 16 TIFY family members across three different tissues of *V. bonariensis* (Figs. 8 and 9). The results demonstrated that *VbTIFY* genes exhibited distinct expression profiles across different tissues of *V. bonariensis*. Specifically, 13 *VbTIFY* genes were expressed in all tissues, while three genes showed no or very low expression in most tissues. *VbTIFY07/01/08/03* were highly expressed in leaves; *VbTIFY05/09/11* exhibited higher expression in stems. Notably, *VbTIFY16* exhibited a marked upregulation in floral tissues relative to other plant organs, implying a potential role in flower development and function. Analysis of the expression patterns of *TIFY* gene family members in *V. bonariensis* under cold stress indicated that the expression levels of 12 members were significantly elevated after low-temperature treatment. Among them, two members (*VbTIFY14/15*) reached peak expression at 3 h; six members (*VbTIFY01/02/05/08/12/13*) peaked at 8 h; two members (*VbTIFY03/09*) peaked at 12 h; and two members (*VbTIFY07/16*) peaked at 24 h. In contrast, four members showed decreased expression levels. A series of abiotic stress-responsive cis-acting elements were detected in the promoter regions of 12 upregulated *VbTIFY* genes, which suggests that these members may play potential regulatory roles in plant responses to cold stress and the development of cold tolerance. Collectively, our results lay a solid theoretical foundation for subsequent research on deciphering the cold stress response mechanism in *V. bonariensis*.

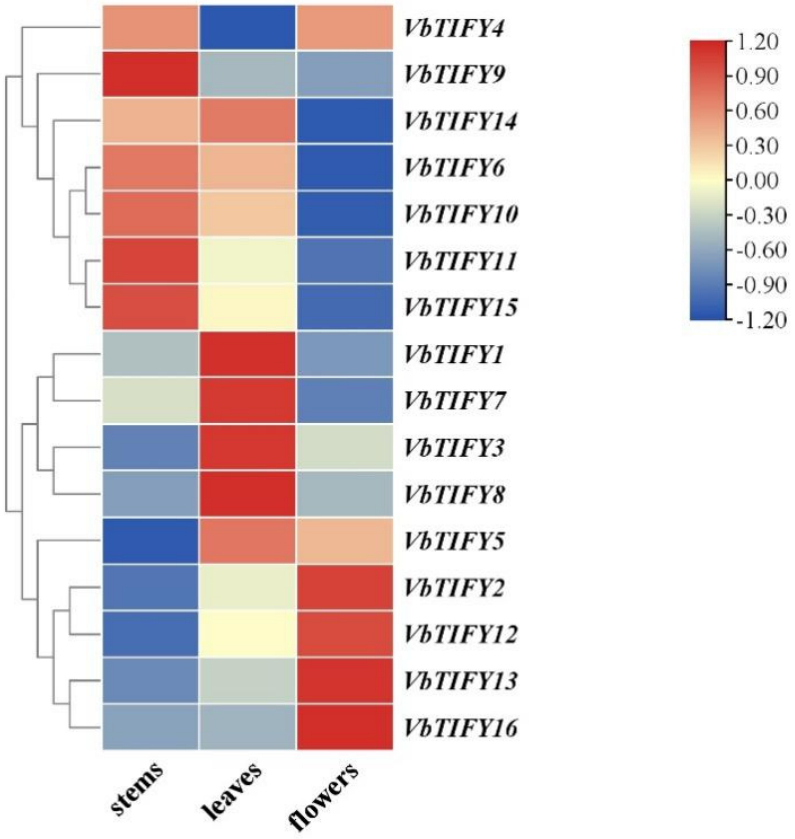

Figure 8: Tissue-specific expression of *TIFY* genes in *V. bonariensis*.

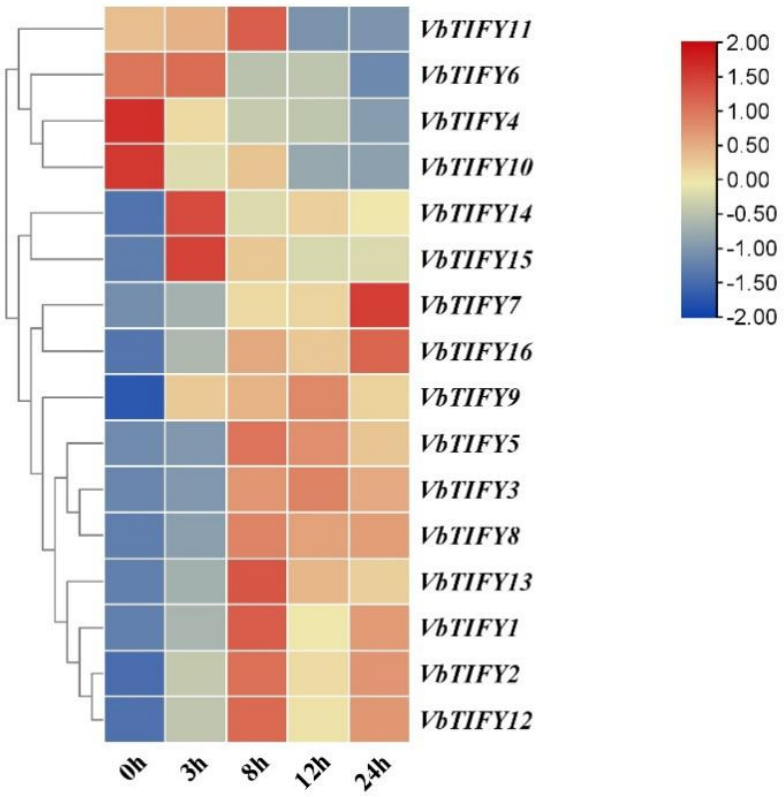

Figure 9: Expression heatmap of Cold stress responsive expression in *V. bonariensis*.

3.7 qPCR-Based Characterization of VbTIFY Gene Expression in Flower Stem and Leaf

To validate the RNA-seq results from different tissues of *V. bonariensis*, qRT-PCR analysis on six (*VbTIFY01, VbTIFY03, VbTIFY05, VbTIFY09, VbTIFY12,* and *VbTIFY16*) randomly selected genes from the RNA-seq dataset (Fig. 10) results demonstrated expression profiles consistent with RNA-seq analysis (see Fig. 8). Notably, *VbTIFY16* showed significant up-regulation in flowers, where its expression level was the highest among all the genes analyzed. Our findings indicated that *VbTIFY16* may play a critical regulatory part in the growth and development processes of *V. bonariensis* flowers.

3.8 Subcellular Localization of VbTIFY16 Protein

To investigate the subcellular localization of *VbTIFY16*, the empty vector pCAMBIA1300-EGFP and recombinant vector pCAMBIA1300-*VbTIFY16*-EGFP were transformed separately. After 2-3 days of low-light incubation, the subcellular distribution of the Protein-GFP fusion protein in the agroinfiltrated leaves was observed under a laser scanning confocal microscope (LSCM) with excitation at 488 nm. The results showed that strong green fluorescent signals of *VbTIFY16*-EGFP were detected in the nucleus (Fig. 1), indicating that VbTIFY16 is localized to the nucleus.

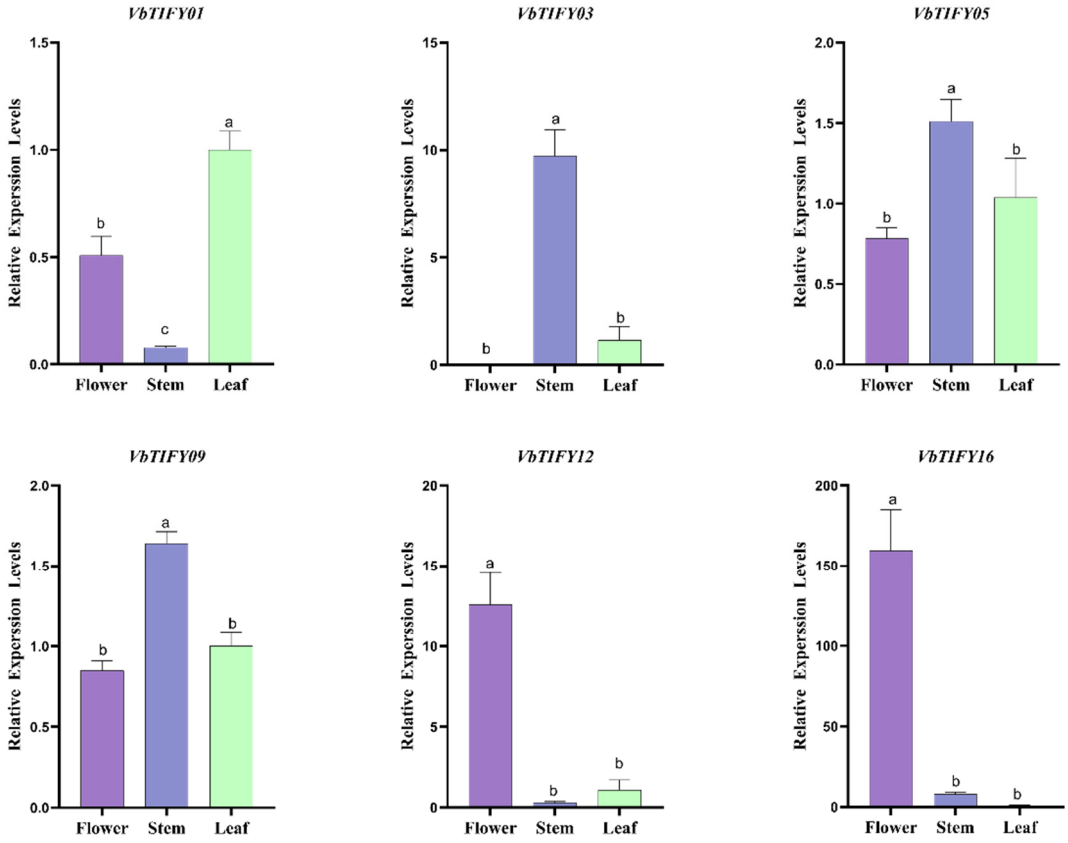

Figure 10: Expression profiling of six *VbTIFY* genes in flowers, stems, and leaves of *V. bonariensis*. Data are presented as mean ± SD, with three biological replicates (independent individuals) and three technical replicates performed in qRT-PCR assays. Distinct lowercase letters denote significant differences as determined by the LSD test ($\alpha = 0.05$).

3.9 Overexpression of VbTIFY16 Negatively Regulates Petal Size in Transgenic Nicotiana Benthamiana

To further elucidate the function of *VbTIFY16*, the 35S: VbTIFY16-GUS construct was introduced into tobacco, leading to the generation of three independent transgenic lines. Observation results showed that at the full petal expansion stage, the *VbTIFY16* OE plants exhibited a significantly reduced inflorescence diameter and shorter petals, with their average petal length being 20.3% lower than that of the wild-type (WT) (Fig. 11C,F,G).

To explore the reasons for shorter petals, sections of petals from both transgenic plants and WT plants were prepared following the method described by Ren, and cytological observations were carried out [32] (Fig. 11D). Morphological observation and quantitative measurement of petal epidermal cells revealed that the cell diameter and cell area of *VbTIFY16*-OE petals were reduced by 20.54% and 40.19% compared with those of WT petals, respectively (Fig. 11I,J). Meanwhile, the total number of epidermal cells in petals showed no significant difference (Fig. 11H). Therefore, we infer that the inhibition of petal growth in *VbTIFY16*-OE plants is mainly attributed to the impaired cell expansion. In addition, the overexpression of *VbTIFY16* transcription factor significantly affected plant growth potential at the vegetative growth stage. Compared with WT plants, *VbTIFY16*-OE plants exhibited a shorter height phenotype with smaller leaves (Fig. 11). Further statistical analysis confirmed that the plant height, leaf length and leaf width of the overexpression plants were all significantly decreased (Figs. 11 and 12).

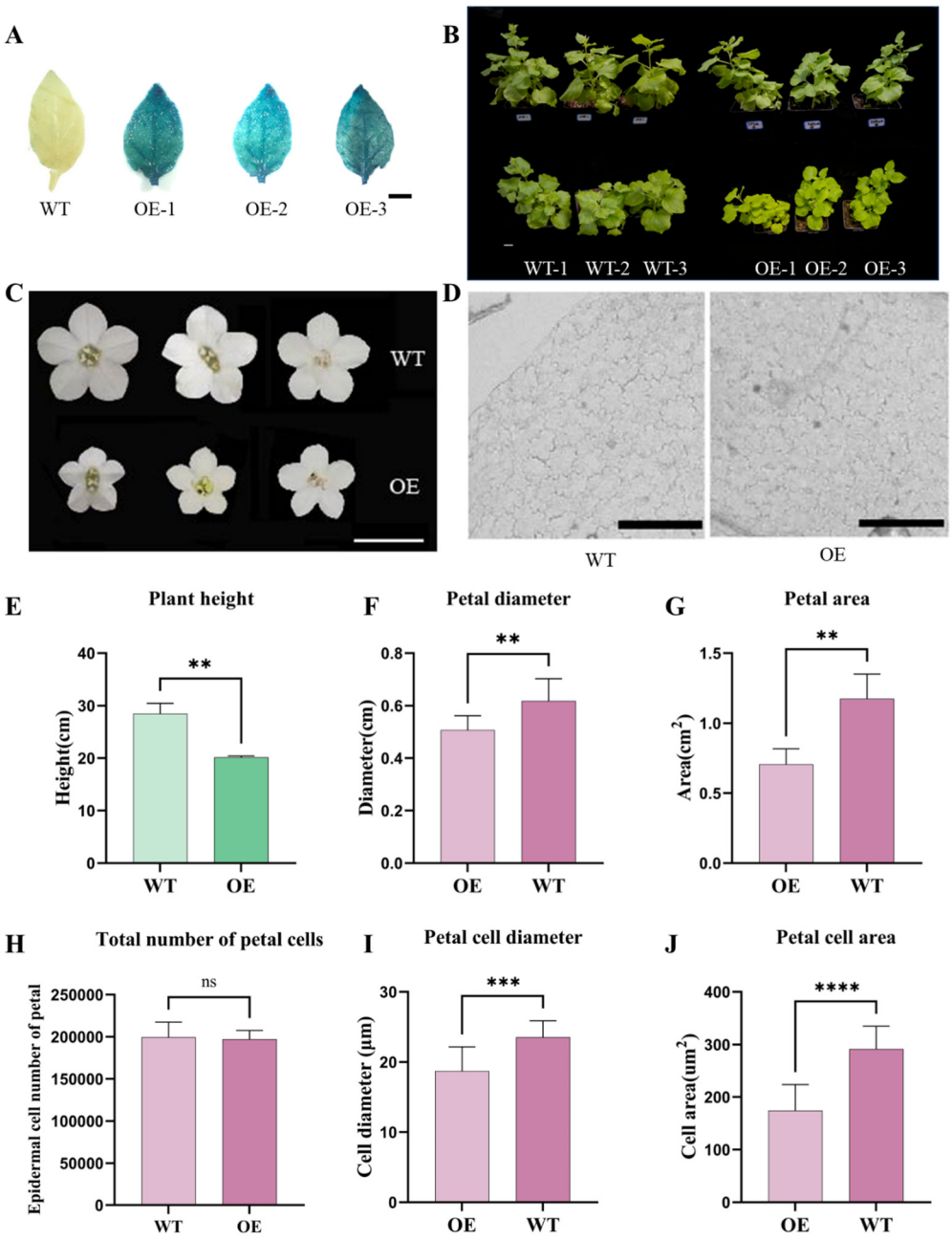

Figure 11: Phenotypic characterization of *VbTIFY16* overexpressing transgenic *Nicotiana Benthamiana.* (**A**) Gus validation of transgenic tobacco (Bar:1 cm) (**B**) Phenotype of WTand *VbTIFY16*-OE transgenic plant in a greenhouse, Bar: 3 cm. (**C**) Petal Comparison Between WT and *VbTIFY16*-OE Plants. Bars: 1 cm. (**D**) The cells in WT and *VbTIFY16*-OE transgenic plants observed using optical microscope (40×). Bars: 50 μm. (**E**) WT and *VbTIFY16*-OE plant heights were measured at 65 days post-transplant. (**F**,**G**) WT and *VbTIFY16*-OE petal length and area: measured at the FB stage. (**H**,**I**) Cell length (**H**) and area (**I**) analysis in WT and *VbTIFY16*-OE petal regions at FB stage. (**J**) Statistical analysis of total number of petal cell in WT and *VbTIFY16*-OE plant. Three independent transgenic lines (OE-1, OE-2, OE-3) were obtained; representative images of each line are shown in (**B**–**D**). (**E**–**J**) Data are means of all three independent lines. (n ≥ 9 plants per line, mean ± SD). All significant differences were determined by Student's *t* test (ns: not significant ($p \geq 0.05$), $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

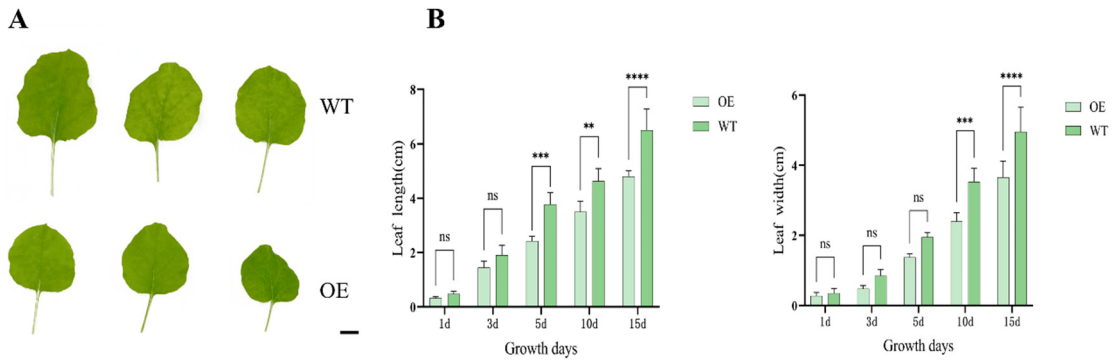

Figure 12: Plant architecture phenotype of *VbTIFY16* overexpression transgenic plants at the vegetative stage. (**A**) Leaf Phenotypes of WT and *VbTIFY16*-OE Transgenic Plants Bars: 1 cm. (**B**) WT and *VbTIFY16*-OE leaf length and width were measured. Data are means of all three independent lines (n ≥ 9 plants per line, mean ± SD). Significant differences were determined by Student's *t* test (ns: not significant ($p \geq 0.05$), $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

4 Discussion

The *TIFY* gene family members (particularly JAZ proteins) represent a class of core regulators induced by jasmonic acid in plants. They typically form regulatory complexes with other proteins or transcription factors, playing pivotal roles in plant growth and development, metabolic synthesis, and responses to environmental stresses [33]. As scientific research continues to advance, the practical application scope of the *TIFY* gene family is anticipated to broaden considerably. As genome sequencing projects for various species reach completion, comprehensive identification of the *TIFY* gene family has been achieved in numerous plant lineages, including 18 in the *A. thalian* [4] and 21 in *Actinidia chinensis* [34]. This study identified 16 *VbTIFY* members from *V. bonariensis*, revealing significant interspecific divergence in the composition of the *TIFY* gene family. Notably, research on the *TIFY* gene family in the *V. bonariensis* genome remains largely unexplored, and their functional roles have not yet been elucidated. Therefore, systematic analysis of the *VbTIFY* gene family will advance our understanding of the specific biological functions of these genes.

The coding sequence lengths of the 16 identified TIFY proteins showed substantial variation, spanning from 136–372 bp. Their relative molecular masses fell within the range of 15,075.11–40,696.34 Da, while the theoretical isoelectric points (pI) of these proteins varied between 4.74–9.75. All members of the *VbTIFY* family were categorized as hydrophilic proteins, a finding that aligns with previous reports on *Lycium ruthenicum* [35]. Furthermore, they were predominantly localized in the nucleus, a result consistent with that observed in birch [36]. Collectively, these data imply that VbTIFY proteins may exert their biological functions mainly through transcriptional regulation in the nucleus.

Based on the constructed phylogenetic tree, the majority of *VbTIFY* genes clustered in a specific clade displayed consistent exon-intron structural patterns and conserved motif profiles, even though the lengths of some internal introns differed considerably. This phenomenon indicates that the intron organization of homologous genes tends to be evolutionarily conserved—and notably, these structural differences also provide a basis for TIFY family classification while implying potential functional divergence among its members.

In addition to structural observation of the phylogenetic tree, a joint phylogenetic tree of *V. bonariensis* and *A. thaliana* was constructed further. All *TIFY* genes were classified into six groups: Group I contained *AtTIFY1/2A/2B* and *VbTIFY10/11/15*, belonging to the ZML subfamily with potential roles in hypocotyl and petiole elongation [37]. Group 2 comprises only *AtTIFY8* and *VbTIFY6*, which may act as repressors of leaf

senescence [38]. Group 3 includes *AtTIFY6A/6B/7* and *VbTIFY7/9/14*, while Group 4 contains *AtTIFY4A/4B* and *VbTIFY4*, members of the PPD subfamily that regulate lamina size and curvature development [39]. Groups 5 and 6 encompass *AtTIFY3B/5A/5B/8A/10A/10B/11A/11B* and *VbTIFY2/3/5/8/12/13/16;* studies on these genes suggest their potential roles in plant defense, growth, and development [40]. Most importantly, the phylogenetic tree analysis provides valuable insight into the functional characterization of different *VbTIFY* genes.

As key regulatory elements governing gene transcription and expression, cis-acting elements are commonly used to predict the potential functions of gene [41]. In this study, a variety of cis-regulatory elements were identified in the promoter regions of *VbTIFY* genes, suggesting that these genes have the activation potential to respond to diverse stress signals. For instance, numerous light-responsive cis-acting elements were identified in the promoters of all members of this gene family, suggesting that the expression of *VbTIFY* genes is modulated by light signals and thus involved in regulating the growth and developmental processes of *V. bonariensis*.

Notably, most *VbTIFY* genes contain both stress- and hormone-related regulatory elements. Cis-element analysis implies that these genes not only help *V. bonariensis* tolerate diverse stresses, but also function as central regulators in plant growth. Existing research indicates that multiple *TIFY* family genes can be induced by abiotic stresses. For example, *OsJAZ* genes are responsive to various abiotic stresses such as drought, cold and salinity, whereas *OsTIFY11a* improves the stress resistance of rice [42]. *GsTIFY10a* is likely to exert a positive function in mediating plant salt tolerance [43]. This study identified *TIFY* genes involved in the low-temperature response in *V. bonariensis*, with 12 *TIFY* genes being upregulated. These findings suggest that these genes may play an active regulatory role in the abiotic stress response of *V. bonariensis*, providing important insights for the subsequent identification of resistance genes in this species.

Members of the TIFY family are involved in the development of plant floral organs. For instance, the PnFL-2 protein of morning glory (*Pharbitis nil*) possesses the conserved TIFY and CCT domains typical of the *TIFY* gene family. Research findings have shown that constitutive overexpression of PnFL-2 in transgenic plants results in a subtle advancement of flowering time under long-day photoperiods, as compared to their wild-type counterparts. Such results suggest that PnFL-2 could be involved in mediating floral induction [44]. Heterologous overexpression of *Saccharum officinarum ScJAZ2* causes early flowering in transgenic *Arabidopsis* [45]. The flowering process and floral organ size are co-determined by cell proliferation and cell expansion. Guan et al. [46] investigated the expression of 13 *TIFY* genes in *Phalaenopsis aphrodite* and found that eight genes showed relatively high expression during the bud stage, with expression gradually decreasing after flower opening. These genes may promote flower opening by regulating cell growth within the buds. In this study, six *VbTIFY* genes (*VbTIFY1/3/5/9/12/16*) were selected, and their expression in three tissues of *V. bonariensis* was validated using qRT-PCR. The results demonstrated that two genes, *VbTIFY12* and *VbTIFY16*, exhibited the highest expression levels in floral tissues, with the expression abundance of *VbTIFY16* being 12.7-fold higher than that of *VbTIFY12*. Phenotypic analysis revealed that both petal size and leaf area of *VbTIFY16*-OE plants were significantly smaller than those of WT plants. Further cytological observations indicated that the petal cell diameter and cell area in *VbTIFY16*-OE lines were lower than those in WT plants. Meanwhile, there was no significant difference in the total number of epidermal cells at the petal base, which suggested that the inhibition of petal size was mainly caused by the reduction of cell expansion. Based on the above findings, we speculate that *VbTIFY16* may participate in the floral development of *V. bonariensis* by regulating cell expansion and growth in floral tissues.

Although this study provides a preliminary bioinformatic analysis of the *TIFY* gene family in a single genome of *V. bonariensis*, certain limitations remain: Firstly, given that *V. bonariensis* is an autotetraploid

species (4n), this study focused exclusively on the analysis of *TIFY* genes within a single subgenome (n) and did not include members from the remaining three subgenomes. Consequently, these findings only reflect the characteristics of this gene family within a partial genomic context, and cannot fully elucidate its overall evolutionary patterns in the tetraploid background, resulting in an incomplete characterization of the complete gene repertoire of the *TIFY* family. Future studies must integrate multi-subgenome data to fully characterize the TIFY family and understand its evolutionary dynamics in this polyploid species. Until such data are available, the conclusions drawn here, particularly regarding gene counts, phylogenetic relationships, and functional predictions, should be interpreted with this genomic context in mind.

Secondly, the depth of functional mechanism analysis remains limited. Although transcriptome profiling, qRT-PCR, and tobacco overexpression experiments confirmed that *VbTIFY16* is most highly expressed in flowers and may lead to reduced petal size. While our overexpression data support a negative regulatory role for *VbTIFY16* in petal size, loss-of-function validation is required to confirm this conclusion. We propose that VIGS (Virus-Induced Gene Silencing)-mediated transient knockdown of *VbTIFY16* in *V. bonariensis* petals represents a feasible next step, as it would enable functional validation in the native species without the need for stable transformation. Such an experiment would predict that *VbTIFY16*-silenced flowers exhibit enlarged petals with increased cell area, directly complementing our overexpression findings. Furthermore, future studies will integrate multi-subgenome data to improve the systematic characterization of the TIFY gene family, and combine protein interaction assays with stable loss-of-function analyses via CRISPR/Cas9 and homologous transgenic systems, aiming to thoroughly reveal the molecular regulatory pathway of *VbTIFY16* during petal development. As a result, the current understanding of its molecular regulatory network is still limited to phenotypic correlations, without thorough dissection at the pathway level. Integrating multi-subgenome data in future investigations will enable a more complete characterization of the entire *TIFY* gene family.

Moreover, mechanistic understanding can be further enhanced through systematic functional experiments, including protein-protein interaction assays to establish regulatory networks and gene editing to verify physiological functions in the endogenous *V. bonariensis* background (rather than in heterologous hosts such as tobacco). These methodological approaches will not only strengthen the reliability of our experimental conclusions but also establish a more holistic analytical framework for deciphering the molecular mechanisms by which the *TIFY* family regulates floral growth and development in polyploid plants.

5 Conclusion

This study provides a comprehensive and integrative analysis of the *VbTIFY* gene family in *V. bonariensis*, uncovering 16 family members. The investigation encompasses gene structural organization, conserve motif/domain analysis, phylogenetic inference, chromosomal localization, and cis-element profiling. Results highlighted the structural conservation of the *VbTIFY* family, which is associated with diverse growth/developmental processes. Expression pattern assays across tissues and cold stress treatments indicated that *VbTIFY* genes are involved in developmental modulation and low-temperature stress tolerance. Notably, six genes (*VbTIFY01*, *VbTIFY03*, *VbTIFY05*, *VbTIFY09*, *VbTIFY12*, and *VbTIFY16*) exerts a positive regulatory effect on different tissues during plant growth and development. Functional validation via *VbTIFY16*-OE in *N. benthamiana* showed negative regulation of petal and leaf size. It is expected that the results of this study can be to further clarify the functional characteristics of *TIFY* family genes in *V. bonariensis* and to use *TIFY* genes to improve the quality of *V. bonariensis* overall.

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Author Contributions: Yan Li designed experiments; Yuan Chen, Hanfei Li and Ju Cai conducted experimental data analysis; Sumeera Asghar and Yuan Chen edited the manuscript; Hanfei Li and Yin You contributed reagents/materials/analytical tools; Yan Li, Sumeera Asghar and Yuan Chen revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The complete genome sequence assembly of *Verbena bonariensis* generated in this research has been submitted to the Genome Warehouse (GWH) repository at the National Genomics Data Center (NGDC, Chinese Academy of Sciences/China National Center for Bioinformation, Beijing) under accession number GWHFWGI00000000.1 (associated BioProject: PRJCA027875). The dataset is freely accessible to the public through the link: https://ngdc.cncb.ac.cn/gwh. For the raw RNA-seq data covering tissue-specific transcriptomes and cold stress response experiments, researchers may request access directly from the corresponding author with a justified application.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

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