



Comprehensive expression analysis of ERF transcription factors during chilling acclimation in Saintpaulia

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Abstract

Saintpaulia (*Saintpaulia ionantha*), a popular indoor ornamental potted plant, is native to the highlands of Kenya and Tanzania where temperatures rarely fall below 4 °C. Chilling injury during cultivation and transportation is a major commercial problem in Saintpaulia. In this study, we investigated chilling acclimation in Saintpaulia ‘Kilauea’. Plants grown at 20 °C (14 h light/10 h dark) displayed rapid and severe chilling injury after 24-h exposure to 4 °C. However, chilling injury at 4 °C could be dramatically reduced by pre-treating the plants at 10 °C but not at 6 °C. From whole genome analysis, 161 ethylene-responsive factors (ERFs) were identified and classified into 12 clades according to existing reports. Among these ERFs, 43, 8, and 4 ERFs were upregulated at 12, 24, and 48 h after 10 °C treatment, respectively. Most of these ERFs had GCC box and/or DRE/CRT core motifs-like sequences in their upstream regions. Finally, we compared the expression of ERFs between the treatments for 24 h at 10 °C, an effective temperature for chilling acclimation, and 6 °C, an ineffective temperature. The results showed that the expression of all six ERFs we investigated was increased by the 10 °C treatment, but not or only barely increased by the 6 °C treatment. This study suggests that Saintpaulia, a subtropical plant, can acclimate to low temperatures and that ERF upregulation is involved in chilling acclimation.

Keywords Chilling stress · Chilling acclimation · Saintpaulia · ERF · CBF/DREB

Introduction

Saintpaulia or African violet (*Saintpaulia ionantha*) is an ornamental plant that belongs to the Gesneriaceae family. It is popular as a potted flower for indoor use because of its high shade tolerance and year-round flowering under low light intensity. Saintpaulia is native to Kenya and Tanzania, where temperatures are relatively stable. Therefore, they

are sensitive to temperature change (Johansson 1978; Baatvik 1993). When exposed to low temperatures, Saintpaulia leaves turn brown and visible damage occurs even within a short period of time (approximately 12 h) (Larcher and Bodner 1980). Sensitivity to low temperatures is a major problem in the transport of Saintpaulia (Chen and Henny, 2009). For chilling sensitive plants, chilling is a significant cause of damage in production, transportation, and interior design (Chen et al. 2004). For this reason, it is necessary to take extreme care during the cultivation and shipment of Saintpaulia under low temperatures during the winter season.

Chilling injuries are caused by non-freezing temperatures (Levitt 1980). Most studies on cold acclimation have investigated responses to freezing stress. Cold acclimation occurs when plants are exposed to suboptimal low temperatures, which do not cause plant death, but enhance their cold tolerance (Levitt 1980; Gilmour et al. 1988; Thomashow 1999). Research has been conducted using model plants native to temperate regions, such as *Arabidopsis thaliana*, to investigate cold tolerance and acclimation. *A. thaliana* is dead at

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freezing temperatures (around -3°C), but exposure to 4°C for some time improves its survival rate at -9°C (Gilmour et al. 1988). Many plants native to tropical and subtropical regions, including Saintpaulia, suffer from chilling injury or die at non-freezing temperatures ($\sim 10^{\circ}\text{C}$). If chilling acclimation is also possible in Saintpaulia, it would allow for safe winter cultivation and commercial transportation.

Extensive genetic studies of cold acclimation have been conducted in *A. thaliana* (Thomashow 1990; Xin and Browse 2000; Yamaguchi-Shinozaki and Shinozaki 2006). The C-repeat binding factor (CBF)/Dehydration responsive element binding (DREB)1 transcription factors, which belong to the APETALA2/ethylene-responsive factor (AP2/ERF) family, are the most well-known genes that are upregulated during cold acclimation and are known to improve cold tolerance. *AtCBF1*, *AtCBF2*, and *AtCBF3* of *A. thaliana* (also known as *AtDREB1B*, *AtDREB1C*, and *AtDREB1A*, respectively) bind to the CRT/DRE element in the promoter region of downstream genes, such as cold-regulated (COR) genes, and contribute to the improvement of cold tolerance by regulating their transcription (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Gilmour et al. 2000, 2004; Yamaguchi-Shinozaki and Shinozaki 2006). *AtCBF2* inhibits *AtCBF1/3*, and cold tolerance is improved in *cbf2* mutants of *A. thaliana*, indicating a complex regulatory mechanism (Novillo et al. 2004). CBF/DREB1 improves cold tolerance not only in *A. thaliana* but also in various other plants, such as rice (*Oryza sativa*), maize (*Zea mays*), and tomato (*Solanum lycopersicum*) (Dubouzet et al. 2003; Qin et al. 2004; Zhang et al. 2004). In addition, several ERFs other than CBF/DREB1 have been reported to contribute to the improvement of cold tolerance in diverse plant species, such as *AtERF102/103/104/105* (*A. thaliana*), *PtrERF9/108/109* (*Poncirus trifoliata*), *MdERF1B*, *MdABI4* (*Malus × domestica*), and *VaERF057, 092* (*Vitis amurensis*) (Sun et al. 2016, 2019; Bolt et al. 2017; Wang et al. 2019, 2021; Illgen et al. 2020; Khan et al. 2021; An et al. 2022; Zhang et al. 2022). Although various transcription factors other than ERFs are involved in cold acclimation, ERF transcription factors play a particularly central role (Guo et al. 2022).

Few studies have been conducted on chilling acclimation of plants native to tropical and subtropical regions such as Saintpaulia. There are also a few reports on whether ERF is involved in the chilling tolerance of subtropical and tropical native plants. In this study, two questions were investigated. First, we investigated whether chilling acclimation occurred in Saintpaulia. Second, we conducted the comprehensive expression analysis of ERFs based on the whole genome sequence and transcriptome analysis during sublethal temperature exposure in Saintpaulia. Finally, we discussed the involvement of ERFs in chilling acclimation of Saintpaulia.

Materials and methods

Plant materials

To eliminate the influence of genetic background on chilling tolerance, adventitious shoots obtained by cutting the leaves of *S. ionantha* ‘Kilauea’ were used in the experiment. After three months of growth under the following conditions, plants with 15–20 leaves were used for all experiments. Plants were grown under conventional conditions at 20°C , 14 h light/10 h dark, using plant growth fluorescent lamps (BioLux-A, 20W, HotaluX, PPFD $82.5\ \mu\text{mol cm}^{-2}\text{s}^{-1}$). Plants were potted in 220-ml plastic pots with Metro-Mix 360 soil (Sun Gro Horticulture, Massachusetts, United States). Temperature data in this study were consistently measured using a data logger (Thermo Recorder Ondotori Jr. TR-50U2, T&D, Japan) and carefully kept within $20 \pm 1^{\circ}\text{C}$.

Identification of chilling injury-causing temperatures

Depending on the environment and cultivar, the temperature at which Saintpaulia exhibits chilling injury varies slightly (Yang et al. 2003). To identify that temperature in this study, Saintpaulia grown at 20°C were transferred to an incubator (MIR-553, SANYO, Japan), and varying temperature treatments were conducted for 24 h (12 h light/12 h dark). The treatment temperatures were 4, 6, 8, 10, 12, 16, and 20°C . Subsequently, electrolyte leakage was measured as an indicator of chilling injury as described below.

The fifth and sixth leaves (5–6 cm in diameter) from the youngest expanded leaves were collected from each plant, and three leaf disks were obtained per leaf using a biopsy punch (Kai Industries, Gifu, Japan. BP-60F. $\phi 6\text{mm}$). The leaf disks were washed with distilled water for 10 s to remove the electrolyte from their cut surface. The disks were then soaked in 3 ml distilled water. To release electrolytes from damaged cells into the water, the disks were shaken at 80 rpm at 20°C in the dark for 24 h. Next, the electrical conductivity (EC) of the water was measured using a compact conductivity meter, LAQUAtwin (HORIBA, Kyoto, Japan). The samples were then autoclaved to destroy all the cells and let the electrolytes leak into the solution. After that, the EC of the solution was measured. Electrolyte leakage was calculated as the ratio of EC before autoclaving to EC after autoclaving and expressed as a percentage. The electrolyte leakage rates were measured in the same manner for all the experiments.

Next, Saintpaulia were maintained at 4°C , which is a stable temperature for chilling injury to occur (described

in the results section), in the dark to mimic transportation conditions. Chilling treatment at 4 °C was conducted for 1, 6, 12, 18, and 24 h. Then, the electrolyte leakage of the leaves was measured to identify when chilling injury occurred.

Investigation of chilling acclimation conditions

To investigate the suitable temperature for chilling acclimation in *S. ionantha* ‘Kilauea’, the plants were placed at 6, 8, 10, 12, 14, 16, 18, and 20 °C for 24 h followed by 24 h treatment at 4 °C. After the treatments, the electrolyte leakage of the leaves was measured, and the best acclimation-inducing temperature was identified.

From the result of the experiment, 10 °C was identified as the acclimation-inducing temperature (described in the results section). To investigate the optimal time for chilling acclimation at 10 °C, plants were placed in a 10 °C incubator for different durations. After 12, 24, 48, 72, and 144 h, plants were exposed to chilling treatment at 4 °C for 24 h. Then, the electrolyte leakage of the leaves was measured. Plant images were captured before and after chilling treatment at 4 °C. The survival rate was measured 20 days after chilling treatment, with or without pretreatment at 10 °C for 144 h.

Genome sequencing, assembly, gene prediction, and functional annotation

Genomic DNA was extracted from young leaves of *S. ionantha* ‘Kilauea’ with the CTAB (Cetyl trimethyl ammonium bromide) method (Dempster et al. 1999). A long-read sequence library was constructed using the SMRTbell Express Template Prep Kit 1.0 (PacBio, Menlo Park, CA, USA) and sequenced on SMRT cells (1 M v3) in a PacBio Sequel system. A short-read library was prepared using the TruSeq Nano DNA Sample Prep Kit (Illumina, San Diego, CA, USA) and sequenced on a MiSeq (Illumina) instrument in paired-end, 301-bp mode.

The Sequel long-reads were assembled by FALCON v1.2.5 (Chin et al. 2016), and the resultant primary contigs and associated contigs were applied to FALCON-Unzip v1.1.5 (Chin et al. 2016) for phasing. The primary contigs were polished by Arrow (<https://github.com/PacificBiosciences/GenomicConsensus>) using Sequel long-reads, and further polished by Pilon (Walker et al. 2014) using Illumina paired-end reads.

Ab initio gene prediction was conducted on the polished primary contigs using BRAKER2 v2.1.5 (Hoff et al. 2019). The quality trimming of RNA-seq reads sampled from petal (1_blue; DRR462688, Kira_P-petal; DRR462690, Kira_Str-Pin; DRR462691, Kira_Str-Pout; DRR462692) and leaf (Kira_P-leaf; DRR462689) samples was performed by PRINSEQ v0.20.4 (Schmieder and Edwards 2011), and

adaptor trimming was further performed by FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit). The trimmed reads were mapped onto the polished primary contigs by HISAT2 v2.2.0 (Kim et al. 2019), and the bam files were used for BRAKER2 as a training set. Similarity searches against the predicted genes were conducted against UniProtKB (The UniProt Consortium 2023), NCBI’s NR (<https://www.ncbi.nlm.nih.gov/refseq/about/nonredundantproteins/>) using DIAMOND (Buchfink et al. 2021) in a more sensitive mode, and the protein sequences in Araport 11 (Cheng et al. 2017) using BLASTP (Camacho et al. 2009). Functional annotation based on orthology relationships against eggNOG was conducted by eggNOG-mapper (<http://eggno-mapper.embl.de/>, Huerta-Cepas et al. 2017).

Determination and classification of candidate ERFs in the *Saintpaulia* genome

Based on the annotation information from the eggNOG-mapper and BLASTP results, 161 *Saintpaulia* ERFs were selected. A molecular phylogenetic tree was constructed using the amino acid sequences of 161 *Saintpaulia* ERFs, 121 *A. thaliana* ERFs amino acid sequences obtained from GenBank, and 137 *S. lycopersicum* ERFs amino acid sequences obtained from PlantTFDB (Guo et al. 2007, <http://plantfdb.gao-lab.org/>). The amino acid sequences were filtered using PREQUAL v1.0.2 (Whelan et al. 2018). Subsequently, multiple sequence alignment was performed using MUSCLE v5.1 (Edgar 2022) with super5 default settings. The aligned sequences were further trimmed using TrimAl v1.4.1 (Capella-Gutierrez et al. 2009). Using trimmed alignment sequences, a maximum likelihood (ML) method-based molecular phylogenetic tree was constructed by IQ-TREE2 v2.2.0.3 (Minh et al. 2020). When constructing, the Ultrafast bootstrap method (UF-boot) and SH-aLRT methods were used 1,000 times, and branches with confidence levels of $\geq 95\%$ and $\geq 80\%$ respectively were considered robust (Anisimova et al. 2011; Hoang et al. 2018). The clade to which the *Saintpaulia* ERFs belonged was estimated according to Nakano et al. (2006).

RNA-seq analysis

RNA-seq analysis was performed to investigate which genes are up-regulated at different times during chilling acclimation at 10 °C. *Saintpaulia* were previously treated at 10 °C for 0 (no treatment as a control), 12, 24, 48, and 144 h. Then, total RNA was extracted from the youngest fully expanded leaves using Sepasol®-RNA I Super G (Nacalai tesque, Kyoto, Japan), according to the manufacturer’s protocol. RNA-seq was performed by Rhelixia (Kanagawa, Japan). RNA-seq libraries were prepared using the NEBNext® Poly(A) mRNA Magnetic Isolation

Module and the NEBNext® UltraTM II Directional RNA Library Prep Kit (New England Biolabs). Paired-end sequencing was performed using the Illumina NovaSeq 6000 with a read length of 150 bp. Adapters and low-quality reads were removed using Trimmomatic v0.39 (Bolger et al. 2014). The assembled genome sequence, SIO_r2.0 genome data were used as a reference sequence and mapping was performed using HISAT2 v2.2.1 (Kim et al. 2019). Transcripts per million (TPM) values of the predicted genes were calculated by Stringtie v2.2.1 (Pertea et al. 2015).

GO enrichment analysis was performed using GO terms of genes whose expression increased more than twofold after treatment at 10 °C. The R package topGO v2.41.0 was used for GO enrichment analysis (with settings; algorithm = “elim”, statistic = “fisher”. Alexa and Rahnenführer 2023). The top ten significant GO terms enriched at each treatment time after 10 °C are shown in the heatmap.

The TPM expression data of 161 Saintpaulia ERFs were used to create a heatmap using the R package ComplexHeatmap v2.14.0 (Gu et al. 2016; Gu 2022) (with the cluster method settings = ward.D2).

Quantitative RT-PCR

The expression levels of some ERFs were confirmed by quantitative reverse transcription-PCR (qRT-PCR). Saintpaulia were previously treated at 10 °C for 0 (no treatment as control), 3, 6, 12, 24, 48, 72 and 144 h. Then, total RNA was extracted from the youngest fully expanded leaves using Sepasol®-RNA I Super G (Nacalai tissue). First-strand cDNA was synthesized from the extracted RNA using ReverTra Ace® (TOYOBO, Osaka, Japan), and qRT-PCR was performed using the cDNA and SYBR THUNDER BIRD® qPCR Mix (TOYOBO) with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad, California, United States). Primers were designed using Primer 3 Plus (Untergasser et al. 2012; Supplementary Table S1). Saintpaulia ERFs that may be associated with chilling tolerance were targeted for qRT-PCR. Relative quantification was performed using the standard curve method. A standard curve with a correlation coefficient of $r \geq 0.99$ was used. *SiActin* (Sio_r2.0_p0205.1.g00590.1) was used as a housekeeping gene.

No injury occurred in the 24 h treatment at 6 °C, nor did chilling acclimation (described in the results section). Therefore, we extracted RNA from leaves treated at 6 °C for 24 h and compared the expression of the ERFs between no treatment (0 h, as control), 6 °C 24 h treatment, and 10 °C 24 h treatment by qRT-PCR. The procedure is the same as described above.

Promoter analysis

Promoter analysis was performed in MEME Suite (Bailey et al. 2015) using the 2 kb upstream of the start codon of the Saintpaulia ERF, which was up-regulated by low temperature. Motif enrichment analysis was performed using MEME (v.5.5.3) with “-mod zoops -minw 5 -maxw 20 -markov_order 0 -objfun classic” settings (Bailey et al. 1994). A motif with an E-value < 0.05 was considered a significant motif. Enriched motifs were used for Tomtom (Gupta et al. 2007), and were used as queries to search for similar motifs in the *A. thaliana* DAP-seq motif database (E-value < 0.05, O'Malley et al. 2016).

Statistical analysis

All statistical analyses were conducted using R software (ver. 4.2.1). Tukey's range test was performed using the stats package, and Dunnett's test was carried out using the multcomp package (Hothorn et al. 2008).

Results

Chilling injury in *S. ionantha* 'Kilauea'

We investigated the chilling injury in *S. ionantha* 'Kilauea'. We exposed the plants to various temperature conditions for 24 h and calculated the electrolyte leakage as an indicator of chilling injury. At temperatures above 6 °C (Fig. 1A), the electrolyte leakage rate was approximately 20% and lower. However, when exposed to 4 °C for 24 h, the electrolyte leakage ratio was approximately 60% (Fig. 1A). We then investigated the relationship between the exposure time at 4 °C and the occurrence of chilling injury using plants grown under the same conditions. The electrolyte leakage was similar to non-treated conditions for up to 12 h after exposure to 4 °C (Fig. 1B), but it increased rapidly after 12 h (Fig. 1B). Plants exposed to 4 °C for 24 h exhibited withered young leaves near their shoot tip and subsequently died (Fig. 2C, D). Based on these results, it was determined that a chilling treatment at 4 °C for 24 h would be used as chilling treatment in subsequent experiments.

Chilling acclimation conditions for *S. ionantha* 'Kilauea'

To investigate the optimal low temperature for chilling acclimation, plants were exposed to different temperatures for 24 h before undergoing chilling treatment at 4 °C. When plants were exposed to temperatures below 8 °C for 24 h before chilling treatment, the electrolyte leakage ratio was around 80%, indicating severe chilling injury (Fig. 2A).

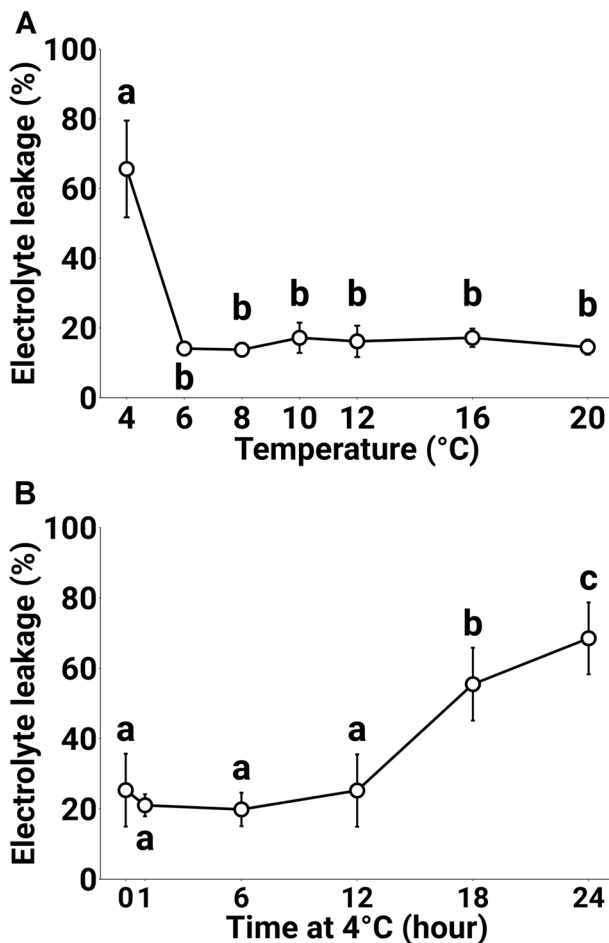


Fig. 1 Electrolyte leakage after low-temperature treatment. **A** electrolyte leakage at different temperatures (4, 6, 8, 10, 12, 16, 20 °C) for 24 h. The error bar represents the mean $\pm$ SD ($n=9$). **B** electrolyte leakage at 4 °C for different durations (0, 1, 6, 12, 18, 24 h). The error bar represents the mean $\pm$ SD ($n=9$). In both figures, each letter indicates significant differences according to the Tukey's range test ($P < 0.05$)

However, when plants were exposed to 10–16 °C for 24 h, the electrolyte leakage was lower than 6 °C or 8 °C treatment, indicating reduced chilling injury (Fig. 2A). Especially, results of comparison the normal growing conditions (20 °C) showed that treatment at both 10 and 12 °C was the most effective. Although both temperatures are effective for chilling acclimation, in this study, 10 °C was chosen as the treatment temperature for chilling acclimation.

To determine the optimal duration of chilling acclimation, the plants were exposed to 10 °C for various periods before chilling treatment. The electrolyte leakage gradually decreased with increasing exposure duration up to 72 h and slightly increased up to 144 h at 10 °C (Fig. 2B). When chilling acclimated plants were subjected to chilling treatment, middle to outer leaves (older than the eighth leaf from the youngest expanded leaf) turned pale but did not wilt. Unlike

non-acclimated plants, the shoot tip stayed alive without withering even after 20 days of returning to the recovery temperature (Fig. 2C). The acclimation treatment prevented leaf and plant death, but leaf bleaching on the outer leaves was observed (Fig. 2C). After 144 h of pretreatment at 10 °C, all plants survived (Fig. 2D).

Genome assembly, gene prediction, and determination of candidate *Saintpaulia* ERF

The statistics of the assembled genome sequences are summarized in Tables 1 and S2. In total, 71.6 Gb of continuous long reads were obtained from 12 SMRT cells. The 12 cells of the Sequel long-reads were assembled by FALCON, and the 2,967 primary contigs and 1,266 associate contigs were constructed. The primary contigs and associated contigs were phased by FALCON-Unzip, and the 2,200 primary contigs and 7,658 haplotigs were constructed. Next, the primary contigs and haplotigs were polished by Arrow and Pilon. The resultant 2,200 primary contigs and 7,658 haplotigs were designated SIO_r2.0. The BUSCO completeness of the primary contigs was 97.9% (Tables 1, S2).

The statistics of the predicted genes are summarized in Tables 1 and S2. The gene prediction was performed against the 2,200 primary contigs by BRAKER2, and 152,177 genes were predicted. Next duplicated genes in splicing variants were removed, and then 145,403 genes were defined as “best genes”. The “best genes” were searched against UniProtKB and NCBI's NR by DIAMOND in the more sensitive mode with an E-value of $1E-100$, identity $\geq 75\%$, and length coverage $\geq 50\%$, and those were searched against the proteins encoded in the genome sequence of *Doroceras hygrometricum* XS01 (Accession: GCA_001598015.1) by BLASTP with E-value of $1E-100$, identity $\geq 60\%$, length coverage $\geq 50\%$ and those in *A. thaliana* provided from Araport 11 (Cheng et al. 2017) with an E-value of $1E-100$, and identity $\geq 40\%$, and length coverage $\geq 50\%$. The “best genes” were also searched against the protein domain database Pfam31.1 by HMMER v3.3 (Eddy 2011) with an E-value of $1e-20$. The expressed genes were searched by mapping of the RNA-seq data applied to the gene prediction by Salmon v1.2.1 (Roberts and Pachter 2013) with a TPM value ≥ 0.4 . The 46,536 displaying similarities against the proteins of UniProtKB and NR related to transposable elements were classified as transposable element (TE) genes. The 37,682 “best genes” displaying similarities and expressions in all of the analyses described above were classified as high-confidence (HC) genes, and the remaining 61,185 genes were classified as low-confidence (LC) genes. The BUSCO completeness of the genes classified into HC was 93.8%. The 37,682 genes classified in HC were used for further analyses as intrinsic genes.

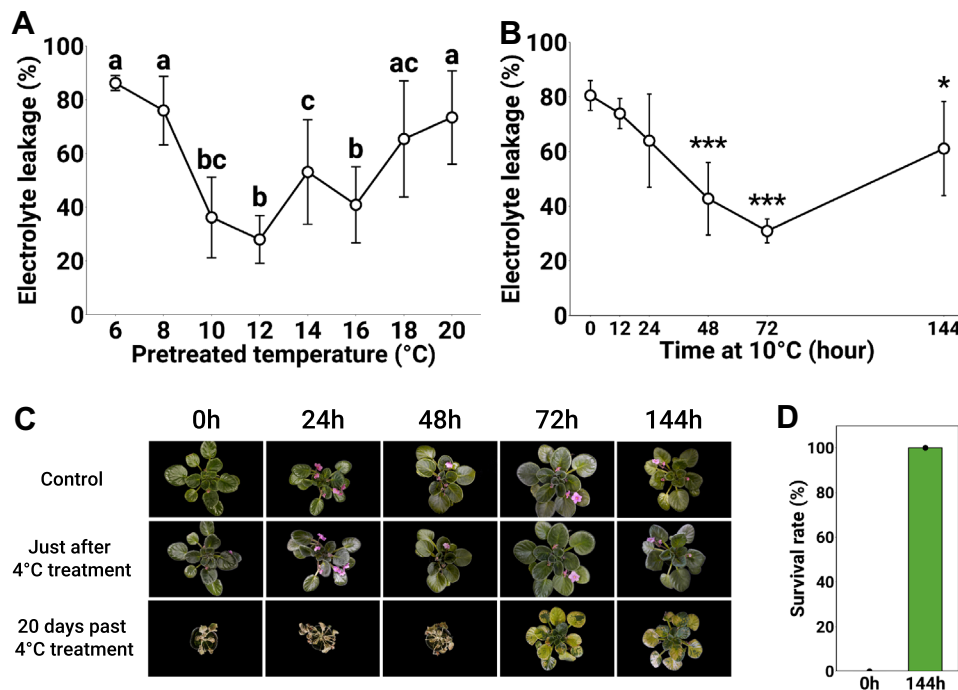


Fig. 2 Chilling acclimation in *S. ionantha* ‘Kilauea’. **A** electrolyte leakage at 4 °C treatment of plants with each temperature pretreatment. The error bar represents the mean $\pm$ SD (n=9). Each letter indicates significant differences according to the Tukey’s range test ($P < 0.05$). **B** evaluation of different pretreatment durations at 10 °C by electrolyte leakage. The error bar represents the mean $\pm$ SD (n=6). The asterisks indicate significant differences according to the Dunnett’s range tests (compared to 0 h, *** $P < 0.001$, ** $P < 0.01$,

* $P < 0.05$). **C** condition of plants before and after 4 °C treatment for 24 h. “Control” indicates the condition before the 4 °C treatment. Each hour indicates the duration of the 10 °C pretreatment. Plants obtained by leaf cutting were grown for 3 months and used for treatment or pretreatment. **D** survival rate after chilling treatment at 4 °C of plants untreated and pre-treated at 10 °C for 144 h (n=10). Whether the plants survived or not was determined 20 days after the 4 °C treatment

Table 1 Statistics of the assembled genome sequences and predicted genes (SIO_r2.0)

	Sio_r2.0 primary	Sio_r2.0 HC
Total		
Number of sequences	2,200	37,682
Total length (bases)	708,683,047	47,273,670
Average length (bases)	322,129	1,255
Max length (bases)	8,369,882	17,298
Min length (bases)	16,884	90
N50 length (bases)	1,262,744	1,656
GC %	35.1	45.0
Complete %	97.9	93.8
BUSCO v5 (embryophyta_odb10;1614)		
Complete and single-copy %	65.1	64.7
Complete and duplicated %	32.8	29.1
Fragmented %	1.0	2.4
Missing %	1.1	3.8

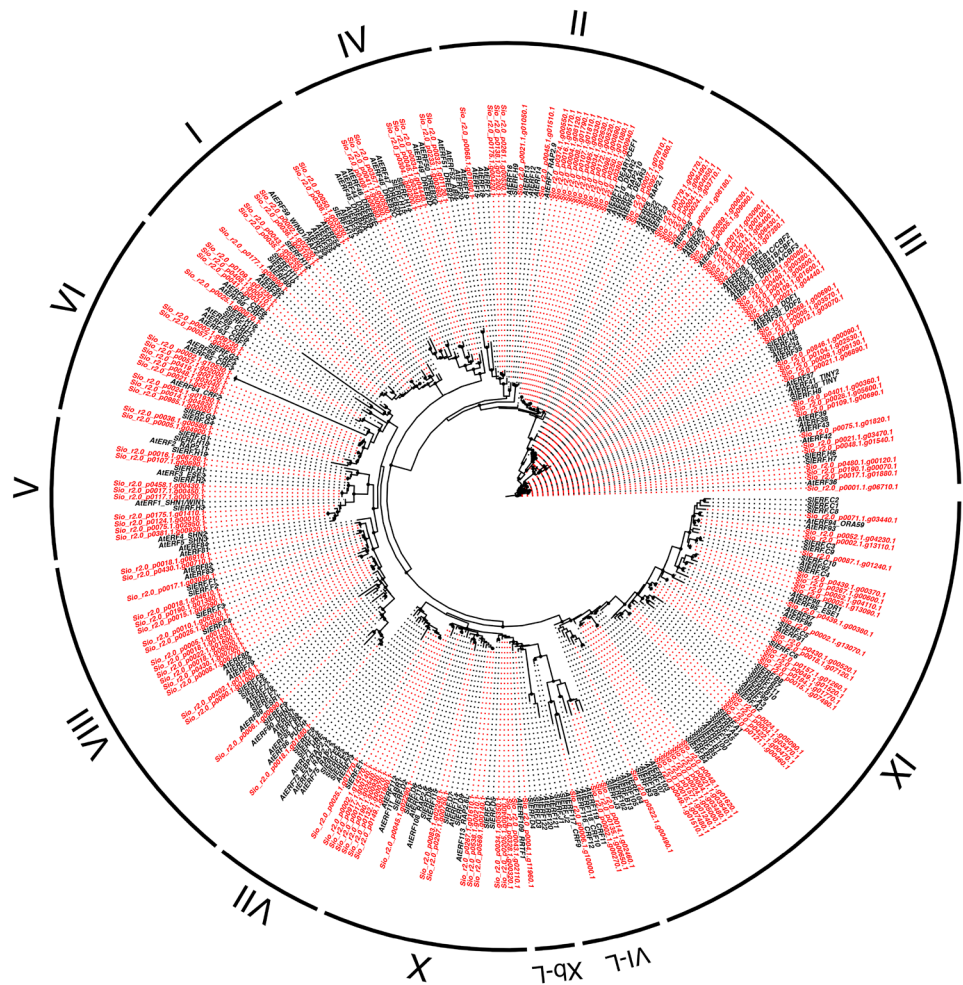
In the *S. ionantha* genome sequence SIO_r2.0, 161 sequences were annotated as ERF transcription factors. Based on previous reports, the ERFs of Saintpaulia were classified into 12 clades (Nakano et al. 2006). The results were as follows: 10 ERFs in clade I, 19 in clade II, 34 in clade III, 10 in clade IV, 9 in clade V, 11 in clade VI, 4 in clade VI-L, 5 in clade VII, 18 in clade VIII, 28 in clade IX, 11 in clade X, 2 in clade Xb-L (Fig. 3). ERFs of III clade were the most abundant, followed by IX clade.

Expression analysis during chilling acclimation and promoter analysis

To confirm the response of the plants during chilling acclimation, GO enrichment analysis was performed on the genes whose expression increased more than twofold after the 10 °C treatment. In Figure S1, the top 10 most significantly enriched GO terms at each treatment time are shown. GO terms at each treatment time included GO terms related to stress response and phytohormone.

The 161 Saintpaulia ERFs found in SIO_r2.0 were clustered based on their expression data during chilling acclimation (Fig. 4A). As a result, some ERFs formed a cluster

Fig. 3 Molecular phylogenetic tree of ERF transcription factors. ERF amino acid sequences from *S. ionantha* (with “Sio_r2.0” ID), *A. thaliana* (with “AtERF”) and *S. lycopersicum* (with “Solyc” ID) were used. The red labels indicate *Saintpaulia* ERF. The GenBank Accession IDs for *A. thaliana* ERF amino acid sequences are listed in Supplemental Table S4. The phylogenetic tree was illustrated using the tidyverse (ver. 1.3.2), treeio (ver. 1.22.0), and ggtree (ver. 3.6.2) packages in the R software

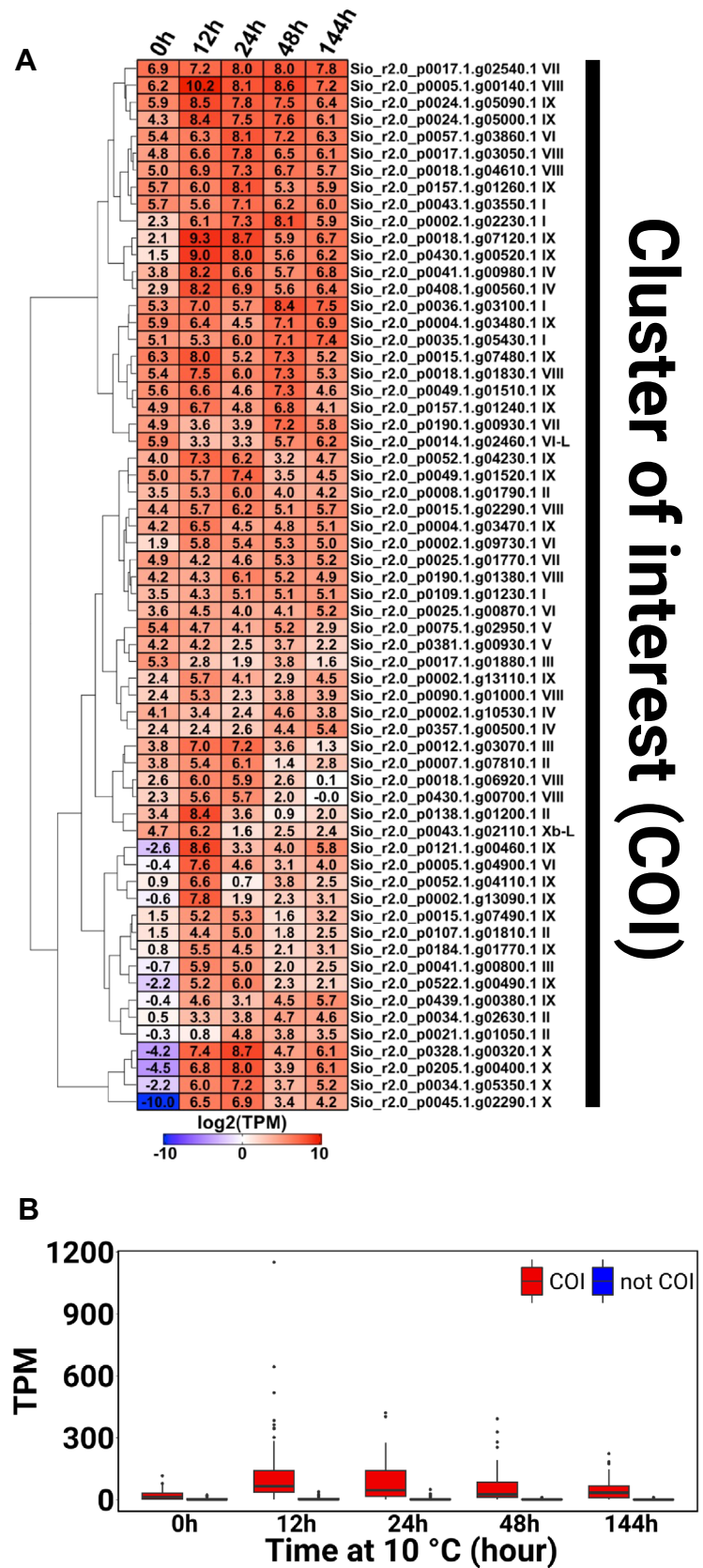


(Fig. 4A). We focused on this cluster and designated it as "cluster of interest (COI)" consisting of 62 ERFs (Fig. 4A). The TPM expression levels of ERFs belonging to the COI were relatively higher than those of ERFs not belonging to the COI after the 10 °C treatment (Fig. 4B). Among them, 43, 8, and 4 ERFs (a total of 55 ERFs) showed more than twofold increase in expression after 12 h, 24 h, and 48 h of treatment at 10 °C, respectively. From the 43 ERFs whose expression increased at 12 h, we compared the expressions of 7 ERFs belonging to different clades that are reported to be involved in cold acclimation by qRT-PCR. In addition, we examined the expression levels of these 7 ERFs at 3 h and 6 h after the 10 °C treatment. The 7 ERFs belonged to the same clades as ERFs reported to be involved in low-temperature acclimation in other plants (Fig. 3). The results of qRT-PCR and RNA-seq were almost identical (Figs. 5, S2), except for Sio_r2.0_p0024.1.g05000.1. Thus, the results of RNA-seq analysis are considered reliable. The expression levels of some ERFs were already increased after 6 h (Fig. 5). Expression of ERFs homologous to CBF/DREB1 was not increased by 10 °C treatment (Fig. S3).

The *Saintpaulia* ERFs whose expression increased greatly at an early stage after the 10 °C treatment are probably involved in the chilling acclimation. Therefore, we analyzed the promoters of 55 ERFs from the COI, whose expression increased more than twofold after 12, 24, and 48 h. As a result, 9 motifs were enriched from ERFs upregulated at 12 h and 2 motifs from ERFs upregulated at 24 h (Tables S5 and S6). No significant motifs were obtained from the ERFs upregulated at 48 h. The 11 enriched motifs were used to investigate the homology with the motifs of *A. thaliana* by Tomtom (Gupta et al. 2007). Except for Motif 2/3, no similar motifs were found. When Motif 2 was used as a query, several motifs of GCC box-like motif, to which ERF transcription factors bind, were hit (with E-value < 0.05, Table S7). When Motif 3 was used as a query, several DRE/CRT core sequences like motif, to which DREB subfamily members bind, were hit (Table S7).

Finally, we compared the expression of ERFs between the treatments for 24 h at 10 °C, an effective temperature for chilling acclimation, and at 6 °C, an ineffective temperature. The results showed that the expressions of all six ERFs we

Fig. 4 A heatmap of *Saint-paulia* ERFs that were highly expressed. From the clustering analysis based on ERFs expression data, clusters with highly expressed ERFs were focused on as “clusters of interest” (COI). Heatmap showing the expression pattern of ERF genes in *Saintpaulia* exposed to 10 °C. On the right side of the heatmap, Sio_r2.0 IDs and the clade to which the ERF belongs are shown. The numbers in the boxes indicate log₂-transformed TPM values. The TPM value of 0 is calculated as 0.001. The heat maps were drawn on R software using the ComplexHeatmap package. **B** comparison of TPM data of COI and not COI by boxplot. At each treatment time, the red boxplot on the left side indicates the TPM data of COI, and the blue boxplot on the right side indicates of not COI



investigated were not or only barely increased by the 6 °C treatment (Fig. 6).

Discussion

Saintpaulia, a subtropical plant, is capable of chilling acclimation

Saintpaulia is extremely sensitive to low temperatures, and once chilling injury occurs, the plant does not re-green (Kratsch and Wise 2000). In this study, the electrolyte leakage of *S. ionantha* ‘Kilauea’ grown at a stable temperature of 20 °C increased rapidly after 24 h of exposure to 4 °C, causing the plant to die (Figs. 1A, B, 2C). In contrast to the 4 °C treatment, electrolyte leakage did not increase after 24 h of treatment above 6 °C (Fig. 1A). A pretreatment at 10 °C before the chilling treatment at 4 °C suppressed the increase in electrolyte leakage, resulting in chilling acclimation (Fig. 2). Even in extremely low temperature-sensitive plants such as mung bean (*Vigna radiata*) and upland cotton (*Gossypium hirsutum*), chilling acclimation can develop chilling tolerance (Kratsch and Wise 2000; Chang et al. 2001; Kargiotidou et al. 2010). Our results indicate *Saintpaulia* can also undergo chilling acclimation and acquire chilling tolerance.

It has been reported that cold water applied to the leaves of *Saintpaulia* results in the death of palisade cells. This disorder is reported to be caused by a sudden drop in leaf temperature. For example, a sudden drop in leaf temperature from 35 to 25 °C also injured palisade cells (Elliot 1946; Maekawa et al. 1987; Yang et al. 2003), which is said to be different from cell death caused by chilling stress. In our study, some leaves of plants that survived the 4 °C treatment by chilling acclimation (at 10 °C for 72–144 h) showed partial bleaching, similar to the injury caused by a sudden temperature drop (Fig. 2C). This bleaching was not observed in plants at 4 °C without acclimation treatment, suggesting that bleaching phenomenon was not caused by a sudden temperature drop in 4 °C treatment. However, the similarity in appearance with the bleaching caused by a sudden temperature drop suggests that the injury caused by a sudden temperature drop and the low-temperature injury have something in common.

GO enrichment analysis showed that the enriched terms included not only ‘response to cold’ but also ‘response to abscisic acid’ and ‘trehalose metabolism in response to stress’ (Fig. S1). Abscisic acid is a well-known stress hormone and is synthesized in response to various stresses, including low-temperature stress (Swamy et al. 1999). Trehalose is also known to respond to various stresses and act as an osmolyte and a protectant of cell membranes and proteins (Iordachescu et al. 2008). The fact that these GO terms were

observed in *Saintpaulia* after 10 °C treatment suggests that abscisic acid and trehalose may contribute to the improvement of low-temperature tolerance, like other plants.

The expression of multiple ERFs is induced during chilling acclimation in *Saintpaulia*

According to the whole genome sequence (Tables 1, S2), 161 ERFs were determined in *Saintpaulia*, with the largest number of ERFs belonging to clade III, followed by clade IX (34 in clade III and 28 in clade IX) (Fig. 3). The increase in AP2/ERF family transcription factors through whole genome and tandem duplication may have contributed to the adaptation of plant ancestors to low-temperature stress caused by climate change (Guo et al. 2022). In addition, it has been suggested that the number of ERFs belonging to clades III and IX was the most expanded by whole genome duplication or tandem duplication (Guo et al. 2022). Also in *Saintpaulia*, whole genome duplication or tandem duplication may be responsible for the increase in the number of ERFs in the various clades, especially in clades III and IX. Clade III includes the CBF/DREB1 transcription factors, which have been extensively studied for their role in cold tolerance in many plants, including *A. thaliana* (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Gilmour et al. 2000, 2004; Dubouzet et al. 2003; Qin et al. 2004; Zhang et al. 2004). In *Saintpaulia*, ERFs similar to *A. thaliana* and *S. lycopersicum* CBF/DREB1 were found (Figs. 3, S3). However, these ERFs did not show an increased expression under low temperatures; instead, their expression levels decreased (Figs. 2, 4A). These results suggest that *Saintpaulia* is unable to induce the expression of CBF/DREB1 in response to low temperatures. Taiwan banana (*Musa acuminata*), native to the Malay Peninsula, and Sea Island cotton (*Gossypium barbadense*), native to the Caribbean Sea, are tropical and subtropical plants growing in areas where the temperature remains stable, around 26 °C. In these plants, the expression of CBF/DREB1 was upregulated by low temperatures (*MaDREB1F*, *GbCBF1*), and the genetic transformation of these CBF/DREB1 improved low-temperature tolerance (Guo et al. 2011; Xu et al. 2023). Thus, CBF/DREB1 is upregulated in response to low temperatures even in tropical plants that do not usually encounter low temperatures, and enhances chilling tolerance by upregulation. As the *cbf2* mutant in *A. thaliana* has enhanced cold tolerance (Novillo et al. 2004), the downregulated expression of CBF/DREB1 like may contribute to the enhanced low-temperature tolerance in *Saintpaulia*. Unlike ERFs homologous to CBF/DREB1 in *Saintpaulia*, other ERFs from various clades exhibited an increased expression under low temperatures (Figs. 4, 6). *AtDDF1* (clade III) and *AtCRF4* (clade VI) in *A. thaliana* are known to express during cold acclimation and improve cold tolerance (Kang et al. 2011; Zwack et al.

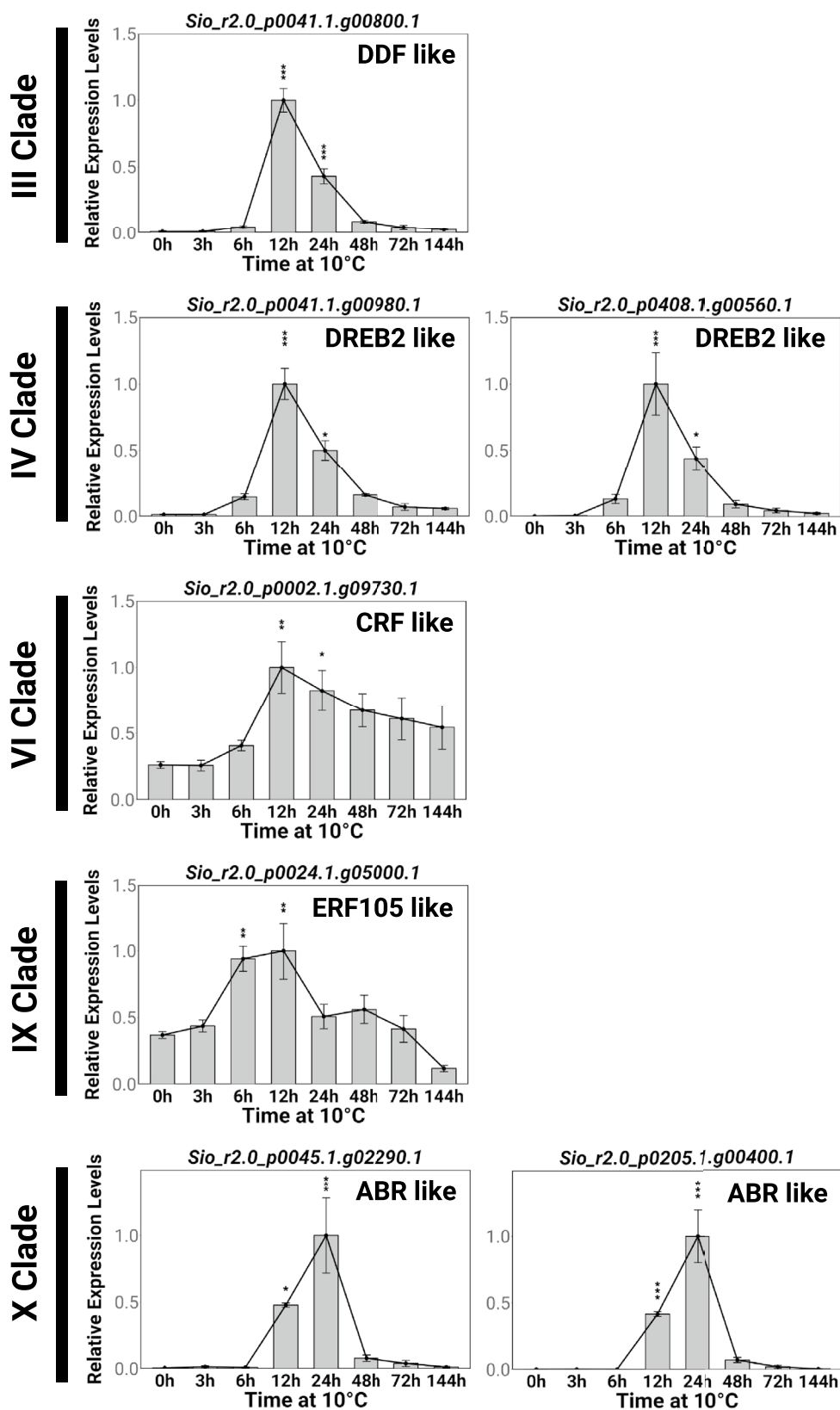


Fig. 5 Expression profiling of *Saintpaulia* ERFs at different durations of acclimation treatment at 10 °C by qRT-PCR. The error bar represents the mean $\pm$ SE (n=3). The asterisks indicate significant differences according to the Dunnett's range tests (compared to 0 h, *** P <0.001, ** P <0.01, * P <0.05). The ERF clades are indicated on the left of each row (see Fig. 3)

2016). Additionally, *VaERF057* (clade VII) in *V. amurensis*, *PtERF9* (clade VIII) and *PtERF108/109* (clade X) in *P. trifoliata* and *MdABI4* (clade IV) in *M. domestica* are also expressed by cold treatment and involved in enhancing cold tolerance (Sun et al. 2016; Wang et al. 2019; Khan et al. 2021; An et al. 2022; Zhang et al. 2022). In many plants, the increased expression of ERFs belonging to various clades has been reported to be involved in the improvement of low-temperature tolerance. Therefore, the increased expression of various clades may also be involved in chilling acclimation in *Saintpaulia*. When the pretreatment duration at 10 °C was 24–48 h, the increase in electrolyte leakage caused by the subsequent 4 °C treatment was suppressed. However, the plants were unable to recover after the 4 °C treatment and eventually withered (Fig. 2C). When pretreated at 10 °C for 72 h or more, the plants survived after the subsequent 4 °C treatment without withering (Fig. 2C, D). The increased expression of ERFs after 12 h of treatment at 10 °C suggests a time lag between that increased expression of ERFs and the acquisition of chilling tolerance by acclimation. Although the expression of several ERFs, which are expected to be associated with chilling acclimation, was increased at 10 °C (Fig. 5), when the plants were exposed to 6 °C, expression levels of these ERFs are not upregulated (Fig. 6). This may suggest that ERF expression is important for *Saintpaulia* chilling acclimation.

The promoter regions of some *Saintpaulia* ERFs whose expression increased during chilling acclimation had cis-elements to which AP2/ERF transcription factors could bind. Thus, the ERFs whose expression is increased by low temperature may regulate the expression of other ERFs by binding to the promoter region of their own or other ERFs.

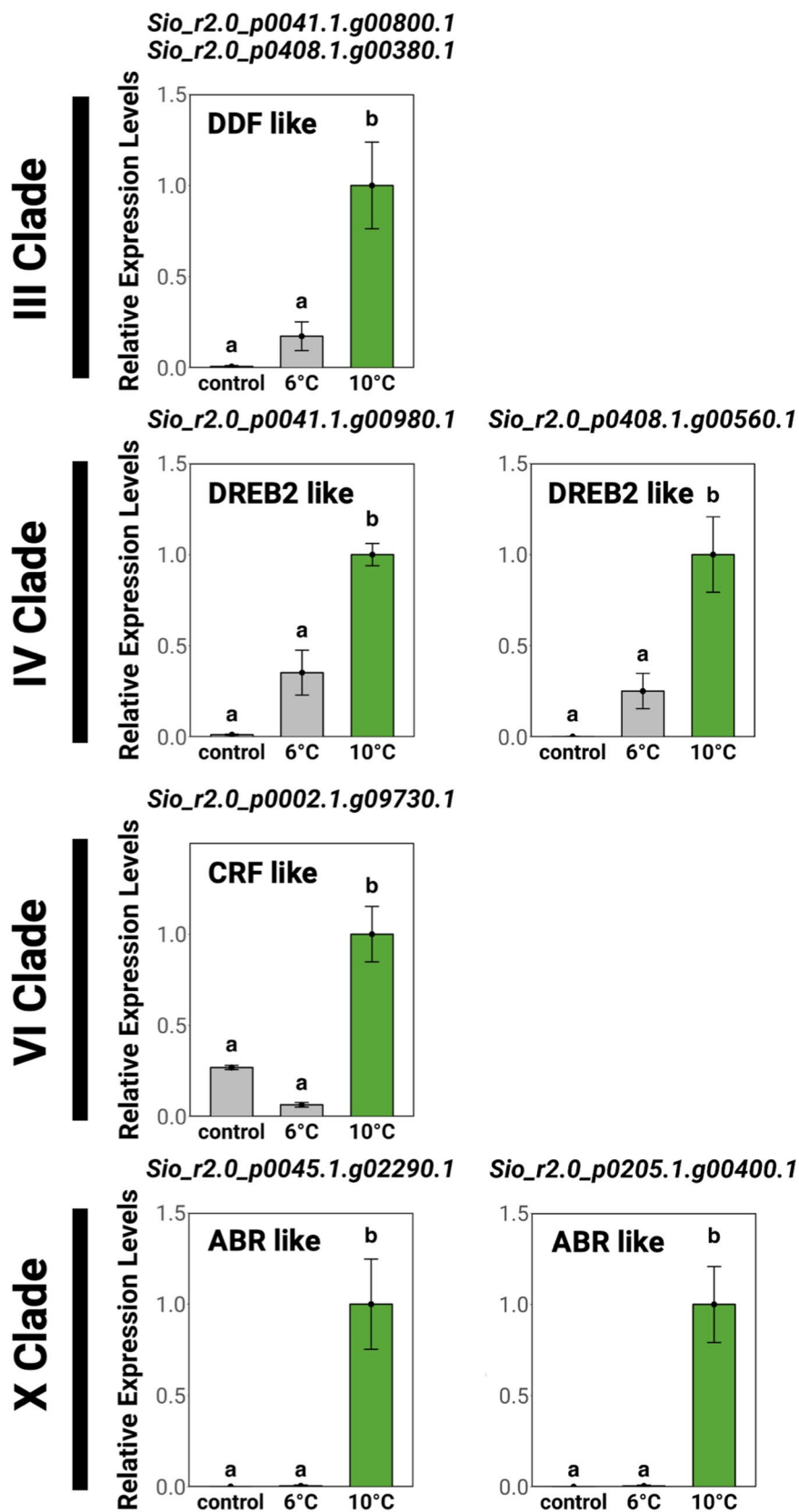
However, since GCC boxes and DRE/CRT core motifs were also found in the promoter regions of ERFs whose expression was not increased by low temperature, the increased expression of multiple ERFs may require other factors. Also, it is reported that the ability of ERFs to bind to cis-elements varies even in the same clade of ERFs (Shoji et al. 2013). Such ability variation is thought to be caused by the difference in the amino acid sequence of the AP2 domain, which is the DNA-binding region. Among the *Saintpaulia* 55 ERFs induced by low temperature, the sequence of each AP2 domain is different (Fig. S4). This suggests that each ERF regulates different downstream genes, and the expression of various ERFs may induce more downstream genes to improve chilling tolerance.

Conclusion

This study revealed that *Saintpaulia* could be chilling acclimatized by exposure to 10 °C for more than 72 h, and can survive at 4 °C for 24 h, which would normally kill the plants. In addition, we constructed the SIO_r2.0 genome data and found that 161 ERF transcription factors, including CBF/DREB1, were present in *Saintpaulia*. The expression of multiple ERFs from various clades was upregulated in response to acclimation at 10 °C.

Chilling injury is a major problem in the production and transportation of *Saintpaulia* in temperate regions. Near perspectives include confirming whether the chilling acclimation of *Saintpaulia* can have a practical effect during cultivation and transportation and optimizing the acclimation conditions by testing various conditions and cultivars. In addition, if the genes associated with chilling acclimation can be identified, the genetic markers or the expression of these genes could be used as indicators to evaluate chilling tolerance in *Saintpaulia* and be helpful with transportation challenges.

Fig. 6 Comparison of expression levels of *Saintpaulia* ERFs during 6 °C or 10 °C treatment for 24 h by qRT-PCR. The error bar represents the mean $\pm$ SE (n=3). Each letter indicates significant differences according to the Tukey's range test ($P < 0.05$). The ERF clades are indicated on the left of each row (see Fig. 3)



Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10725-024-01181-7>.

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Author contributions Daichi Kurata: data curation, investigation, resources, visualization, writhing-original draft. Kento Fukutomi: investigation, resources. Kanae Kubo: investigation, resources. Kenta Shirasawa: funding acquisition, data curation, formal analysis, writing-original draft. Hideki Hirakawa: funding acquisition, data curation, formal analysis, writing-original draft. Munetaka Hosokawa: funding acquisition, supervision, project administration, conceptualization, writing-review & editing.

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Data availability The genome sequence and raw data are available under the BioProject PRJDB15710. The accession numbers of the raw data are summarized in Table S3. The accession numbers of the amino acid of *A. thaliana* ERFs used in the phylogenetic tree construct are summarized in Table S4. The cds and pep files of the predicted genes (HC, LC, and TE) and gff3 files were provided from FigShare (<https://doi.org/10.6084/m9.figshare.24548854>).

Declarations

Conflicts of interest The authors declare that they have no conflicts of interest.

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