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A guayule C-repeat binding factor is highly activated in guayule under freezing temperature and enhances freezing tolerance when expressed in *Arabidopsis thaliana*

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Running title: PaCBF4 enhances freezing tolerance in Arabidopsis.

Abstract

Natural Rubber (NR)-producing guayule (*Parthenium argentatum* Gray) has been developed as an alternative crop to diversify NR production. Guayule NR is mainly synthesized in its stem and is upregulated by cold temperatures. A guayule *C-repeat binding factor 4* (*PaCBF4*) was highly expressed in cold-treated stem tissue, coinciding with active rubber biosynthesis and accumulation. Sequence alignments of PaCBF4 with other CBFs indicated that PaCBF4 contains DNA-binding domains responsible for regulating cold-regulated (COR) gene expression. Spatial gene expression profiling of *PaCBF4* revealed that stems had the highest expression level among different organs examined. We further confirmed the function of *PaCBF4* as regulator of cold-signaling processes by expressing it in the model plant Arabidopsis under a constitutive ubiquitin promoter from potato. The resulting transgenic Arabidopsis lines expressing *PaCBF4* turned on expression of a set of Arabidopsis COR genes under both room (24˚C) and cold (4ºC) temperatures, in contrast to the wild-type Arabidopsis that expressed these COR genes solely upon cold treatment. Furthermore, the transgenic plants displayed enhanced freezing tolerance at -5ºC, exhibiting a survival rate of 88–98% compared with 0% survival rate of wild-type plants. Our results suggest that *PaCBF4* is a functional member of the guayule CBF gene family and plays a significant role in cold and freeze tolerance. Interestingly, overexpressing *PaCBF4* in Arabidopsis did not affect the normal phenotype of the plant during vegetative and inflorescence growth, but the gene led to more undeveloped siliques after flowering.

Keywords: guayule, *Parthenium argentatum*, C-repeat binding factor, dehydration responsive element binding factor1, *Arabidopsis thaliana*, gene expression, freezing tolerance.

1. Introduction

Natural rubber (NR) production from the hevea rubber tree (*Hevea brasiliensis)* faces numerous challenges, including susceptibility to diseases, limited germplasm diversity, land use constraints, and geopolitical uncertainties in certain Southeast Asian nations (Guyot and Le Guen, 2018; Vaysse et al., 2012). To address the challenges, the cultivation of alternative rubber-producing crops such as guayule (*Parthenium argentatum* Gray), a perennial woody shrub, and rubber dandelion (*Taraxacum kok-saghyz*) are being explored to diversify global rubber supply (Cornish, 2017; Kuluev et al., 2023; Rasutis et al., 2015; Rousset et al., 2021; Salehi et al., 2021; van Beilen and Poirier, 2007). Despite its potential, guayule NR production remains less cost-effective compared to Hevea rubber. Efforts have been undertaken to increase NR yield in guayule through germplasms utilization and agricultural practices (Abdel-Haleem et al., 2018; Cruz et al., 2022; Foster and Coffelt, 2005; Ilut et al., 2017; Placido et al., 2021; Rasutis et al., 2015; Ray et al., 1999; Sulas et al., 2020). Extensive studies explored the molecular mechanisms underlying NR biosynthetic pathways and rubber particle accumulation to elucidate the genes and pathways involved in NR synthesis (Amerik et al., 2021; Cherian et al., 2019; Dong et al., 2021; Kwon et al., 2023; Men et al., 2018; Nelson et al., 2019; Stonebloom and Scheller, 2019; Yamashita and Takahashi, 2020). Nonetheless, despite attempts to overexpress genes related to NR biosynthesis and rubber particle accumulation, tangible enhancements in NR production in guayule field crops have yet to be achieved (Chen et al., 2023; Dong et al., 2013; Placido et al., 2020; Placido et al., 2019; Ponciano et al., 2018; Veatch et al., 2005). Therefore, identifying new targets for genetic engineering of guayule with increased NR production remains a pressing objective.

NR biosynthesis in guayule is highly upregulated by cold temperatures (Allen et al., 1987; Benedict et al., 2008; Bonner, 1943; Bucks et al., 1985; Cornish and Backhaus, 2003; Dong et al., 2021; Downes and Tonnet, 1985; Hunsaker et al., 2019; Miyamoto and Bucks, 1985; Nelson et al., 2019; Ponciano et al., 2012; Veatch-Blohm et al., 2007). To unravel the mechanisms of cold-induced NR production in guayule, several studies have examined gene expression and enzyme activities in rubber biosynthetic pathways (Cornish and Backhaus, 2003; Ponciano et al., 2012). Given that NR production requires transcriptional activation of gene expression, a transcriptome study was conducted to identify differentially expressed genes in cold-treated guayule stems (Stonebloom and Scheller, 2019). Among these genes, the guayule C-repeat binding factor transcriptional activator (*PaCBF4*), also known as *dehydration responsive element binding factor 1D* (*DREB1D*) was found to be highly induced in cold-treated stem tissue where active rubber synthesis and accumulation occurred (Stonebloom and Scheller, 2019). The potential of *PaCBF4* as a novel target for genetic engineering to increase NR production in guayule has since piqued interest. The CBF/DREB1 transcription factor family has been extensively studied, especially in Arabidopsis, including members like *AtCBF1/DREB1B, AtCBF2/DREB1C, AtCBF3/DREB1A* and *AtCBF4/DREB1D* (Agarwal et al., 2017; Hwarari et al., 2022; Liu et al., 2019; Mehrotra et al., 2020; Shi et al., 2018; Zhang and Xia, 2023). While *AtCBF1*, *AtCBF2*, and *AtCBF3* are well known for their major roles in cold and freezing tolerance (Gilmour et al., 2000; Jaglo-Ottosen et al., 1998; Novillo et al., 2004), *AtCBF4* was initially associated with drought tolerance (Haake et al., 2002), but has also shown significance for cold and freezing tolerance (Liu et al., 2021; Oh et al., 2007; Tillett et al., 2012; Wang and Hua, 2009; Welling and Palva, 2008). The CBFs are considered the main components in early phases of cold signaling pathways, involving Inducer of CBF Expression (ICE), CBF, and cold-

regulated (COR) genes (Hwarari et al., 2022; Liu et al., 2019). Cold stress is perceived by receptor proteins, triggering signal transduction, and leading to activation of ICE, which subsequently regulates the expression of CBF genes. The CBFs then bind to the C-repeat/dehydration responsive element (CRT/DRE) of COR gene promoters, initiating the ICE-CBF-COR transcriptional cascade for activating cold and freezing responses (Hwarari et al., 2022; Liu et al., 2019). The CBFs belong to the superfamily of *APETALA2/Ethylene Responsive* (*AP2/ERF*) transcription factors containing a 60-amino acid consensus AP2/ERF domain present in numerous plant proteins (Nakano et al., 2006; Sakuma et al., 2002; Xie et al., 2019; Xu et al., 2011). CBF/DREB1 sub-family has unique CBF signature sequences, PKK/KPAGRxKFxETRHP and DSAWR, located at the N-terminal or C-terminal of the AP2/EFR domain (Figure 1B) (Canella et al., 2010; Gilmour et al., 1998; Medina et al., 2011; Stockinger et al., 1997). In Arabidopsis, these CBF signatures have been shown to be important for CBF to bind the DRE/CRT cis-acting element (Canella et al., 2010). In addition to responding to the abiotic stresses, CBFs expression is also affected by hormones (Zhang and Xia, 2023) and circadian clock (Fowler et al., 2005).

Arabidopsis has over 200 COR genes that are either activated or repressed by CBFs (Li et al., 2020). Among these genes, some encode key enzymes involved in osmolyte biosynthesis and regulation for maintaining hydrophobic interactions, ion homeostasis, cryoprotective proteins, and soluble sugars that stabilize cells and membranes to prevent damage caused by freezing temperatures (Meng et al., 2021; Okawa et al., 2008; Ramachandra Reddy et al., 2004; Shi et al., 2018; Wang et al., 2003). COR genes are directly regulated by CBF transcription factors, making COR transcripts useful markers for assessing the function of CBFs (Jia et al., 2016; Shi et al., 2017; Zhao et al., 2016). Arabidopsis *COR15a* and *KIN1* (Cold-Inducible 1) are well-established

indicators of CBF functions (Artus et al., 1996; Kurkela and Franck, 1990; Lin and Thomashow, 1992; Meng et al., 2015; Shi et al., 2017; Wang et al., 1994). The *COR15a* gene encodes a 15 KDa protein with high amino acid sequence similarities to Late Embryo Abundant Protein (LEA) proteins, which accumulate in plants in response to cold stress (Lin and Thomashow, 1992). COR15a is located in the stromal compartments of chloroplasts, protecting chloroplastic enzymes from freeze-induced inactivation and contributing to protecting membrane function against low temperature stress (Artus et al., 1996). Arabidopsis KIN1 encodes a 6.5 KDa kinesin protein with sequence similarity to anti-freeze proteins, playing a role in cold/freeze tolerance by stabilizing cellular compartments in plants (Wang et al., 1994; Wang et al., 2014; Wang and Hua, 2009). Functional evaluation of *CBF/DREB1* genes has been conducted through their overexpression in transgenic plants, often resulting in higher survival rates than controls when exposed to cold/freezing temperatures, drought, high salinity, and other abiotic stress (Agarwal et al., 2017; Zhang and Xia, 2023). However, in some cases the overexpression of certain *CBF/DREB1* genes resulted in retarded growth (Agarwal et al., 2017; Zhang and Xia, 2023). There are reports where the constitutive overexpression of *CBF/DREB1* genes caused few or no negative growth changes in transgenic plants. For example, overexpressing a BB-*CBF* from blueberry (*Vaccinium corymbosum*) enhanced freezing tolerance in Arabidopsis and native blueberry without affecting growth (Polashock et al., 2010; Walworth et al., 2012). Similarly, transgenic Arabidopsis lines overexpressing a *NnDREB1* from lotus (*Nelumbo nucifera*) (Cheng et al., 2017) or a *GthCBF4* from cotton (*Gossypium hirsutum*) (Liu et al., 2021) grew normally but exhibited increased drought (Cheng et al., 2017) or cold (Liu et al., 2021) tolerance compared to wild type. Transgenic paper mulberry (*Broussonetia papyrifera*) lines constitutively

expressing a *FaDREB1* from tall fescue (*Festuca arundinacea*) exhibited no growth retardation and had higher salt and drought tolerance than wild-type plants (Li et al., 2011).

Given the pronounced increase in guayule's NR production under cold conditions during the winter, we hypothesize that a master transcriptional regulator could upregulate the entire NR biosynthestic pathway via the cold signaling cascade. To better understand rubber synthesis through cold-mediated signaling in guayule, we isolated and characterized the *PaCBF4* gene. This study considers the sequence, organ-specific expression, functionality, and influence of the *PaCBF4* gene on *COR15a* and *KIN1* gene expression. The study of cold tolerance in transgenic Arabidopsis aims to show the potential of using *PaCBF4* for NR production enhancement.

2. Materials and Methods

2.1. Sequence and phylogenetic analysis

The PaCBF4 (Genbank ID, GFTW01034449.1) protein sequence was used as a query to search the protein databases using the BLASTP method with an E-value threshold of <1E-20. The protein databases were downloaded from NCBI. If a gene had multiple isoforms, the longest protein was selected to represent the gene. Some CBFs/DREBs from crop species were included as they are better studied for their biological function. In addition, the presence of the AP2-domain was examined using the hmmscan function of HMMER3 v3.3.2 (http://hmmer.org) (Eddy, 2011) with AP2 domain profile (PF00847) used as a query. The protein sequences were excluded from further consideration if the AP2 domain was incomplete, or the AP2 domain match E-value was greater than 1E-5. Multiple protein sequences were aligned using Clustal

Omega (Sievers and Higgins, 2018). with default parameters. The phylogenetic tree was generated based on the alignment using the Neighbor-Joining method in MEGAX (Kumar et al., 2018) with default parameters. These alignments were then used to infer phylogenetic relationships by using the Maximum Likelihood method and JTT+G matrix-based model (Jones et al., 1992) and 100 bootstraps (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed.

2.2. Plasmid construction, plant material, plant transformation, and growth conditions

The *PaCBF4* sequence used in this study is the same gene (TR78450_c1_g1_i1 or Genbank ID GFTW01034449.1) as described previously (Stonebloom and Scheller, 2019). The guayule genotype is the industrial standard cultivar AZ2 (www.ars-grin.gov/npgs) (Dierig et al., 1989; Ray et al., 1999). The other genes in the T-DNA cassette in plasmid *pND_PaCBF4* (Suppl Figure. S1A) were constructed as described previously (Dong et al., 2013).

Shoot tip clones derived from one seedling of AZ2 germplasms, designated as AZ2-D, were maintained in tissue culture as described previously (Dong et al., 2013). Newly sub-cultured shoot tips usually generated 3-5 roots within 1-2 weeks. Regenerated plantlets were carefully removed from the tissue culture medium and transplanted into 4-inch pots for continued growth in a chamber set at 24°C, with 12 h light (500 µmol/m2) and 12 h dark cycle. Guayule plants reached the flowering stage after three months in the chamber. Various tissues from 3-month-old plants were harvested for analysis. Low-temperature treatments were conducted by placing separate sets of 3-month-old guayule plants at 4°C in a refrigerator, or at -5°C in a freezer for 6 h in dark. Control plants were incubated in a dark chamber at 24°C for 6 h.

Arabidopsis transformation was carried out in wild-type (Col-0) genotype using a floral dip method as described previously (Clough and Bent, 1998). Arabidopsis were grown in a chamber under 24°C, continuous light (24 h light, 100 µmol/m^2). Leaf tissues from the T_3 and T_4 generations of the transformed lines were used in all experiments. For Arabidopsis cold or freezing treatment, 23-day-old plants were exposed to 4°C for 12 h or -5°C for 24 h, respectively. The survival rate was scored 5 days after the freezing-treated plants were returned to their normal growth conditions. Cold and freezing experiments were repeated three times. Soil mix, growth conditions, and plant care were performed in accordance with previously described methods (Placido et al., 2019).

2.3. Genomic DNA extraction and confirmation of PaCBF4 integration

Genomic DNA from T_3 transgenic Arabidopsis lines and PCR reaction mixtures were prepared following the instruction described (REDExtract-N-Ampa Plant PCR kit (Sigma-Aldrich, Carlsbad, CA, United States). PCR primers spanning the 409 promoter (5'-AACCCTATGAGGCGGTTTC-3') and *PaCBF4* (5'- CCTCTTAAGCGGAGCACCAA-3') region were used to amplify the genomic DNA with predicted amplicon size of 892 bp (Suppl Figure S1). PCR primers of Arabidopsis *Actin2* gene (Genbank ID, AY087751), forward (5'-CTGCTGGAATCCACGAGACA-3') and reverse (5'-CCTGCCTCATCATACTCGGC-3') were used as internal control to the genomic DNA with predicted amplicon size of 371 bp (Suppl Figure S1B). The PCR reaction and gel electrophoresis were performed as described previously (Chen et al., 2005).

2.4. RNA extraction, cDNA synthesis and quantitative PCR (qPCR)

Tissues were collected from 3-month-old guayule plants. Arabidopsis leaf leaves were collected from homozygous T4 generation plants grown in a chamber as described above. Samples were immediately frozen in liquid nitrogen after collection and stored at -80 ºC until RNA extraction using Total RNA Isolation Kit (Ambion, Pittsburg, PA, United States). Guayule young leaves were the first three leaves from shoot tip in size between 25–40 mm. Mature leaves were near shoot tips and newly reached full-size of 50–70 mm. The cDNA samples and qPCR reactions were performed as described previously (Kim and Chen, 2015). Genes and their primer sequences are listed in Suppl Table S1. *PaEF1a* or *AtACT2* was used as an internal control to normalize gene expression in guayule or Arabidopsis. Relative gene expression was calculated according to the Pfaffl model (Pfaffl, 2001).

2.5. Light Microscope

The flowers were photographed using a stereoscopic microscope (Leica MZ16F, Leica Microsystems Inc., Buffalo Grove, IL). Digital images were collected using a MicroPublisher6 color camera (Qimaging, Surrey, BC, Canada) and Image-Pro software (Media Cybernetics, Rockville, MD).

3. Results and Discussion

3.1 Sequence analysis of guayule CBF4 gene

The full length of PaCBF4 (or PaDREB1D) encodes a small protein with 199 amino acid residues (Genbank ID, GFTW01034449.1). Using PaCBF4 as a query, we retrieved 17 CBF/DREB1 protein family members from 9 plant species and conducted a phylogenetic analysis. The resulting phylogeny tree can be divided into four groups (Figure 1A). Arabidopsis CBF1 to CBF4 and a Hevea rubber tree HbDREB1A were grouped in Group I (Figure 1A). PaCBF4 showed the greatest identity (80.8%) with a sunflower (*Helianthus annuus*) HaDREB1D, followed by rubber dandelion TkCBF6 (65.7%) and TkCBF1 (59.4%), all of which are Asteraceae, clustered to group II (Figure 1A, Suppl Table S2). Compared to members in group I, PaCBF4 was more closely related to HbDREB1A (58.8%), followed by AtCBF4 (55%) (Figure 1A, Suppl Table S2). Group III and Group IV contain members from dicot woody species, such as cottonwood (*Populus trichocarpa*), tea (*Camellia sinensis*), apple (*Malus domestica*), peach (*Prunus persica*) and Eucalyptus (*Eucalyptus grandis*). Alignment of CBF sequences allowed us to compare three important domains: the AP2/ERF domain, and two CBF signature domains (Figure 1B). The PaCBF4 protein had 100% conservation with the Arabidopsis CBF Signature Sequence I, PKKPAGRKKFRETRHP. Regarding the CBF Signature Sequence II, DSAWR, there was a valine in PaCBF4 resulting in DSVWR, while this position is alanine in all AtCFBs (Figure 1B). The substitution of alanine with valine was also found in many other species, including CaDREB, HaDREB1D, TkCBF1, and TkCBF6 (Figure 1B), as well as five bilberry species (Oakenfull et al., 2013) and two blueberry species (Polashock et al., 2010). One of the blueberry CBF sequences, BB-CBF derived from northern cultivar Bluecrop (*Vaccinium corymbosum*), was demonstrated to activate COR gene expression in transgenic Arabidopsis (Polashock et al., 2010) and in southern blueberry cultivars Legacy (*V.*

darrowii and *V. virgatum*) (Walworth et al., 2012). Therefore, it is evident that the change from the alanine to valine does not impede the binding of CBF to the DRE/CRT cis-acting element. In general, the CBF Signature Sequences in PaCBF4 are highly conserved compared to known CBF sequences.

3.2 Expression of PaCBF4 in guayule

To study the potential role of *PaCBF4*, we first characterized its organ-specific expression in guayule. Under normal growth condition (24°C, 12 h light, 12 h dark), the relative expression levels of *PaCBF4* were quantified in guayule samples taken from 3-month-old plants after light was on for 6 h. We calculated the relative expression of *PaCBF4* in each organ by comparing with the level of *PaCBF4* in stem (set at 100) under light and 24°C. As shown in Figure 2A, the expression of *PaCBF4* was more than 90% higher in stems than in leaves, peduncles, flowers and roots. As most *CBFs*, including those from Arabidopsis, are expressed only under low-temperature or other stress conditions, it is intriguing to investigate if the constitutive expression of *PaCBF4* in stems is associated with NR synthesis in guayule. We also examined samples from plants placed in dark chambers (24°C) for 6 h. The spatial expression pattern of *PaCBF4* remained consistent with that observed under light conditions (Figure 2A, 2B). However, the transcript levels in all examined organs decreased to lower levels, showing 80% reduction in stem and residual levels in the other organs (Figure 2B). These findings suggest that *PaCBF4* expression may be regulated by light or circadian clock. The regulatory mechanisms *PaCBF4* expression in guayule is currently under investigation.

We conducted cold (4°C) and freezing (-5°C) treatments in parallel with the controls in the dark, as temperature drops during nighttime in winter. Under cold temperature, *PaCBF4* exhibited a slight increase in expression in stems (1.2-fold). In contrast, in peduncle and root, *PaCBF4* transcript levels increased 7.6-fold and 8.9-fold respectively (Figure 2C). The results suggest that stem, peduncle and root are important organs in protecting and reviving guayule from cold stress. The differentially expression of *PaCBF4* between stems and leaves under cold conditions is consistent with the previous report (Stonebloom and Scheller, 2019). We are currently conducting experiments to measure *PaCBF4* expression under longer period of cold-treatment. Upon exposure to freezing temperatures, dramatic increases of *PaCBF4* expression occurred in all organs. Notably, the stem exhibited the highest level of induction with a 238-fold increase compared to the control. This was followed by peduncles, roots, leaves and flowers, which exhibited increases between 16% and 44% (Figure 2D). These data indicated that *PaCBF4* was induced by freezing temperature and thus may regulate the freezing stress responses. In the future, we will examine the expression profile of *PaCBF4* under light and cold, or light and freezing to understand whether light participates in the regulation of *PaCBF4* expression under low temperatures.

3.3. PaCBF4 induced COR gene expression in Arabidopsis

To investigate the mechanisms underlying *PaCBF4*-mediated responses to low temperatures, we introduced *PaCBF4* into Arabidopsis, a well-established model for studying *CBF* gene regulation. Multiple transgenic Arabidopsis lines constitutively expressing *PaCBF4* under the control of the potato ubiquitin 409 promoter (Placido et al., 2019; Rockhold, 2008) were

298 generated and 37 independent transgenic T_1 lines were identified by kanamycin (Km) selection.
299 Among 20 lines analyzed, 11 T_2 lines had typical segregation for one-locus T-DNA insertion and
300 were selected for further analysis. PCR-based confirmation of *PaCBF4* integration into the
301 Arabidopsis genome was achieved by amplifying a region spanning part of the potato ubiquitin
302 409 promoter and part of *PaCBF4*. This produced 892 bp amplicons in all the transgenic lines
303 and a positive *pND_PaCBF4* plasmid control, while the wild-type plants lacked these amplicons
304 (Suppl Figure S1B). All samples, except for *pND_PaCBF4* plasmid, produced a predominant
305 PCR band for the Arabidopsis endogenous gene, *Actin2*, (371 bp) (Suppl Figure S1B). These
306 PCR results confirmed the presence of *PaCBF4* in all the 11 transgenic lines. The relative
307 expression levels of *PaCBF4* were quantified in T_3 homozygous population of these 11 lines
308 using qPCR. As expected, *PaCBF4* transcripts were not detectable in WT but were detected in
309 all transgenic samples (Figure 3). Line 5 had the highest *PaCBF4* transcript abundance, followed
310 by line 8, line 4 and line 2 (Figure 3). In contrast, line 1, line 6, line 7, and lines 9 to 12 had
311 relatively low transcript levels, ranging from 2% to 20% of that observed in line 5 (Figure 3).
312 It is well known that Arabidopsis CBFs can be induced by cold and bind to the promoter
313 regions of downstream COR genes, including *COR15a* and *KIN1* (Gilmour et al., 2000; Jia et al.,
314 2016; Seki et al., 2001; Shi et al., 2017; Thomashow, 1999, 2001; Wang and Hua, 2009; Zhao et
315 al., 2016). To assess the transcriptional activation function of *PaCBF4* on COR genes, we
316 selected lines L2, L4, L5 and L8 that showed relatively high constitutive expression levels of
317 *PaCBF4* (18- to 50-fold higher than that in the lowest L9) and measured the transcript levels of
318 *AtCOR15a* and *AtKIN1* in these lines. Intriguingly, constitutive overexpression of *PaCBF4*
319 resulted in induction of *COR15a* and *KIN1* transcripts in all of these transgenic lines, even in the
320 absence of exposure to cold temperature, when compared to the WT plants (Figure 4). When

plants were exposed to cold temperature (4°C for 12 h), both *COR15* and *KIN1* transcripts were detected in WT, and their levels were elevated in all cold-treated transgenic samples (Figure 4). These results indicate that *PaCBF4* functions as an active member of the guayule *CBF* gene family and operates in a manner conserved between guayule and Arabidopsis. The enhanced transcript levels of *AtCOR15a* and *AtKIN1* under cold temperature were highly likely induced by both *AtCBFs* and *PaCBF4* in the transgenic lines (Figure 4).

3.4 PaCBF4 increased freezing tolerance in Arabidopsis.

Overexpression of functional CBFs in Arabidopsis or other species leads to the constitutive expression of downstream COR genes, resulting in constitutive freezing tolerance (Mehrotra et al., 2020; Shi et al., 2018; Shi et al., 2017; Zhang and Xia, 2023). We observed that *PaCBF4* strongly activated the expression of COR genes in Arabidopsis (Figure 4), and drastically increased its transcript levels in various organs of guayule under freezing temperature (-5°C) (Figure 2D). These results prompted us to investigate the role of *PaCBF4* in freezing tolerance. We selected lines L2 and L5 to determine whether the transgenic lines were more freezing tolerant than the WT. As shown in Figure 5, 23-day-old plants were subjected to freezing treatment at -5°C for 24 h and then returned to normal growth conditions at 24°C. Five days later, all of the WT plants had died, whereas most of L2 and L5 plants had recovered from the freezing treatment, with survival rates of 87.5% and 97.9%, respectively (Figure 5C). Although the mechanism of *PaCBF4*-mediated freezing tolerance requires further investigation, it is likely that *PaCBF4* induced the expression of a set of genes known as the CBF-regulon (Seki et al., 2001; Shi et al., 2017; Thomashow, 2001) in guayule. The increase in *PaCBF4* transcript levels

in various guayule organs under freezing temperatures (-5°C) (Figure 2D) suggests that similar mechanisms may exist in guayule.

3.5 Overexpression of PaCFB4 did not affect vegetative growth but affected silique development

During the initial 30 days of growth under controlled conditions (24°C), the transgenic plants appeared phenotypically normal, indistinguishable from the WT, and initiated bolting, marking the transition from vegetative to reproductive growth. (Figure 5A, 5B). However, upon development of multiple inflorescences around day 45 of growth, it became apparent that lines L2 and L5 had many undeveloped siliques, even though their inflorescence and branch growth appeared normal (Figure 6A). These undeveloped siliques were devoid of seeds. Further examination of the flowers of WT and L2 revealed that the stigmas of WT flowers were almost completely covered with pollen, whereas many L2 stigmas had little or no pollens (Figure 6B). It is therefore likely that the undeveloped silique phenotype was caused by insufficient pollination. Interestingly, L2 and L5 occasionally developed normal siliques and seeds at random, suggesting that *PaCBF4* might affect pollen desiccation in these lines, leading to unopened anthers. The precise mechanisms of pollination and silique development associated with the *PaCBF4* expression in Arabidopsis are currently under investigation. It should be noted that seed production in guayule is usually desirable, rubber yield in unaffected.

4. Conclusions

PaCBF4 possesses an AP2 domain and CBF signature sequences, which are widely conserved features among known *CBF* family members. The high expression level of *PaCBF4* in guayule stems indicate its important role in cold and freezing tolerance and association with NR synthesis and accumulation. We demonstrated that *PaCBF4* is a functional member of *CBF/DREB1* family by expressing it in Arabidopsis. The results support that *PaCBF4* is a promising candidate for overexpression in guayule, potentially boosting NR production without the need for cold stress induction. To prevent any impact on reproductive development in guayule, as seen in Arabidopsis, a suitable promoter could be employed to ensure robust expression exclusively in rubber-producing tissues, such as stem.

Author contributions

GC designed the experiments, participated the experiments, data collection and analysis, and wrote the manuscript; ND, KJ, CD, DFW and TW performed experiments and data analysis; HVS participated in Discussion and edited the manuscript; All authors read and approved the final manuscript.

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Figure legends

Figure 1. Comparison of 17 CBF/DREB1 protein family members from 9 plant species. A, A phylogenetic analysis constructed using the Maximum Likelihood method with bootstrap score (100 replicates) shown next to the branches. PaCBF4 gene ID is indicated by a rectangle. B, Alignment of AP2/EFR domains marked with a solid line and flanking CBF signature sequences marked with dotted lines. PaCBF4 sequences are indicated by rectangles. The species are Arabidopsis (At), Rubber Tree (Hb: *Hevea brasiliensis*); Eucalyptus (Eg: *Eucalyptus grandis*); Apple (Md: *Malus domestica*); Cottonwood (Pt: *Populus trichocarpa*); Sunfower (Ha: *Helianthus annuus*); Tea plant (Ca: *Camellia sinensis*); Dandelion (Tk: *Taraxacum kok-saghyz*); Guayule (Pa: *Parthenium argentatum*). Genbank ID of each sequence was listed in square brackets.

Figure 2. qPCR analysis of *PaCBF4* transcript abundance in various organs of guayule. Bar charts show *PaCBF4* expression level from samples collected under light at 24°C (A), under dark at 24°C (B), under dark at 4°C (C), and under dark at -5°C (D). Relative expression in each

organ was compared with stem (set at 100) collected under light at 24°C. Numbers in parentheses indicate relative expression levels. Data are representative of three independent experiments. Error bars represent ± SD of three technical replicates.

Figure 3. qPCR analysis of *PaCBF4* transcript abundance in Arabidopsis. WT, wild type. ND, not detected. Relative expression of each T3 line was compared to transgenic line 2 (L2) set at 100. Data are representative of three independent experiments. Error bars represent ± SD of three technical replicates.

Figure 4. qPCR analysis of *COR* gene expression in Arabidopsis. Bar charts show *COR15a* (A) and *KIN1* (B) expression levels from samples collected under 24°C (open bar) and 4°C (solid bar). WT, wild type. Numbers in parentheses indicate relative expression levels. Relative expression of each T4 line was compared to the transgenic L2 sample (set at 100). Data are representative of three independent experiments. Error bars represent ± SD of three technical replicates.

Figure 5. Freezing tolerance of wild-type and transgenic L2 and L5 plants. (A) Photos of 23-day-old plants growing under normal 24°C before freezing treatment. (B) Photos of plants exposed to -5°C for 24 h and then returned to 24°C for 5 recovery days. (C) Survival rate, scored as the percentage of plants showing healthy leaves after 5 days recovery from the freezing treatment (solid bar). Non-freezing controls were grown under 24°C (open bar). Number in parenthesis indicate survival rate of 0% for the WT. Data are mean ± SD of three independent experiments. Each treatment had 64 individuals.

Figure 6. Inflorescence of wild-type and transgenic L2 plants showing reduced size of siliques and unpollinated flowers. 45-day-old plants were grown under normal 24°C continuous light conditions. Examples of reduced siliques are indicated by red circles (A). Unpollinated flowers are displayed in L2 (B).

Supplementary figure legend

Figure S1. Schematic presentation of the T-DNA construct in *pND_PaCBF4* plasmid (A) and genomic DNA PCR identification of *PaCBF4* (B). Black solid arrows indicate the primers locations for amplifying a PCR product (892 bp). The Arabidopsis *Actin2* gene was used as an internal control with a PCR product (371 bp).

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768

Figure 1

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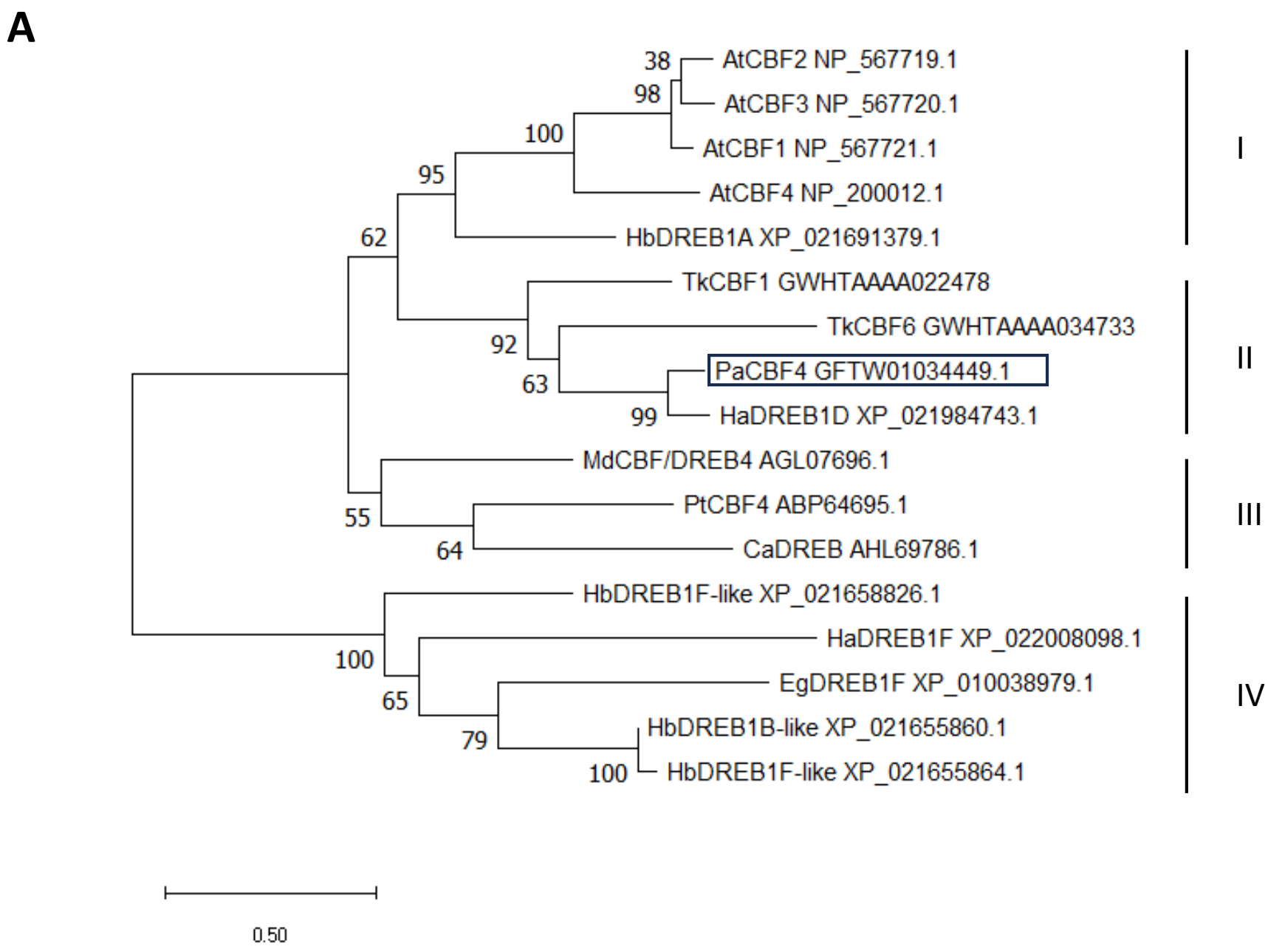

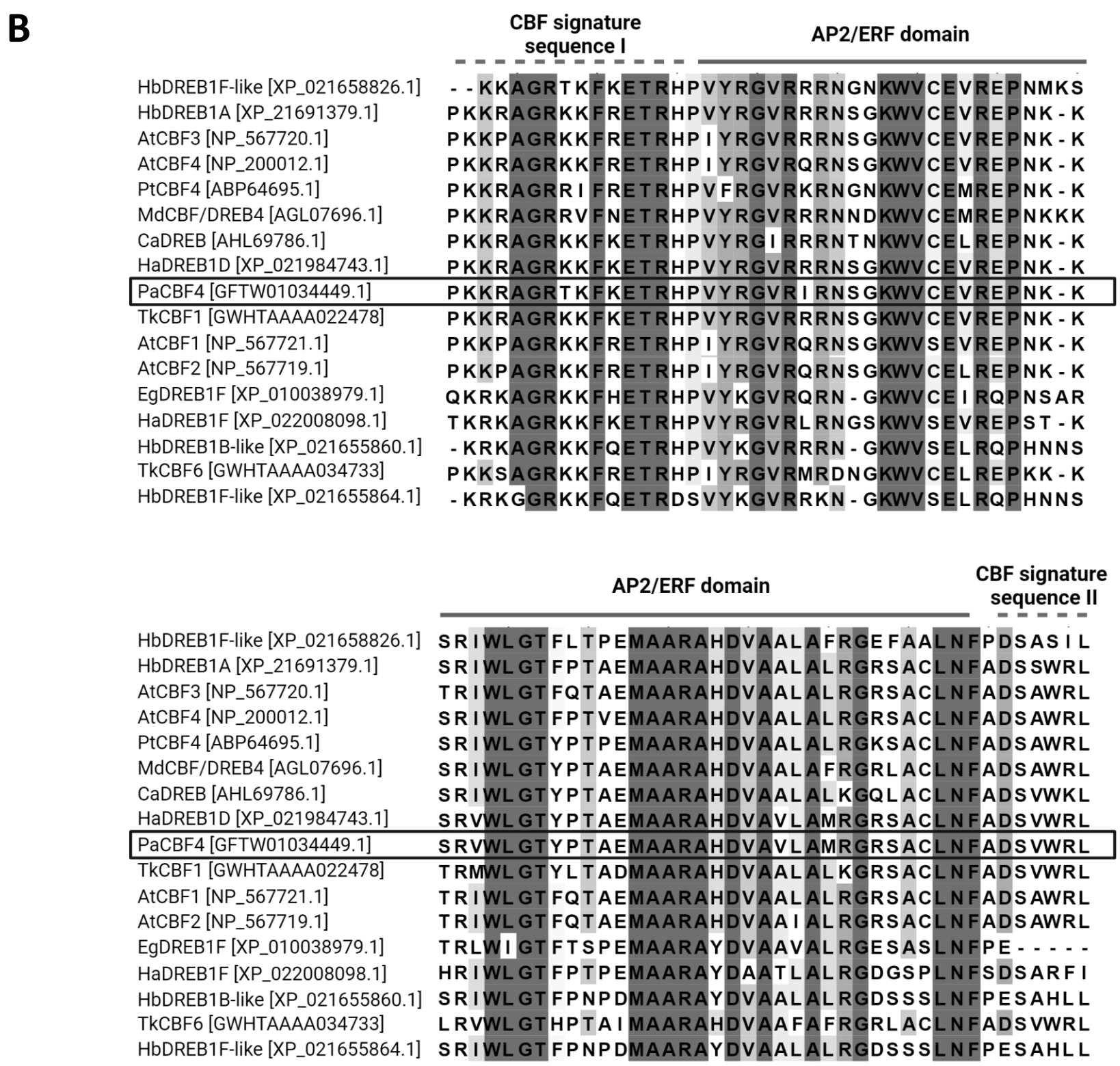

Fig. 1

Figure 1. Comparison of 17 CBF/DREB1 protein family members from 9 plant species. A, A phylogenetic analysis constructed using the Maximum Likelihood method with bootstrap score (100 replicates) shown next to the branches. PaCBF4 gene ID is indicated by a rectangle. B, Alignment of AP2/EFR domains marked with a solid line and flanking CBF signature sequences marked with dotted lines. PaCBF4 sequences are indicated by rectangles. The species are Arabidopsis (At), Rubber Tree (Hb: *Hevea brasiliensis*); Eucalyptus (Eg: *Eucalyptus grandis*); Apple (Md: Malus domestica); Cottonwood (Pt: *Populus trichocarpa*); Sunfower (Ha: *Helianthus annuus*); Tea plant (Ca: *Camellia sinensis*); Dandelion (Tk: *Taraxacum kok-saghyz*); Guayule (Pa: *Parthenium argentatum*). Genbank ID of each sequence was listed in square brackets.

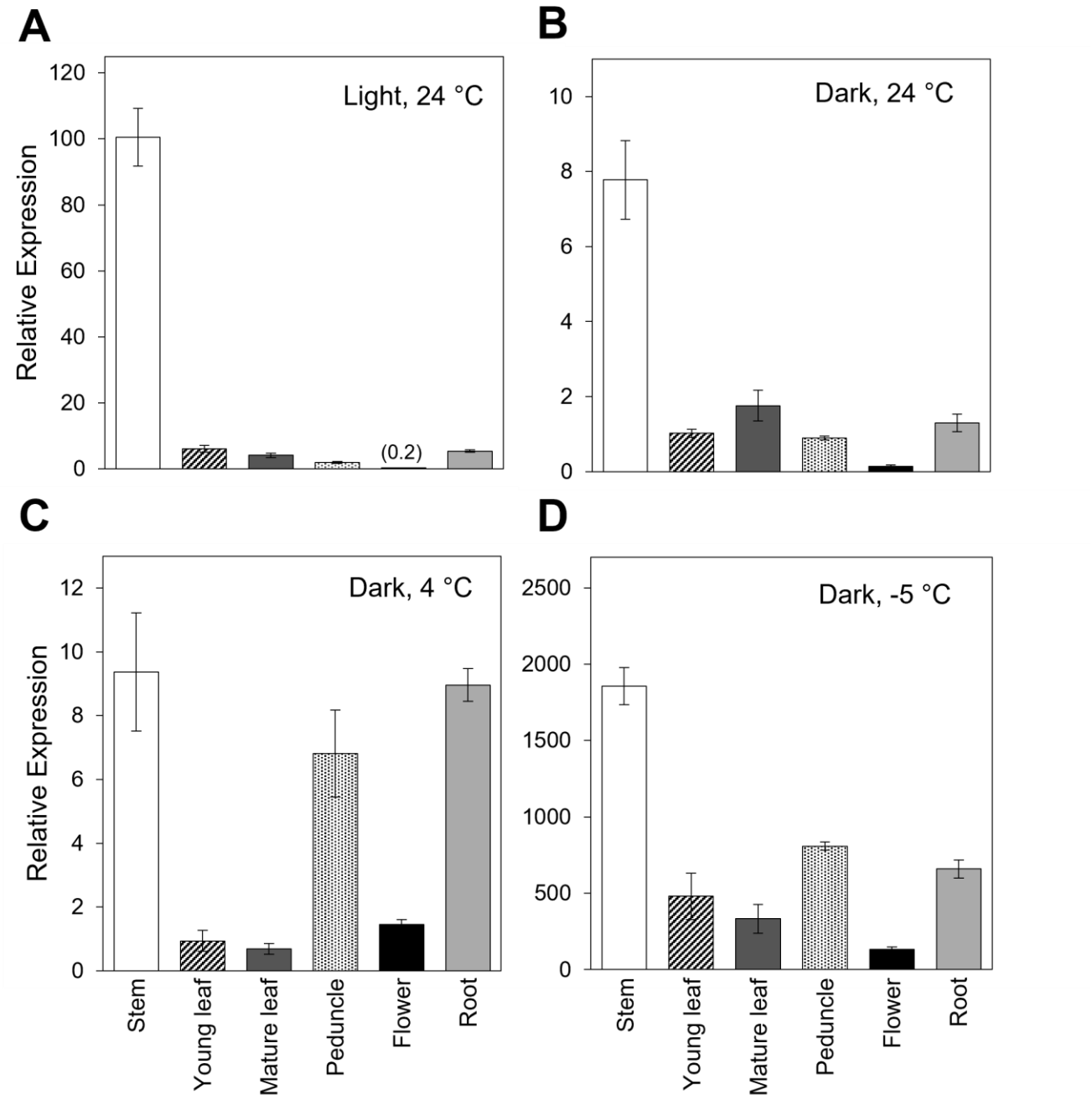

Figure 2. qPCR analysis of PaCBF4 transcript abundance in various organs of guayule. Bar charts show *PaCBF4* expression level from samples collected under light at 24°C (A), under dark at 24°C (B), under dark at 4°C (C), and under dark at -5°C (D). Relative expression in each organ was compared with stem (set at 100) collected under light at 24°C. Numbers in parentheses indicate relative expression levels. Data are representative of three independent experiments. Error bars represent ± SD of three technical replicates.

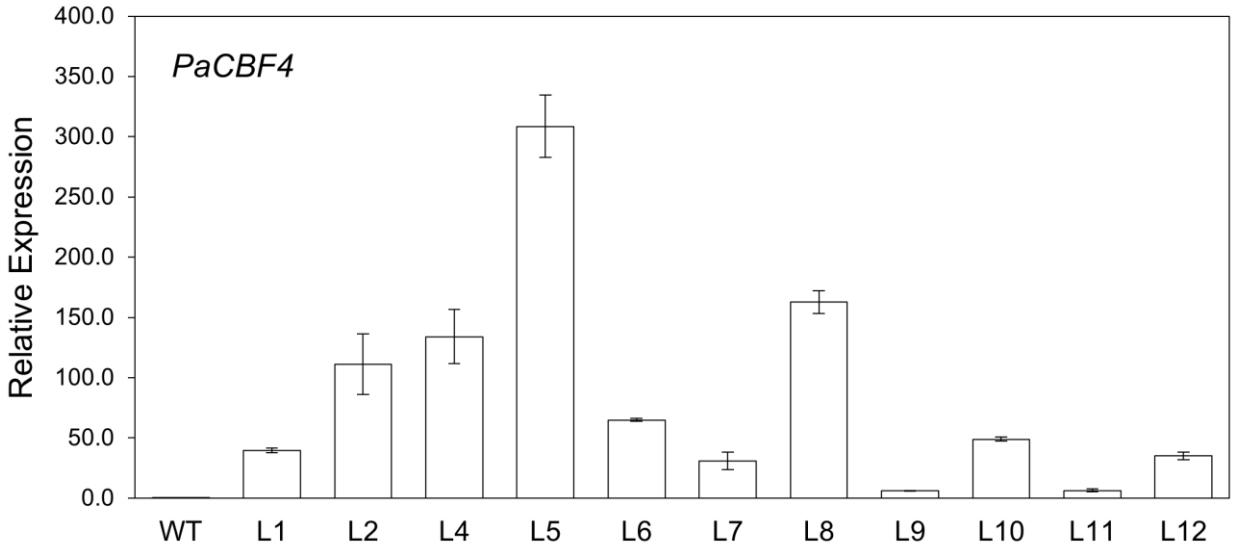

Figure 3. qPCR analysis of *PaCBF4* transcript abundance in Arabidopsis. WT, wild-type. ND, not detected. Relative expression of each T_3 line was compared to transgenic line 2 (L2) set at 100. Data are representative of three independent experiments. Error bars represent ± SD of three technical replicates.

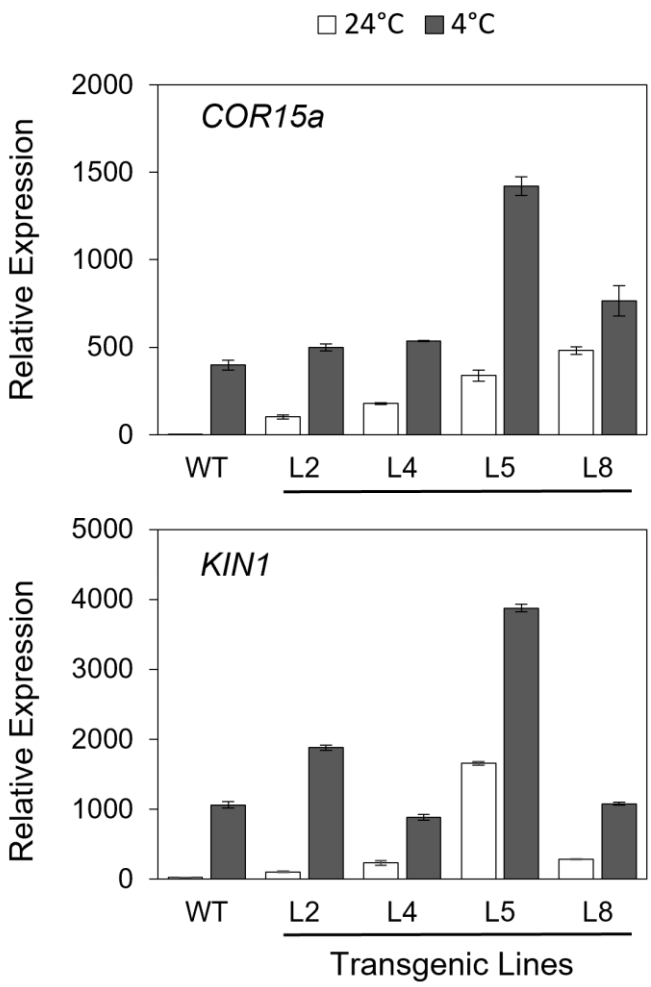

Figure 4. qPCR analysis of *COR* gene expression in Arabidopsis. Bar charts show *COR15a* (A) and *KIN1* (B) expression level from samples collected under 24°C (open bar) and 4°C (solid bar). WT, wild-type. Numbers in parenthesis indicate relative expression level. Relative expression of each T_4 line was compared to transgenic L2 sample (set at 100). Data are representative of three independent experiments. Error bars represent ± SD of three technical replicates.

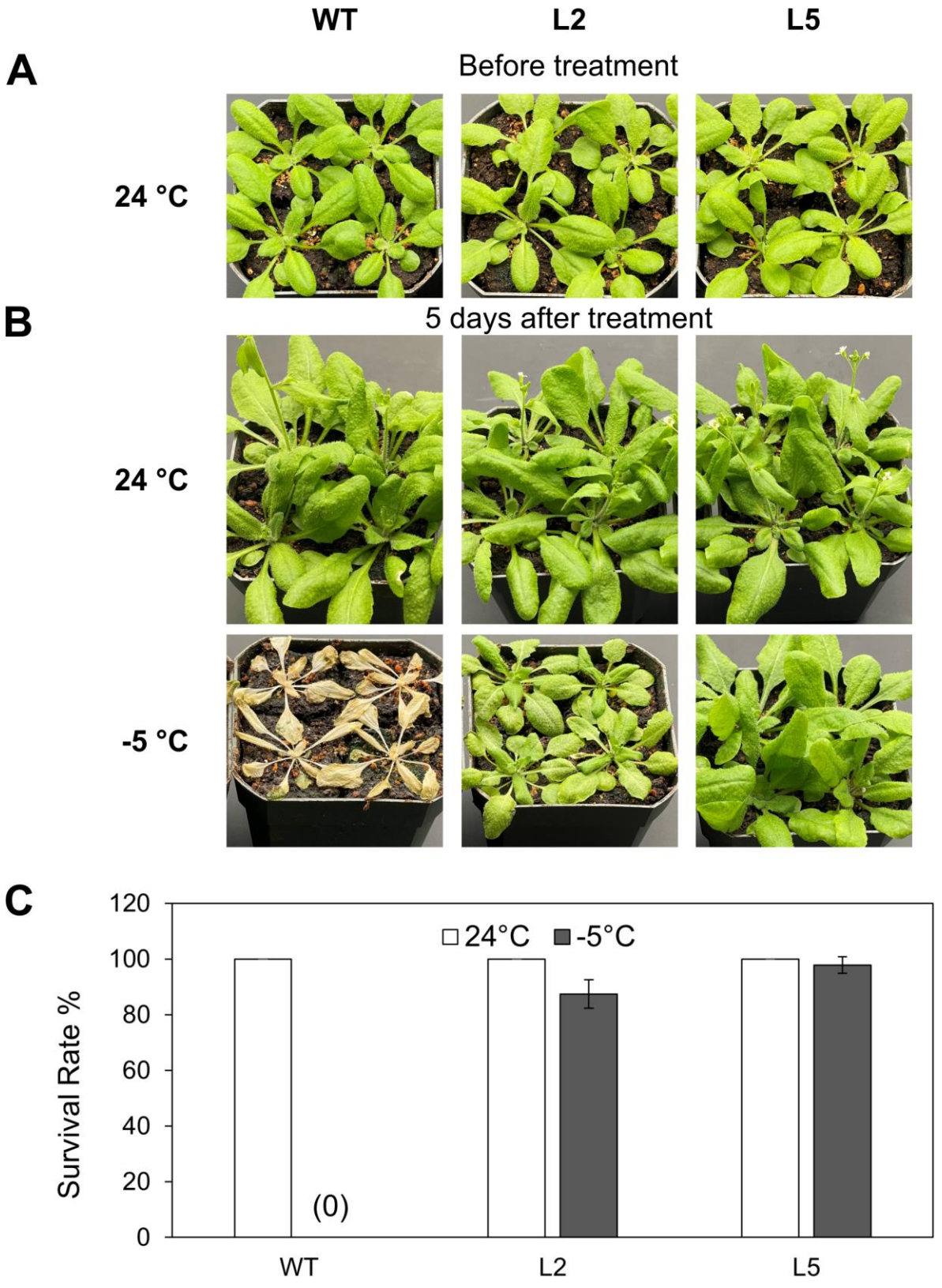

Figure 5. Freezing tolerance of wild-type and transgenic L2 and L5 plants. (A) Photos of 23-day-old plants growing under normal 24°C before freezing treatment. (B) Photos of plants exposed to -5°C for 24 h and then returned to 24°C for 5 recovery days. (C) Survival rate, scored as the percentage of plants showing healthy leaves after 5 days recovery from the freezing treatment (solid bar). Non-freezing controls were grown under 24°C (open bar). Number in parenthesis indicate survival rate of 0% for the WT. Data are mean ± SD of three independent experiments. Each treatment had 64 individuals.

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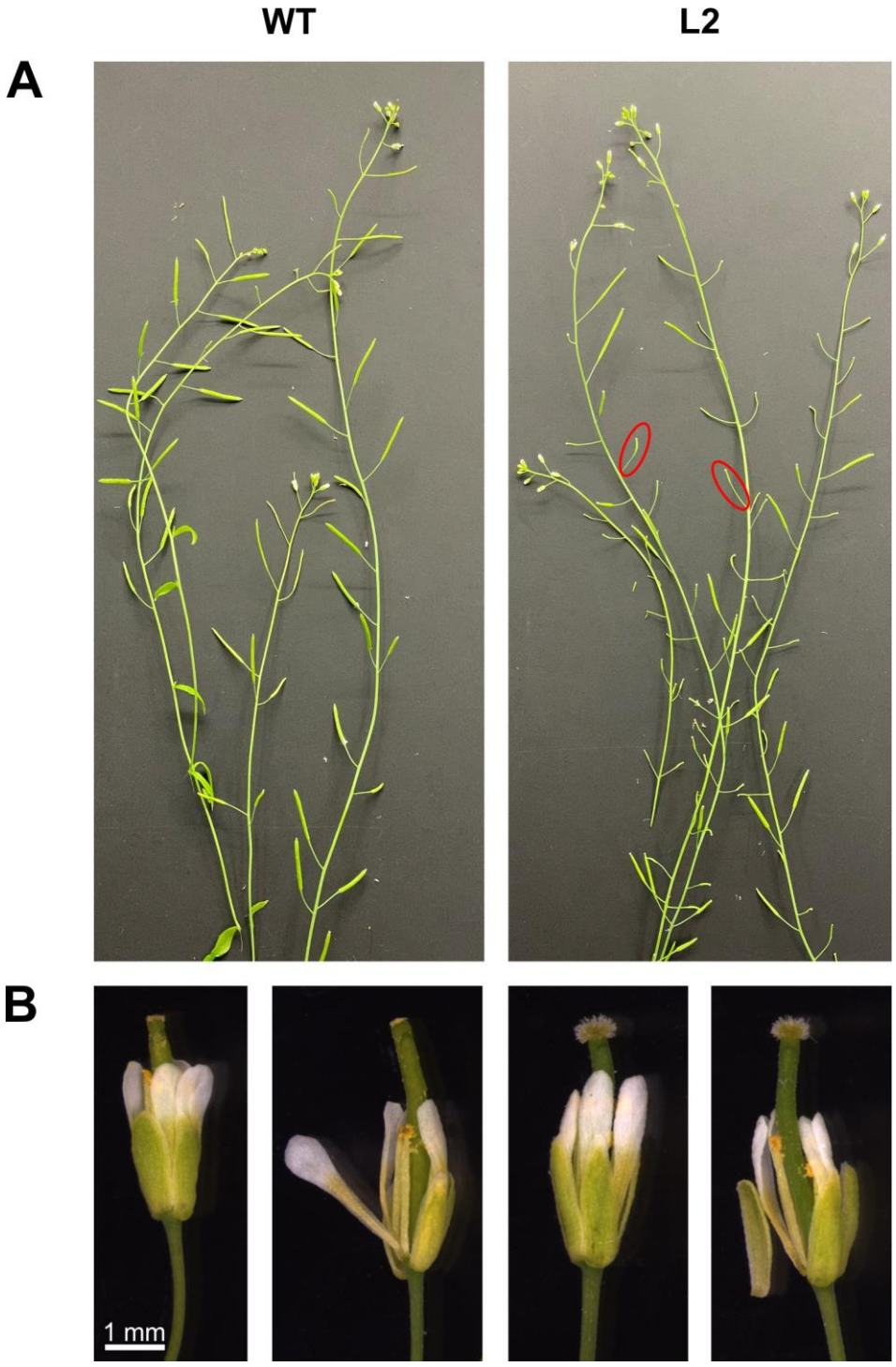

Figure 6. Inflorescence of wild-type and transgenic L2 plants showing reduced size of siliques and unpollinated flowers. 45-day-old plants were grown under normal 24°C continuous light conditions. Examples of reduced siliques are indicated by red circles (A). Unpollinated flowers are displayed in L2 (B).

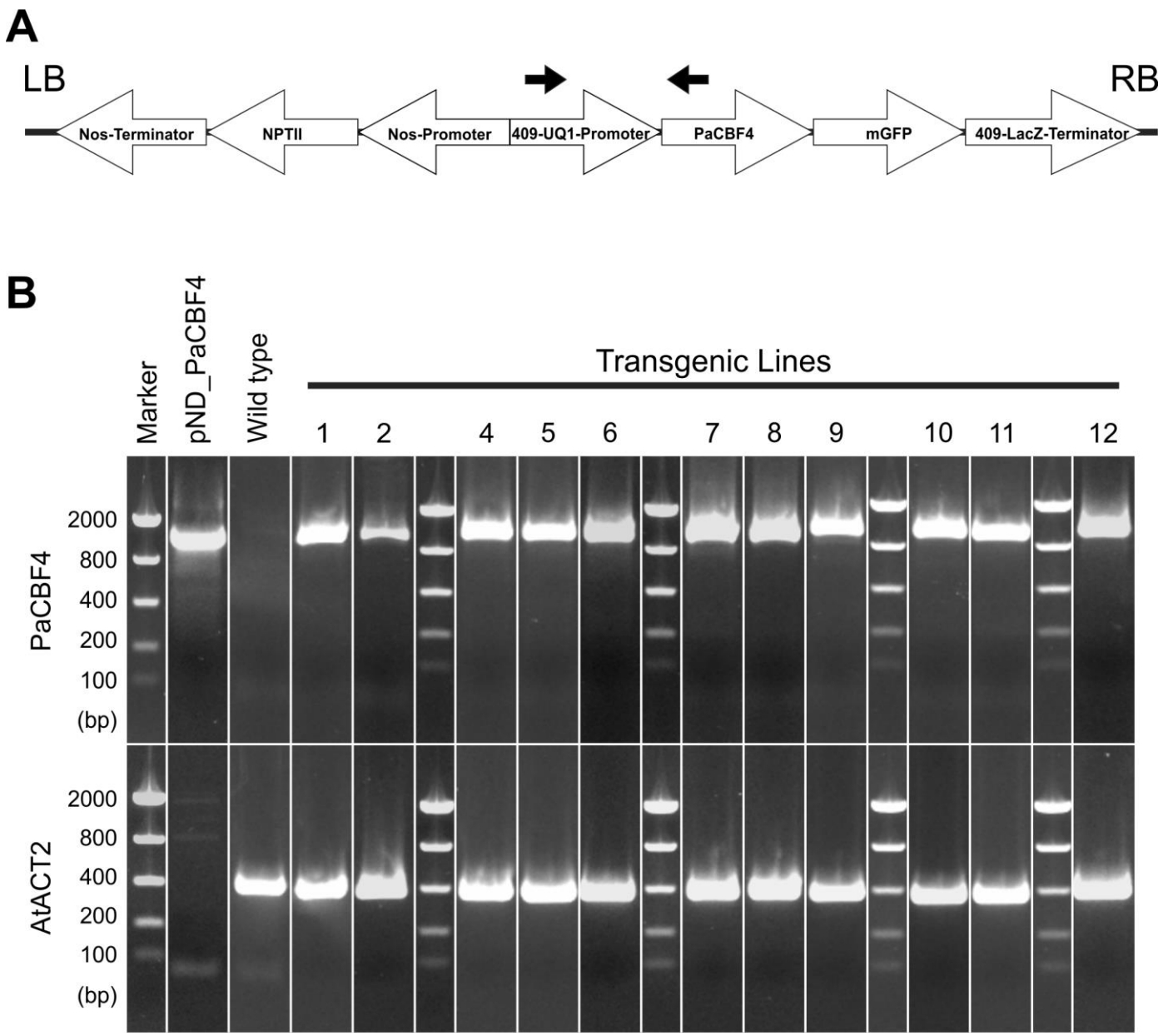

Figure S1. Schematic presentation of the T-DNA construct in *pND_PaCBF4* plasmid (A) and genomic DNA PCR identification of *PaCBF4* (B). Black solid arrows indicate the primers' locations for amplifying a PCR product (892 bp). Arabidopsis actin2 gene was used as an internal control with a PCR product (371 bp).

Table S1. Primer information for qPCR

Gene and primer name	Genbank ID	primer pairs (5' to 3')
PaEF1a-F	KU176069.1	CACAGCAAACCGACCAAGTG
PaEF1a-R		CGACAGACGATCCGGTAAGG
PaCBF4-F	GFTW01034449.1	TGCAGCACCGGGAAACTAAT
PaCBF4-R		CCCAGCCACACTCTCGATTT
AtACT2-F	NM_112764.4	GGTAACATTGTGCTCAGTGGTGG
AtACT2-R		AACGACCTTAATCTTCATGCTGC
AtCOR15-F	AY057640.1	GTCGTCGTTTCTCAACGCAAGA
AtCOR15-R		GCTTTCTCAGCTTCTTTACCCA
AtKIN1-F	NM_121601.3	ATGCCTTCCAAGCCGGTCAGAC
AtKIN1-R		CCGGTCTTGTCCTTCACGAAGT

amplican length (bp)	Eff%
142	91.06
170	94.64
109	98.68
213	97.41
170	98.27

supplemental Table S2

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	AtCBF2 [N	AtCBF3 [N	AtCBF1 [N	AtCBF4 [N	PaCBF4 [G	TkCBF1 [G
AtCBF2 [NP_567719.1]		87.16	87.10	65.78	52.75	52.75
AtCBF3 [NP_567720.1]	12.89		86.24	65.33	55.30	55.76
AtCBF1 [NP_567721.1]	13.02	12.55		65.78	54.63	54.84
AtCBF4 [NP_200012.1]	36.88	37.55	36.62		55.00	54.22
PaCBF4 [GFTW01034449.1]	57.87	54.84	53.83	52.96		65.70
TkCBF1 [GWHTAAAA022478]	61.62	56.71	56.98	59.87	41.79	
TkCBF6 [GWHTAAAA034733]	65.43	66.88	63.93	62.68	49.61	54.10
PtCBF4 [ABP64695.1]	58.45	58.02	55.55	55.55	51.27	50.71
MdCBF/DREB4 [AGL07696.1]	59.91	58.22	57.75	62.29	57.81	54.78
CaDREB [AHL69786.1]	70.24	67.48	66.99	69.31	60.94	59.91
EgDREB1F [XP_10038979.1]	90.85	92.15	89.26	95.04	86.22	76.85
HbDREB1A [XP_21691379.1]	50.62	46.81	47.38	48.03	49.61	49.03
HbDREB1B-like [XP_21655860.1]	86.75	84.40	80.59	83.24	80.44	73.09
HbDREB1F-like [XP_21655864.1]	92.89	90.39	86.44	89.16	86.50	78.74
HbDREB1F-like [XP_21658826.1]	86.75	80.97	81.73	84.73	75.91	69.31
HaDREB1F [XP_22008098.1]	96.14	92.37	86.07	91.87	82.00	78.34
HaDREB1D [XP_21984743.1]	44.75	39.61	39.92	38.34	9.10	27.74

Upper: Percent Identity
Lower: Evolutionary Divergence

TkCBF6 [C	PtCBF4 [Al	MdCBF/DF	CaDREB [A	EgDREB1F	HbDREB1	HbDREB	HbDREB1F	HbDREB1F	HaDREB1F
48.86	53.23	48.96	48.72	39.07	57.80	43.06	41.51	39.22	38.07
47.49	54.23	49.79	50.00	38.60	58.99	44.23	41.83	40.95	40.55
47.91	54.23	51.05	49.13	41.04	60.93	44.50	41.63	41.08	42.33
46.46	54.37	48.98	50.00	42.15	59.91	43.72	41.86	39.22	42.15
59.42	57.81	48.74	51.29	39.51	58.80	43.48	41.63	40.43	44.44
56.76	59.69	51.41	51.82	42.08	59.23	44.93	42.51	41.18	46.08
	51.83	47.20	44.00	37.31	48.50	41.06	38.65	37.77	37.27
62.38		58.11	54.84	40.84	61.81	46.77	44.28	42.54	44.00
62.92	43.48		51.37	36.60	54.76	44.16	41.56	45.38	40.59
78.85	45.95	62.42		37.12	54.76	42.67	40.44	42.45	39.06
96.44	81.39	95.75	101.94		41.59	51.87	49.53	45.45	45.54
64.28	47.22	54.03	60.84	89.03		45.89	43.00	44.02	44.44
82.15	73.35	81.22	89.44	51.79	80.47		95.63	50.21	52.15
87.02	79.41	86.87	93.12	56.36	86.25	4.47		48.09	50.24
82.97	64.30	71.78	83.44	64.66	77.15	57.21	60.78		48.51
94.95	74.47	87.08	89.28	73.05	81.34	62.19	65.92	67.37	
35.40	47.76	39.93	44.67	67.24	33.90	59.84	64.78	56.80	64.84

HaDREB1D [XP_21984743.1]

54.50
57.14
56.08
55.21
80.77
70.79
63.84
56.59
50.70
50.49
44.32
62.23
44.74
43.16
40.09
43.16

Table. Estimates of Evolutionary Divergence between Sequences

The number of amino acid substitutions per site from between sequences are shown. Analyses were conducted using the Poisson correction model [1]. This analysis involved 18 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 305 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2]

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Declaration of Interest Statement

The authors declare no conflicts of interest.