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Cold Response in *Phalaenopsis aphrodite* and Characterization of *PaCBF1* and *PaICE1*

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Phalaenopsis* is a winter-blooming orchid genus commonly cultivated in tropical Asian countries. Because orchids are one of the most economically important flower crops in Taiwan, it is crucial to understand their response to cold and other abiotic stresses. The present study focused on gene regulation of *P. aphrodite* in response to abiotic stress, mainly cold. Our results demonstrate that *P. aphrodite* is sensitive to low temperatures, especially in its reproductive stage. We found that after exposure to 4°C, plants in the vegetative stage maintained better membrane integrity and photosynthetic capacity than in the flowering stage. At the molecular level, *C-repeat binding factor1* (*PaCBF1*) and its putative target gene *dehydrin1* (*PaDHN1*) mRNAs were induced by cold, whereas *inducer of CBF expression1* (*PaICE1*) mRNA was constitutively expressed. PaICE1 transactivated MYC motifs in the *PaCBF1* promoter, indicating that up-regulation of *PaCBF1* may be mediated by the binding of PaICE1 to MYC motifs. Overexpression of *PaCBF1* in transgenic Arabidopsis induced *AtCOR6.6* and *RD29a* without cold stimulus and maintained better membrane integrity after cold stress. Herein, we present evidence that cold induction of *PaCBF1* transcripts in *P. aphrodite* may be transactivated by PaICE1 and consequently protect plants from cold damage through up-regulation of *cold-regulated* (*COR*) genes, such as *DHN*. To our knowledge, this study is the first report of the isolation and characterization of *CBF*, *DHN* and *ICE* genes in the Orchidaceae family.

Keywords: C-repeat binding factor (CBF) • Cold stress • Dehydrin (DHN) • Inducer of CBF expression (ICE) • MYC motif • *Phalaenopsis aphrodite*.

Abbreviations: ABRE, ABA-responsive element; ACT, actin; AP2/ERF, apetala 2/ethylene response factor; bHLH, basic helix–loop–helix; BLAST, basic local alignment search tool; CAM, crassulacean acid metabolism; CaMV, cauliflower mosaic virus; CBF, C-repeat binding factor; COR, cold-regulated; DHN, dehydrin; DREB, dehydration responsive element binding protein; EGFP, enhanced green fluorescent protein; GUS, β-glucuronidase; ICE, inducer of CBF expression; MS, Murashige and Skoog; 4-MUG, 4-methylumbelliferyl glucuronide; ORF, open reading frame; PEG, polyethylene glycol; qRT–PCR, quantitative real-time reverse transcription–PCR; RACE, rapid amplification of cDNA ends; R stage, reproductive stage; RLuc, *Renilla* luciferase; RT–PCR, reverse transcription–PCR; SUMO, small ubiquitin-like modifier; TSS, transcription start site; UTR, untranslated region; V stage, vegetative stage.

The nucleotide sequences reported in this paper have been submitted to the GenBank database under the accession numbers KF574003 (*PaCBF1*), KJ586125 (*PaDHN1*) and KF574004 (*PaICE1*).

Introduction

Phalaenopsis aphrodite Rchb.f. subsp. *formosana*, one of the most popular orchids in the trade, belongs to the family Orchidaceae, subfamily Epidendroideae, tribe Vandeae, subtribe Aeridinae, and genus *Phalaenopsis* (Christenson 2001). It is an ornamental species of great economic importance. Official export statistics show that sales of *Phalaenopsis* brought >US$88 million per year to the Taiwan flower industry during the past 3 years. The genus *Phalaenopsis* is adapted to tropical and subtropical climates, though variation in environmental conditions in habitats exists among species. It is classified as a crassulacean acid metabolism (CAM) plant (Endo and Ikusima 1989).

Low temperatures adversely affect the development of *P. aphrodite* (Lee et al. 2007) and significantly reduce the commercial value of this winter-blooming orchid. In early winter, the reduction in temperature and temperature difference between day and night induce inflorescence production in *P. aphrodite*. Inflorescences of *Phalaenopsis* are commonly induced when plants are under a 25/20°C or 20/15°C temperature regime for 1 month, and this strategy is often used in the orchid flower industry. However, temperatures lower than 10°C during the coldest months in Taiwan and the dramatic temperature fluctuations during export to temperate-zone markets may cause severe damage to orchid plants. *Phalaenopsis* species which originate in tropical or subtropical areas have been cultivated in temperate regions through advances in agronomic management, but are less tolerant to low temperatures due to evolutionary differences in their genetic make-up. In addition to the improvement of agricultural facilities and transportation, a better understanding of the effects of cold temperatures on orchid growth may provide new strategies to reduce damage

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to plants. Chilling temperatures between 0 and 15°C in tropical and subtropical regions usually result in growth retardation, discoloration, lesions on leaves, tissue breakdown and/or senescence (Lyons 1973). Leaves from chilling-injured plants showed membrane injury (Willing and Leopold 1983, Chen et al. 2008), inhibition of photosynthesis (Tjus et al. 1998, Allen and Ort 2001), accumulation of reactive oxygen species (ROS) (Bowler et al. 1992, Airaki et al. 2012), slower carbohydrate translocation and lower respiration rates (Bunce 2007).

In response to low temperature, signaling pathways regulate gene expression and post-translational modification. A *C-repeat* (*CRT*) or *dehydration responsive element* (*DRE*) is commonly found in the promoter regions of cold-regulated and drought-responsive genes, and is bound by CRT binding factor (CBF)/DRE binding (DREB) protein. CBF/DREBs belong to the apetala 2/ethylene response factor (AP2/ERF) protein family (Miura and Furumoto 2013). The Arabidopsis CBF/DREB1 family consists of six paralogs, but only *CBF1* (*DREB1B*), *CBF2* (*DREB1C*) and *CBF3* (*DREB1A*) are cold inducible and physically linked in chromosome 4 (Gilmour et al. 1998). Through the AP2/ERF domain, CBFs bind to *CRT/DRE* elements (G/ACCGAC) in the promoter of *cold-regulated* (*COR*) genes and induce their expression, which contributes to low temperature tolerance in Arabidopsis (Baker et al. 1994, Yamaguchi-Shinozaki and Shinozaki 1994, Stockinger et al. 1997, Nylander et al. 2001). CBFs also elevate the levels of proline and total sugar (Gilmour et al. 2000) to protect proteins and membranes from freezing damage (Pearce 2001). Overexpression of *AtCBF1* or *AtCBF3* genes in *Arabidopsis thaliana* enhances plant tolerance to freezing and drought (Jaglo-Ottosen et al. 1998, Liu et al. 1998), and overexpression of *OsDREB1* in *Oryza sativa* plants enhances tolerance to freezing and high salinity (Dubouzet et al. 2003). However, Arabidopsis CBF2 is a negative regulator of *CBF1* and *CBF3* (Novillo et al. 2004).

In Arabidopsis, *CBF/DREB* genes are positively regulated by inducers of CBF expression (ICEs) through specific binding to the MYC elements in the promoter (Chinnusamy et al. 2003). The *ice1* mutation blocks *AtCBF3* expression and significantly reduces plant tolerance to low temperatures, and overexpression of *AtICE1* and *AtICE2* enhances the expression of *AtCBF3* and *AtCBF1* under cold stress and improves freezing tolerance (Chinnusamy et al. 2003, Fursova et al. 2009). ICE proteins which belong to the MYC family of transcription factor have a basic helix–loop–helix (bHLH) domain for binding to the MYC consensus sequence, CANNTG (E-box), in the promoter region. In plants, 74% of the analyzed bHLH proteins have a critical E9 residue (Pires and Dolan 2010) that contacts the CA nucleotides of the hexanucleotide sequence (Ferré-D'Amaré et al. 1993). AtICE1 protein is degraded through an ubiquitination–proteasome pathway mediated by a RING finger ubiquitin E3 ligase HOS1 (high expression of osmotically responsive gene 1) (Dong et al. 2006, Miura et al. 2011). On the other hand, cold-induced sumoylation of AtICE1 is mediated by a SUMO E3 ligase AtSIZ1, and activates CBF3-dependent cold signaling and represses MYB15 transcription (Miura et al. 2007).

Plants modify their gene expression for the products which directly protect cells against environmental stresses. Of the low temperature-responsive genes characterized to date, several have been predicted to encode highly hydrophilic proteins, dehydrins (DHNs), which were classified as group II of late embryogenesis abundant (LEA) proteins (Rorat 2006). All DHNs have a distinctive conserved sequence, lysine-rich 15 amino acid domain, EKKGIMDKIKEKLPG, named the K-segment. DHNs are found in nearly all the vegetative tissue in response to cellular dehydration during drought, low temperature and salinity. A wheat DHN (WCOR410) associated with the plasma membrane protects the freeze-labile structure of the plasma membrane (Danyluk et al. 1998), and overexpression of WCOR410 improves freezing tolerance in transgenic strawberry (Horde et al. 2004).

Genome information is currently available on model plants such as Arabidopsis and rice. The non-model plant *Phalaenopsis* has unique biological characteristics which enable different aspects of plant physiology to be studied, and two gene expression databases of orchid were published in 2013. The Orchidstra database contains transcriptome information for five orchid species and one commercial hybrid, with assigned gene descriptions and functional annotation to protein-coding genes (Su et al. 2013). The OrchidBase 2.0 contains a collection of floral transcriptomes derived from 10 orchid species across five subfamilies of Orchidaceae (Tsai et al. 2013). These databases provide rich genomic information for investigations of orchid gene function. The aim of this study was to examine the cold-stress responses in *P. aphrodite* through analyses of physiological processes and to identify important cold-induced genes. Here we present the first analysis of the expression of a *Phalaenopsis CBF* gene, its potential regulator PaICE1 and one putative downstream gene *PaDHN1*. Our results showed that low temperature affects cell membrane integrity and photosynthesis efficiency, and accumulates *PaCBF1* transcripts. This study concluded that the *P. aphrodite PaCBF1* is a cold stress-inducible gene, which may be activated by PaICE1 and may induce the expression of *PaDHN1* and other *COR* genes to enhance tolerance to cold.

Results

Exposure to 4°C decreased cell membrane stability and photosynthetic efficiency in *P. aphrodite* plants

To study the effect of low temperature on *P. aphrodite*, plants in reproductive (R) and vegetative (V) stages were subjected to 4°C for different time periods, and then allowed to recover at 25°C for 2 d. Necrosis resulting from exposure to 4°C for 12–24 h was detected in leaves after 24 h recovery at 25°C (**Fig. 1D** vs. **A**). The growth rate of root tips decreased after 8 h cold treatment (**Fig. 1F** vs. C) and they withered after 12 h at 4°C (**Fig. 1G** vs. C). The floral buds and fully opened flowers were undamaged up to 48 h at 4°C, and wilted 2 d after recovery (**Fig. 1E** vs. **B**).

The effect of 4°C on the cell membrane stability of *P. aphrodite* plants at the two different stages was estimated by ion leakage to assay chilling injury. Low temperature did not cause significant damage to flower petals at the R stage, nor to leaves at the V stage up to 48 h (**Fig. 2A**). However, electrolyte leakage in

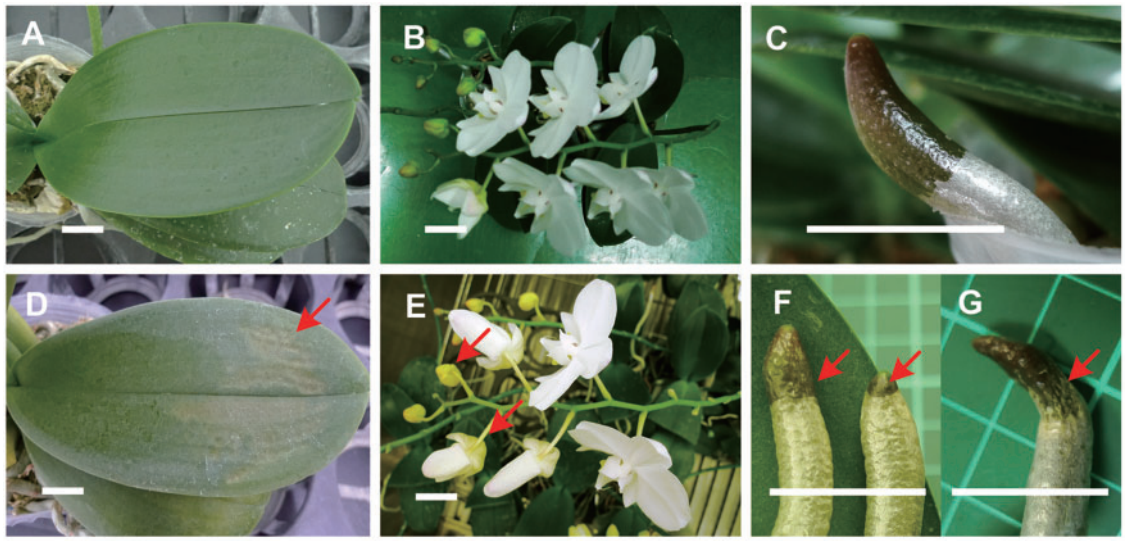

Fig. 1 Symptoms of low temperature damage to *P. aphrodite* plants. Plants were exposed to 4°C for different time periods, and then allowed to recover at 25°C for 2 d. Control plants were kept at 25°C (A–C). Cold stress caused damage to leaves (D, 4°C for 24 h), flowers (E, 4°C for 48 h) and root tips (F, 4°C for 8 h; G, 4°C for 12 h). Images were recorded with a digital camera and cold damage is indicated by arrows. Scale bar = 2.5 cm.

leaves during the R stage was significantly increased by exposure to 4°C in a time-dependent manner (**Fig. 2A**, $P < 10^{-22}$, $n = 3$), with a strong positive correlation between cold-induced injury and exposure time (**Fig. 2A**, $y = 0.577x + 16.322$, $r^2 = 0.884$). The extent of cell membrane damage reached a maximum (56.5 ± 1.9%) at 72 h, and then recovered to a basal level after returning to 25°C for 24 h. Our results showed that the cell membrane stability of *P. aphrodite* leaves at the R stage is more sensitive to low temperature than at the V stage (**Fig. 2A**). Nevertheless, full recovery of membrane stability in cold-stressed R stage leaf tissue was achieved.

The F_v/F_m ratio (maximal quantum yield of PSII), which evaluates photosynthetic capacity as a stress indicator (Krause and Weis 1991, Ferrante et al. 2007), was used to monitor low temperature effect on photosynthetic efficiency of the CAM plant *P. aphrodite*. In general, the efficiency of the maximal quantum yield in C_3 is similar to that in C_4 or CAM plants (Björkman and Demmig 1987). In our experiments, *P. aphrodite* plants grown at 25°C displayed a quantum yield of 0.75 ± 0.01 with little or no detectable variation during daytime, whereas blooming plants (R stage) grown at 25°C showed a greater quantum yield (0.77 ± 0.01, **Fig. 2B**) than V stage plants ($P < 10^{-4}$, $n = 28$). Exposure to 4°C for 2 h significantly lowered the F_v/F_m ratios of R stage (0.67 ± 0.05, $n = 4$) and V stage plants (0.70 ± 0.04). The reduction in quantum yield at 4°C was time dependent from 2 to 48 h and was linear after logarithmic transformation of F_v/F_m. The linear equation is $y = -0.068 \times \log x + 0.723$ for R stage plants, and $y = -0.038 \times \log x + 0.704$ for V stage plants (**Table 1**). The decrease in quantum yield was more pronounced in R stage plants than in V stage plants ($P < 0.01$). The F_v/F_m ratio of R stage plants decreased to 0.47 ± 0.05 after 48 h at 4°C, and those at V stage decreased to a lesser extent (0.53 ± 0.02). The quantum yield of V stage plants was restored to the original level after recovery for 24 h at 25°C (**Fig. 2B**); however, R stage plants displayed a lower F_v/F_m value (0.67 ± 0.01, $P < 0.05$) after recovery. The 13% decrease in the F_v/F_m ratio indicates that only partial recovery occurred in R stage plants, and 4°C caused irreversible damage of PSII in *P. aphrodite* leaves.

Isolation and characterization of *PaCBF1* and *PaICE1* genes from *P. aphrodite*

Thus far we have shown that 4°C caused important physiological responses in *P. aphrodite* plants including tissue necrosis, cell membrane injury and decreased photosynthesis. Induction of *CBF* expression by low temperatures has been found in various plant species (Lata and Prasad 2011), which raised the question of whether low temperature regulates the *CBF* genes in *Phalaenopsis*. Using reverse transcription–PCR (RT–PCR) amplification with a degenerate primer pair (CBFP1 and P2, **Supplementary Table S1**) designed according to the conserved regions among the available monocot *CBF* cDNAs, a partial cDNA fragment of 543 bp was produced. Then a gene-specific primer (CBFP3, **Supplementary Table S1**) was used to obtain the 3′ end of *CBF* with RT–PCR. The 606 bp *CBF* open reading frame (ORF) was completed through 5′-rapid amplification of cDNA ends (RACE) and designated *PaCBF1*. The upstream sequence of *PaCBF1* was obtained by genomic DNA walking. A 1,007 bp product was amplified from genomic DNA using primer pairs at the 5′- and 3′-untranslated regions (UTRs) of *PaCBF1* (**Supplementary Table S1**), and comparative analysis of the cDNA sequence with genomic sequence confirmed that *PaCBF1* is an intronless gene. In the 5′-RACE experiments, the transcription start site (TSS) was determined to be located 74 bp upstream of the start codon and 35 bp downstream of a putative TATA box (AATTTAAA) (**Supplementary Fig. S1**). To determine the copy number of the *PaCBF1* gene in *P. aphrodite*, Southern hybridization was performed with

restriction enzyme-digested genomic DNA using the *PaCBF1* cDNA as a probe (**Supplementary Fig. S2**). *Eco*RI, which has no cutting site in the *PaCBF1* gene, produced only one fragment. *Bss*SI-digested products showed three major bands, i.e. fragments Bs1 (623 bp, sites 2527 and 3150), Bs2 (1,023 bp, sites 2137 and 3150) and Bs3 (~2,200 bp, site 3150 and a downstream site). Our results suggest that only one copy of *PaCBF1* is present in the *P. aphrodite* genome. The nucleotide sequence of *PaCBF1* cDNA is 99.0% identical to the *PATC128464* sequence in the Orchidstra database (Su et al. 2013), and 26.2–63.7% identical to another 12 homologs (**Supplementary Table S2**). The predicted PaCBF1 protein is a 22.5 kDa peptide of 201 amino acids (pI 5.26) and shares 100% identity with the deduced PATC128464 and 22.2–58.9% identity with the other 12 *P. aphrodite* homologs (**Supplementary Table S2**). Our results suggest that *PaCBF1* and *PATC128464* cDNAs are the same genes since they only contain six different nucleotides. The highly conserved AP2/ERF DNA-binding domain is located between amino acids 38 and 88 in PaCBF1 (**Supplementary Fig. S1**) by using the Pfam database (Punta et al. 2012) at an E-value cut-off of 10^{-12}. The CBF signature sequences, PKK/RPAGRxKFxETRHP and DSAWR, which bracket the AP2/ERF DNA-binding domain (Jaglo et al. 2001), exhibit more species diversity in monocots than in dicots. The eighth residue of the PKK/RPAGRxKFxETRHP sequence shows a strong preference for T in monocots and for K in dicots (**Supplementary Fig. S3A**). PATC072278 and PATC073314 that contain the CBF signature sequences share 58.9% and 57.4% identity with PaCBF1. Moreover, PaCBF1 protein shares 54.3% and 42.9% identity with *Hordeum vulgare* HvCBF1 (AAL84170, Xue 2002) and HvCBF3 (AAG59618, Choi et al. 2002), 56.0, 47.8, 42.8 and 34.1% identity with rice OsDREB1C (BAA90812), OsDREB1B (AAN02488) OsDREB1A (AAN02486) and OsDREB1D (BAD67595), respectively, and 48.1, 47.3, 46.4 and 45.9% identity with Arabidopsis CBF4 (NP_200012), CBF3 (NP_567720), CBF1 (NP_567721) and CBF2 (NP_567719), respectively. Phylogenetic analysis was performed using *P. aphrodite* CBF homologs and AP2/ERF proteins that have been functionally studied. PaCBF1 and PATC128464 are clustered with CBF/DREB1 proteins of monocots including *O. sativa*, *H. vulgare*, *Triticum aestivum* and *Zea mays* (group III-1, **Supplementary Fig. S3B1**), and the dicot CBF/DREB1s are grouped into III-2. PATC152393, PATC124683, PATC129187 and PATC127074 are in group II with AtERFs. PATC127020, PATC149783, PATC130017, PATC038797 and PATC068318 are grouped with AtTINYs. Low bootstrap values were found within the branches containing PATC072278, PATC073314 and PATC127667 (**Supplementary Fig. S3B2**), indicating that the clustering of PATC072278, PATC073314 and PATC127667 might be less significant. Removal of these three proteins from the phylogenetic analysis supports the dendrogram with high bootstrap values, suggesting an elevated reliability of the pattern (**Supplementary Fig. S3B1**). It appears the AP2 family

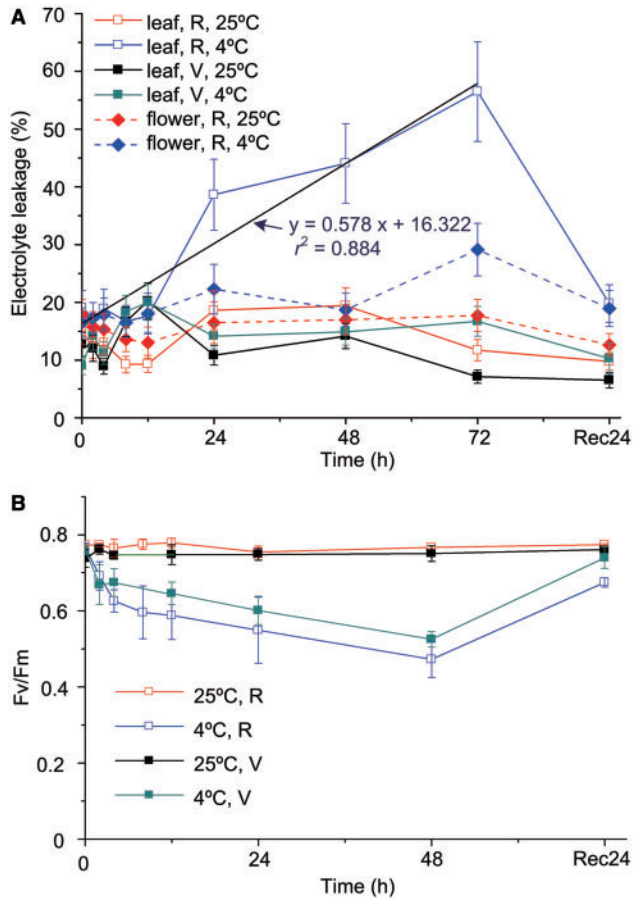

Fig. 2 Effects of cold stress on cell membrane stability and photosynthesis of *P. aphrodite*. (A) Plants were grown at 4 and 25°C at the R stage (R) and the V stage (V). Leaves or flowers were harvested at 0, 2, 4, 8, 12, 24, 48 and 72 h after cold treatment. After 72 h, plants were moved to 25°C for 24 h and are referred to as recovery samples (Rec). The control plants were kept at 25°C and samples were harvested at the same time points as the cold-treated samples. The percentage electrolyte leakage of blooming leaves vs. time was analyzed using linear regression. (B) The photosynthesis efficiency (F_v/F_m ratio) was measured with PAM-2100 using plants as described above after exposure to 4 and 25°C for different time intervals (0, 2, 4, 8, 12, 24 and 48 h). After 48 h cold stress, plants recovered for 24 h (Rec). Bars indicate the means ± SD of at least three independent measurements.

Table 1 Statistical analysis of the F_v/F_m ratio vs. time in cold-stressed *P. aphrodite* plants

Coefficients	R stage, $y = -0.068 \times \log x + 0.723$			V stage, $y = -0.038 \times \log x + 0.704$		
	Estimate	SE	*P*-value	Estimate	SE	*P*-value
Intercept (y)	0.723	0.021	2.35×10^{-16}	0.704	0.024	3.10×10^{-13}
log (time)	−0.068	0.008	5.05×10^{-7}	−0.038	0.009	1.28×10^{-3}
R^2	0.802			0.563		

is less complex in *Phalaenopsis* than in Arabidopsis or rice. Among the 12 homologs extracted from the Orchidstra database, only PaCBF1/PATC128464 was grouped in CBF/DREB1 proteins with a high bootstrap value (>60%).

To isolate the *P. aphrodite ICE* gene, a primer pair (ICEP1 and P2, **Supplementary Table S1**) was designed according to the conserved bHLH domain for RT–PCR amplification and a 380 bp fragment was produced, then a fragment of 3,840 bp was obtained by genomic DNA walking and designated *PaICE1*. The *PaICE1* gene contains four exons and three introns (**Supplementary Fig. S4**), and the predicted TSS of *PaICE1* is 170 bp upstream of the start codon and 36 bp downstream of a putative TATA-box (TTTTATA). The nucleotide sequence of *PaICE1* cDNA shares 99.1% identity with *PATC134808*, and is 32.9–52.2% identical to the other 13 homologs (**Supplementary Table S3**) in the Orchidstra database (Su et al. 2013). The sequences of *PaICE1* and *PATC134808* have one different nucleotide in the 68 bp 5′-UTR and are identical in the 241 bp 3′-UTR (**Supplementary Fig. S5**); however, *PATC134808* has a single nucleotide insertion at both positions 300 and 337 downstream of the translational start codon, which shifts the reading frame of the gene and might be sequencing errors. Our results suggest that *PaICE1* and *PATC134808* are the same genes with 16 different nucleotides in their ORFs, and these differences could be due to variations in plants. The predicted PaICE1 protein contains 527 amino acids (56.4 kDa, pI 5.01) and shares 98.9% identity with the deduced PATC134808 and 14.3–41.0% identity with the other 13 *P. aphrodite* homologs (**Supplementary Table S3**). PaICE1 contains three potential sumoylation sites at residues 295, 368 and 426 using SUMOsp 2.0 (Ren et al. 2009) prediction (**Supplementary Fig. S4**). The most conserved sumoylation site in ICE proteins was found only in PaICE1 (K426) but not in the other 13 *P. aphrodite* homologs. PaICE1 shares 55.3% and 54.4% identity with AtICE2 (NP_172746) and AtICE1 (NP_189309), and 55.3% and 47.6% identity with TaICE41 (ACB69501, Badawi et al. 2008) and TaICE87 (ACB69502). The bHLH domain is located between amino acids 343 and 385 (**Supplementary Fig. S4**), classified by the Pfam database (Punta et al. 2012) at an E-value cut-off of 10^{-8}, and is highly conserved among species (**Supplementary Fig. S6A**). PaICE1 peptide was subjected to a homology search in NCBI using the DELTA-BLAST program to collect the MYC homologs with known function (E-value cut-off of 10^{-16}). Phylogenetic analysis permitted classification of PaICE1/PATC134808 with ICEs of monocots including the *Musa* AB Group and *T. aestivum* (group VI-1, **Supplementary Fig. S6B**). Although a complex MYC protein family exists in *P. aphrodite*, only one *P. aphrodite ICE* gene (PaICE1/PATC134808) has been identified thus far. To isolate the *DHN* gene, a pair of degenerate primers (DHNP1 and P2, **Supplementary Table S1**) was designed based on the consensus sequences of barley (X71362) and rice (AY786415) DHNs to obtain *DHN* with RT–PCR using a cDNA library constructed from cold-treated *P. aphrodite* as template. The partial *DHN* cDNA contains 217 bp encoding 72 amino acids, and a 2,380 bp fragment including full-length *DHN* was obtained by genomic DNA walking and designated *PaDHN1*. The comparative analysis of the cDNA sequence with the genomic sequence confirmed that *PaDHN1* is an intronless gene. The upstream sequence of *PaDHN1* was searched against the database of Plant Cis-acting Regulatory DNA Elements (PLACE; Higo et al. 1999), and we found three CBF-binding sites and nine MYC elements in the *PaDHN1* promoter (**Supplementary Table S4**). The predicted PaDHN1 protein contains 145 amino acids (15.5 kDa, pI 8.11) and shares 47.7% identity with the deduced PATC155965 and 37.7% identity with PATC157534 in the Orchidstra database (Su et al. 2013). PaDHN1 shares 53.9% identity with HvDHN7 (X71362), 53.4% identity with DcCAISE1 (AB105039), 51.5% identity with LeESI18-3 (AF031248) and 50.4% identity with CcDH1b (DQ323988) (**Supplementary Table S5**). Two K-segments (KIKEKLPG) and one S-segment (polyserine) are found in PaDHN1 (SK_2 type) (**Supplementary Fig. S7**).

Prediction with PSORT (Nakai and Kanehisa 1992), NLStradamus (Nguyen Ba et al. 2009), cNLS Mapper (Kosugi et al. 2009) and LOCtree (Nair and Rost 2005) programs indicated that the PaCBF1 contains a putative nuclear localization signal (NLS) at residues 21–33 (**Supplementary Fig. S1**). PaICE1 was predicted to contain two possible NLS motifs at residues 260–264 and 331–352 (**Supplementary Fig. S4**). To examine the subcellular localization of PaCBF1 and PaICE1, we introduced chimeric enhanced green fluorescent protein (EGFP) fusion constructs under the control of the cauliflower mosaic virus (CaMV) 35S promoter into the protoplasts of *P. aphrodite* flowers (**Fig. 3**). PaCBF1–EGFP and PaICE1–EGFP fusion proteins were detected mainly in the nucleus, implying that PaCBF1 and PaICE1 may be involved in regulation of gene expression. The EGFP (26.9 kDa) protein which can passively diffuse into the nucleus showed a whole-cell distribution, while PaCBF1–EGFP (50.2 kDa) and PaICE1–EGFP (82.9 kDa) may enter the nucleus with the help of their NLS.

Cold stress significantly increased the transcript level of *PaCBF1* and *PaDHN1* but not *PaICE1*

PaCBF1 forms a monophyletic clade with other monocot CBF/DREB1 proteins, which raised the question of whether PaCBF1 responds to cold. Gene-specific primer pairs were designed based on non-conserved regions in homologous genes for RT–PCR analysis using *PaACT1* as an endogenous control for relative quantification of the total amount of mRNA (**Supplementary Table S1**). Cold induction of *PaCBF1* mRNA was found in *P. aphrodite* leaves (**Fig. 4A**). *PaCBF1* transcripts at the R stage (**Fig. 4B**) and V stage (**Fig. 4C**) responded to cold up to 48 h in quantitative real-time RT–PCR (qRT–PCR). In R stage plants, *PaCBF1* transcripts increased 12.0 ± 1.5-fold at 4 h (**Fig. 4B**, $P < 10^{-11}$, $n = 3$), then increased to 76.6 ± 8.4-fold at 12 h and followed a decline to 16.8 ± 2.5-fold at 24 h. Subsequently, the *PaCBF1* transcripts decreased to 1.9 ± 1.1-fold after 48 h cold stress. *PaDHN1* transcripts accumulated slowly and reached the highest level at 24 h (4.8 ± 1.3-fold, $P < 10^{-5}$, $n = 3$). Similarly, cold induction of *PaCBF1* and *PaDHN1* was detected in V stage plants (**Fig. 4C**) with a maximum at 12 h (15.6 ± 2.7-fold, $P < 10^{-7}$, $n = 3$) and at 24 h (3.6 ± 1.3-fold, $P < 10^{-3}$), respectively, and decreased thereafter.

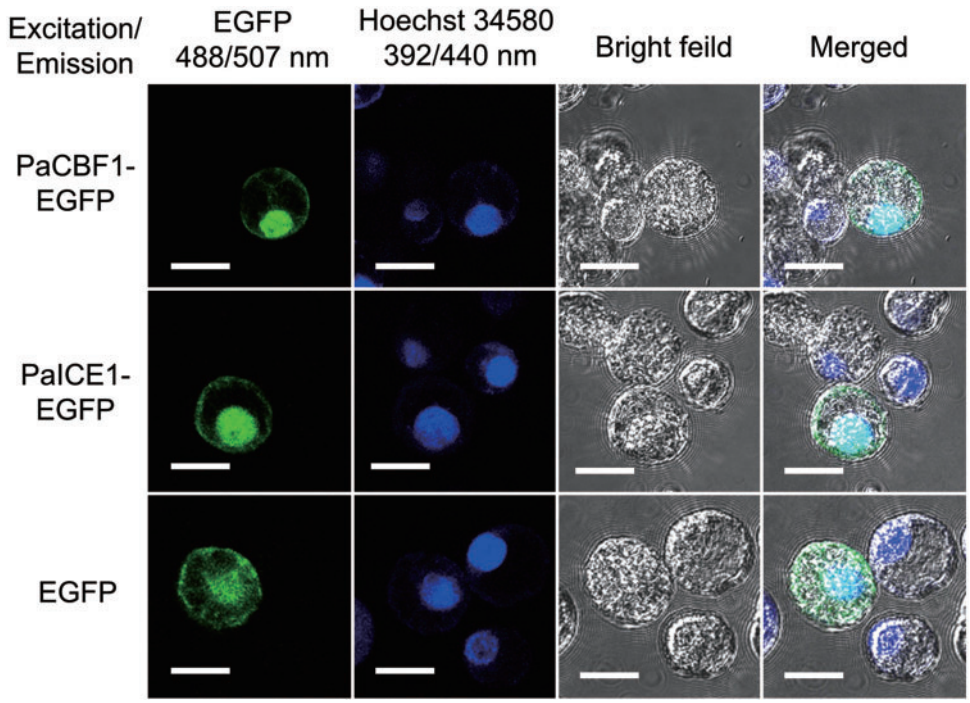

Fig. 3 Subcellular localization of the PaCBF1 and PaICE1 proteins. PaCBF1–EGFP (50.2 kDa) or PaICE1–EGFP (82.9 kDa) fusion proteins driven by the CaMV 35S promoter were transiently expressed in the protoplasts made from *P. aphrodite* petals. The expressed PaCBF1–EGFP and PaICE1–EGFP fusion proteins were localized within the nucleus, which was stained by the nuclear dye Hoechst 34580 (Invitrogen). Images were captured with a confocal microscope (LSM510, Carl Zeiss). Scale bar = 20 μm.

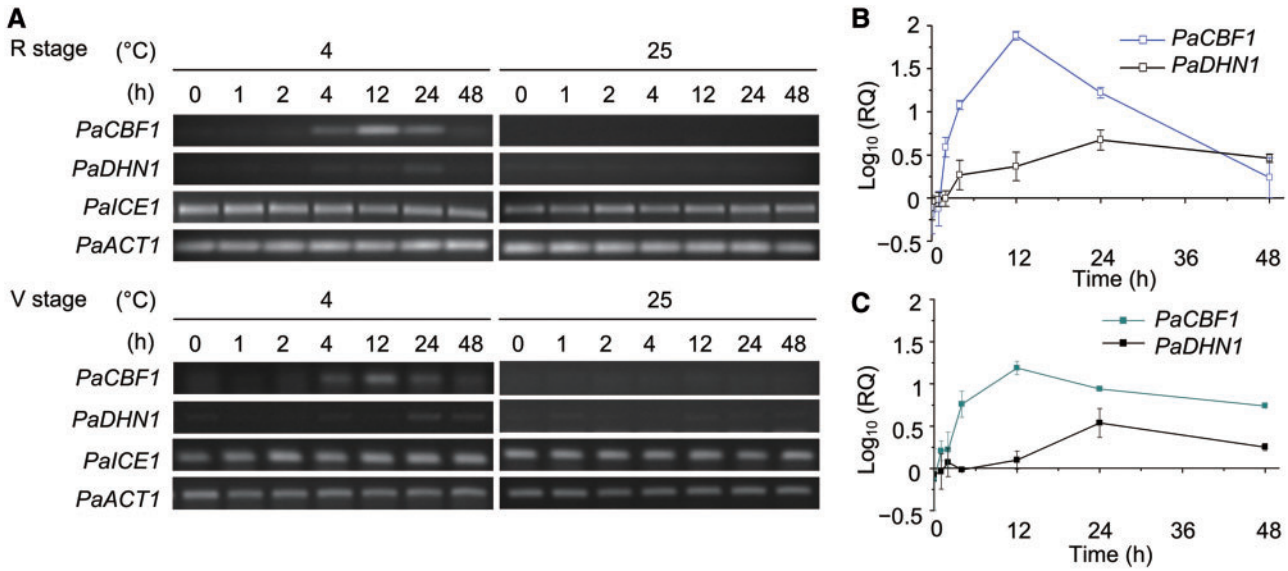

Fig. 4 Transcript levels of *PaCBF1*, *PaDHN1* and *PaICE1* in leaves of *P. aphrodite* in response to 4°C at different times. Leaves of plants at the R stage were exposed to 4°C and harvested at the indicated times in comparison with plants at 25°C. The transcripts were amplified by RT–PCR and resolved in a 2% agarose gel (A), and quantified using qRT–PCR (B and C). qRT–PCR analysis was normalized to the expression values of *PaACT1* which was used as an internal control. Results shown are the mean ± SD from three replicates.

Undetectable or very low levels of *PaCBF1* and *PaDHN1* mRNAs were found in plants at 25°C. These results showed that *PaCBF1* transcripts accumulated in response to cold in a time-dependent manner within 12 h and the cold effect on *PaDHN1* mRNA levels was less significant than that of *PaCBF1*.

We further explored the tissue specificity of the *PaCBF1* mRNA levels in different organs after exposure to 4°C for 10 and 24 h. Cold stress induced the levels of *PaCBF1* transcripts in root, flower and leaf tissues, while *PaCBF1* mRNA was very low or not detected in any examined tissue under 25°C in both R

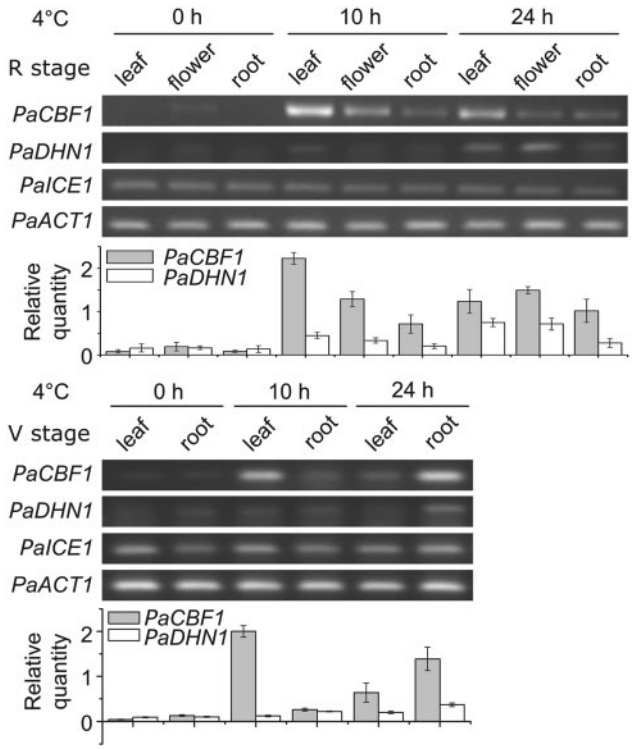

Fig. 5 Transcript levels of *PaCBF1*, *PaDHN1* and *PaICE1* in different tissues of *P. aphrodite* in response to cold stress. After 10 and 24 h exposure to 4°C, leaf, root and flower tissues were harvested and total cellular RNA was extracted for semi-quantitative RT–PCR analysis. The expression values of *PaACT1* are used as an internal control. A representative result of three independent experiments with similar results is shown. Bars are the mean ± SD.

stage and V stage plants (**Fig. 5**). The highest accumulation of *PaCBF1* transcripts in R stage plants occurred in leaves exposed to 4°C for 10 h, followed by flowers and roots. A longer exposure to 4°C (24 h) did not increase the level of *PaCBF1* mRNA in any tissue. In V stage plants after 24 h at 4°C, *PaCBF1* mRNA accumulated at a higher level in roots. Cold-induced ubiquitous expression of *PaCBF1* in plants at either the R or V stage and the *PaCBF1* transcripts decreased after prolonged exposure, except for root tissues of V stage plants. In addition, the *PaDHN1* transcripts reached a maximum level at 24 h in all the examined tissues of plants at either stage. In contrast to *PaCBF1* and *PaDHN1*, *PaICE1* mRNA is constitutively and ubiquitously expressed in *P. aphrodite* under various conditions.

PaCBF1 and its promoter was activated by abiotic stresses and *PaICE1*, and overexpression of *PaCBF1* regulated *AtCOR* genes in transgenic Arabidopsis plants

Because PaCBF1 peptide shares high sequence similarity with stress-inducible CBFs (Lata and Prasad 2011), expression of *PaCBF1* in response to various abiotic stresses was examined using qRT–PCR. In V stage leaves, the level of *PaCBF1* mRNA was induced by 4°C (13.9 ± 1.8-fold, $P < 10^{-12}$, $n = 3$) and salinity (0.3 M) treatment (8.2 ± 1.2-fold) (**Supplementary Fig. S8A**). *PaCBF1* transcripts also accumulated 5.6 ± 0.5-fold when leaves were dehydrated for 4 h, and 4.0 ± 0.8-fold in heat (37°C) treatment. Compared with alcohol treatment (1.2 ± 0.14-fold), *PaCBF1* mRNA was slightly induced by ABA (2.7 ± 0.7-fold). However, *PaCBF1* mRNA scarcely or did not respond to 10% polyethylene glycol (PEG; 1.2 ± 0.1-fold). The expression of *PaDHN1* was induced by 0.1 and 1 mM ABA (21.0 ± 2.7- and 23.7 ± 3.2-fold, respectively, $P < 10^{-12}$, $n = 3$), dehydration (15.2 ± 1.4-fold) and salinity (13.1 ± 2.4-fold). On the other hand, the *PaICE1* mRNA is constitutively expressed and not affected by any of the stresses mentioned above. Moreover, a 1,311 bp fragment of the *PaCBF1* promoter was fused to the *β-glucuronidase* (*GUS*) reporter gene in pCAMBIA1301 to form construct *p1311::GUS* for analysis of promoter efficiency. The GUS activity was induced by different forms of abiotic stresses ($P < 10^{-10}$, $n = 5$), including cold (2.0 ± 0.1-fold), salinity (1.9 ± 0.1-fold), ABA (1.5 ± 0.1-fold), dehydration (1.5 ± 0.2-fold) and mannitol (1.4 ± 0.1 fold) for 6 h treatments in transgenic Arabidopsis plants (homozygous T_4) (**Supplementary Fig. S8B**). An increase in GUS activity ($P < 10^{-44}$, $n = 5$) was observed in *p1311::GUS* transgenic plants during exposure to 4°C, 2.0 ± 0.1-fold at 6 h and 1.4 ± 0.1-fold at 8 h (**Fig. 6A**). The *PaCBF1* promoter was searched against the PLACE database (Higo et al. 1999), and the predicted transcription factor-binding elements include two ABA-responsive elements (ABREs) and seven MYC recognition sites (**Supplementary Table S6**). The MYC motifs sharing the consensus sequence CANNTG (Meshi and Iwabuchi 1995) in the *AtCBF3* promoter can be bound by AtICE1 (Chinnusamy et al. 2003). PaICE1 has a highly conserved bHLH domain (**Supplementary Fig. S6A**) which may recognize and bind regulatory elements in the *PaCBF1* promoter.

Transient gene expression analysis was carried out to analyze the effect of PaICE1 on the expression of *PaCBF1* by using co-transformation of *p1311::GUS* and *35S::PaICE1* (**Fig. 6B**) into Arabidopsis mesophyll protoplasts. The *PaCBF1* promoter showed cold-inducible activity (1.5 ± 0.3-fold, $n = 7$), and co-transformation with *35S::PaICE1* resulted in an increase (1.9 ± 0.2-fold) in *PaCBF1* promoter activity without cold stimuli (**Fig. 6C**). The *PaCBF1* promoter contains MYC motifs, including CATTTG (MYC_{TT}) at −1,112 to −1,117, CACTTG (MYC_{CT}) at −1,030 to −1,035 and CAAATG (MYC_{AA}) at −766 to −771, but no CACATG (MYC_{CA}) motif which is found in the *AtCBF3* promoter (−172 to −167 and −1,432 to −1,427). Since AtICE1 prefers binding to the MYC_{CA} element rather than to the MYC_{TT} element (Chinnusamy et al. 2003), the existence of the MYC_{TT} element in the *PaCBF1* promoter led to the question of whether this element is mediated by PaICE1. To investigate the transactivation ability, the effector (*35S::PaICE1*) was co-transformed with a GUS reporter construct containing a 4×MYC or 4×GCC element in front of the CaMV 35S minimal promoter (**Fig. 6B**). Relative GUS activity showed that MYC and GCC regulatory elements may bind PaICE1 more efficiently at 4°C compared with 25°C ($P < 10^{-7}$, $n = 3$) (**Fig. 6D**). At 4°C, transactivation of PaICE1 increased expression of the reporter gene containing 4×MYC_{TT}, 4×MYC_{AA} and 4×MYC_{CT} to 4.2 ± 0.5-, 3.9 ± 0.3- and 3.8 ± 0.7-fold, respectively over the basal level, while the

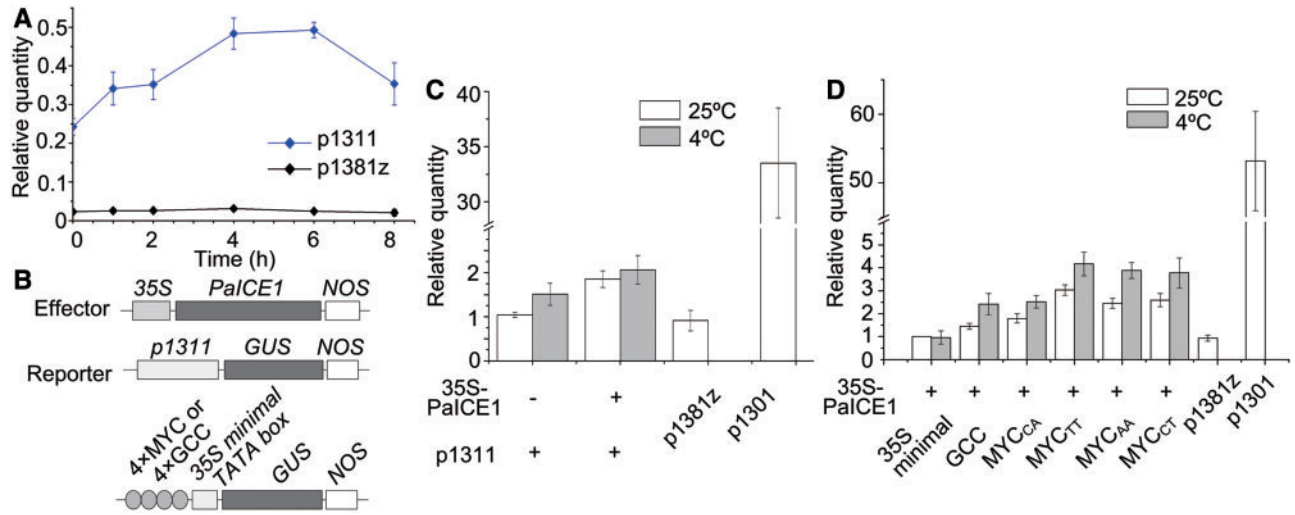

Fig. 6 Quantitative analysis of cold stress effects on the *PaCBF1* promoter and transactivation of the *PaCBF1* promoter and MYC box mediated by PaICE1. (A) Two-week-old transgenic Arabidopsis plants carrying *p1311*::*GUS* were exposed to 4°C for different time periods, and harvested for quantitative analysis. A promoter-less *GUS* in p1381z was used as a negative control. The relative GUS activity of the *PaCBF1* promoter was compared with that of *CaMV35S*::*GUS* (p1301), which was set as 1. Bars are mean ± SD of five replicates. (B) Schematic overview shows the effector and reporter constructs used in the transient assay. To analyze the transactivation by PaICE1, the vector carrying *35S*::*PaICE1* was used as an effector. The *GUS* reporter gene was driven by the *PaCBF1* promoter (p1311), the 4×MYC or 4×GCC box, the CaMV 35S minimal promoter and the TATA-box in tandem. NOS is the terminator signal of the nopaline synthase gene. (C and D) The reporter constructs specified above were co-transformed with or without *35S*::*PaICE1*, and half of the samples were moved to 4°C for 4 h before harvest. The relative GUS activities were compared with the basal transcriptional level. Bars are the mean ± SD of three replicates.

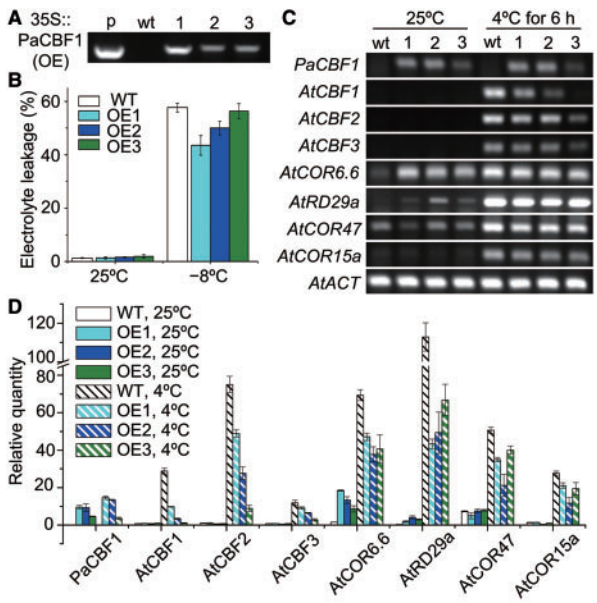

Fig. 7 Expression patterns of stress-responsive genes and freezing tolerance analysis in wild-type (WT, Col-0) and *35S*::*PaCBF1* transgenic Arabidopsis plants (OE) in response to cold stress. (A) Genomic DNAs of three transgenic lines were amplified using primers corresponding to the CaMV 35S promoter and *PaCBF1*. (B) Freezing tolerance was evaluated using electrolyte leakage with leaves of wild-type or *35S*::*PaCBF1* plants after exposure to −8°C. Bars are the mean ± the confidence interval of 14 replicates. (C, D) Two-week-old seedlings under control conditions or after 6 h of exposure to 4°C were collected and semi-quantitative RT–PCR (C) was applied for ImageJ evaluation (D). Similar results were obtained from three biological replicates. Bars are the mean ± SD.

reporter constructs containing 4×MYC_{CA} and 4×GCC elements only increased 2.5 ± 0.3- and 2.4 ± 0.5-fold, respectively. Our results suggest that PaICE1 may bind to specific MYC elements in the *PaCBF1* promoter to induce transcription.

PaCBF1 is cold inducible and has a highly conserved AP2/ERF DNA-binding domain which may activate CRT/DREs in the *COR* gene promoter. To analyze the function of PaCBF1 under cold stress, we overexpressed *PaCBF1* in Arabidopsis plants. Three transgenic lines overexpressing *PaCBF1* (T_4 plants, *35S*::*PaCBF1* OE) were verified by genomic DNA PCR (**Fig. 7A**). Cold tolerance of mature plants was evaluated using electrolyte leakage analysis (**Fig. 7B**), and less membrane damage was observed in transgenic lines, OE1 and OE2 (43.5 ± 3.7% and 50.0 ± 2.6%, respectively), than in wild-type plants (57.7 ± 1.6%, $P < 10^{-14}$, $n = 14$). Thus, we examined the expression of *PaCBF1* and its potential target genes in transgenic Arabidopsis plants (**Fig. 7C**) and found similar *PaCBF1* mRNA levels at 25 and 4°C. Interestingly, the *AtCOR6.6* and *AtRD29a* transcripts in *35S*::*PaCBF1* plants were 5- to 12-fold ($P < 10^{-4}$, $n = 3$) thode in wild-type plants at 25°C (**Fig. 7D**). The cold induction of the trasncripts of the *COR* genes *RD29a* and *AtCBF* genes in *35S*::*PaCBF1* plants was lower than that in control plants.

Discussion

Phalaenopsis aphrodite is a commercially important ornamental crop in Taiwan. For this reason, understanding its response to environmental stress is essential. In this study, we

investigated the stress responses of *P. aphrodite* plants and characterized the regulation of *PaCBF1* and *PaICE1* transcripts. Cold stress resulted in various symptoms including whole-plant wilting, necrosis and chlorosis (**Fig. 1**), as well as damage to cell membranes and reduction in photosynthesis efficiency (**Fig. 2**). Our results demonstrated that root tips are more sensitive to cold injury than other tissues. In addition to lesions and membrane injury, low temperatures may slow down biochemical reactions (Georlette et al. 2004). Soluble sugar has the property of stabilizing enzymes and cellular structures (Krasensky and Jonak 2012). Our experiments showed that ion leakage from leaf tissues of blooming *P. aphrodite* plants increased linearly with duration of cold stress. However, membrane injury was not detected in floral tissues (**Fig. 2A**), because flowers of blooming *P. aphrodite* plants contain higher levels of starch and soluble sugars than leaves (Chang et al. 2003). Total soluble sugars in *Phalaenopsis* leaves declined after floral spikes emerged (Kataoka et al. 2004), and leaf tissues at the R stage are more susceptible to cold stress which may be associated with reduced sugar content. Lowering the temperature decreases reaction rates and reduced the F_v/F_m ratio (**Fig. 2B**), similar to the results found in soybean (Wang et al. 1997) and cotton (Zhao et al. 2012). The severity of plant damage at different developmental stages may be partially due to carbohydrate content.

In this study, *PaCBF1* and *PaICE1* genes were isolated from *P. aphrodite*, which show high sequence similarity to their homologs. *PaCBF1* and *PaICE1* genes are considered to be identical to *PATC128464* and *PATC134808*, respectively. The minor differences between *PaCBF1* and *PATC128464* (in six of 606 nucleotides) and *PaICE1* and *PATC134808* (in 16 of 1,584 nucleotides) might have resulted from single-nucleotide polymorphism in different plants. *PaCBF1*, *PaDHN1* and *PaICE1* are the first characterized *CBF*, *DHN* and *ICE* genes in the Orchidaceae family, to the best of our knowledge. The level of *PaCBF1*, but not *PaICE1* mRNA, is up-regulated by cold, salinity and drought (**Supplementary Fig. S8**). The *PaDHN1* mRNA is up-regulated by ABA, drought and salinity after 4 h treatment. Cold induction in transgenic Arabidopsis plants harboring *PaCBF1 promoter::GUS* (**Fig. 6A**) was consistent with *PaCBF1* transcript levels in *P. aphrodite* plants (**Fig. 4**). After cold induction, ubiquitous and high levels of *PaCBF1* transcripts were found in *Phalaenopsis* plants, similar to *AtCBF1/2/3* in Arabidopsis (Shinwari et al. 1998) and *OsDREB1A/1B* in rice (Dubouzet et al. 2003). *PaCBF1* transcripts increased 4 h after exposure to 4°C, reached a maximum level at 12 h and decreased thereafter (**Fig. 4**). The decline of *PaCBF1* mRNA after 12 h may be mediated by negative regulators, such as AtCBF2 (Novillo et al. 2004) or AtMYB15 (Agarwal et al. 2006). Alternatively, the decrease in *PaCBF1* mRNA may be attributed to less sumoylated PaICE1, and further studies are needed to understand the mechanism. Both *PaCBF1* and *OsDREB1A* are induced by cold and salinity, *OsDREB1B* is induced by cold but not salinity (Dubouzet et al. 2003). Interestingly, the deduced PaCBF1 protein shares higher sequence identity to the constitutively expressed OsDREB1C than to OsDREB1A and OsDREB1B. The Arabidopsis *AtCBF3/1/2* genes are cold inducible, while only *AtCBF2* responds to salinity (Shinwari et al. 1998, Lata and Prasad 2011). Although sharing higher sequence identity to OsDREB1C and AtCBF3, *PaCBF1* responds to both salt and cold stress, which suggests its involvement in multiple stress signaling pathways.

The maximum accumulation of *PaCBF1* transcripts was detected at 12 h (**Fig. 4**), followed by that of *PaDHN1* at 24 h, which implied that PaCBF1 might be an upstream regulator of *PaDHN1* in response to cold stress. CBFs may regulate multiple *COR* and *RD* genes in response to cold stress in plants. Overexpressing *CBF* genes in transgenic Arabidopsis plants showed activation of *KIN1*, *COR6.6*, *COR15a*, *COR47* and *RD29a* transcripts without a low temperature stimulus and enhanced tolerance to freezing stress (Kasuga et al. 1999, Gilmour et al. 2000, Li et al. 2014, Siddiqua and Nassuth 2011). In our results, the significant increase in *AtCOR6.6* and *RD29a* transcripts in the *35S::PaCBF1* plants under control conditions (**Fig. 7D**) implies that *COR6.6* and *RD29a* might be the *PaCBF1* target genes under non-stress conditions in the transgenic plants. The up-regulation of stress-responsive genes in the non-stress conditions increased stress tolerance of *35S::PaCBF1* plants as indicated by better maintenance of membrane integrity. These results suggested that *PaCBF1* played an important protective role in cold response.

PaICE1 is a nuclear-localized protein (**Fig. 3**) containing a highly conserved bHLH domain (**Supplementary Fig. S4**), which may activate *PaCBF1* expression at low temperatures through binding to MYC motifs as AtICE1 does. AtICE1 positively regulates the expression of the *AtCBF3* gene and exhibits a higher binding affinity for MYC_{CA} elements (Chinnusamy et al. 2003). Within the −1,311 bp upstream sequence of the *PaCBF1* gene, only MYC_{TT}, MYC_{CT} and MYC_{AA} elements exist, not the MYC_{CA} element. PaICE1 significantly activated the *GUS* gene controlled by the promoter containing $4\times MYC_{TT}$, MYC_{CT} and MYC_{AA} elements (**Fig. 6D**). Our data suggest that PaICE1 may activate *PaCBF1* through the MYC elements in the *PaCBF1* promoter under cold temperatures. However, *PaCBF1* mRNA is stress inducible, whereas *PaICE1* mRNA is constitutively expressed (**Figs. 4**, **5**). Then a specific mechanism should exist to induce *PaCBF1* through either the level or the activity of PaICE1 without affecting its mRNA level. PaICE1 contains three putative sumoylation sites, suggesting that the protein may be constantly degraded by ubiquitination in *Phalaenopsis* plants, except that the protein is stabilized by sumoylation during cold treatment like AtICE1. Alternatively, the *PaCBF1* promoter may not be available to ICE in the absence of a cold treatment, and more studies are needed to understand the mechanism.

In conclusion, low temperature causes cell membrane injury and a decrease in photosynthesis efficiency in *P. aphrodite*, with more severe damage in plants at the R stage. We have characterized *PaCBF1* as a cold-inducible gene, its potential inducer *PaICE1* as a constitutively expressed gene, and a downstream candidate gene, *PaDHN1*. Transcripts of *PaCBF1* are at a low or undetectable level at 25°C and increase ubiquitously in different plant tissues at 4°C with subsequent expression of *PaDHN1*. The MYC elements found in *PaCBF1* promoters are transactivated by PaICE1. We hypothesize that the PaICE1 protein activates

PaCBF1 expression through MYC elements and further up-regulates *PaDHN1*, which plays a protective role at 4°C in *Phalaenopsis*, with the assumption that changes in transcript levels are correlated with changes in protein concentration and/or activity. Overexpression of *PaCBF1* in transgenic Arabidopsis showed better freezing tolerance and increased *AtCOR6.6* and *RD29a* transcripts, which may also improve tolerance to drought, salt and heat stress as mentioned in other studies using transgenic or cisgenic plants (Kasuga et al. 1999, Lata and Prasad 2011, Miura and Furumoto 2013). In addition, overexpressing *AtICE1* and multiple *DHN* genes improved freezing tolerance in transformed Arabidopsis (Chinnusamy et al. 2003, Puhakainen et al. 2004). Therefore, overexpression of *PaCBF1*, *PaDHN1* and/or *PaICE1* through genetic engineering is conceivable to enhance the protection of *Phalaenopsis* plants against abiotic stress during plant cultivation and benefit the flower industry.

Materials and Methods

Plants materials and stress treatments

The *P. aphrodite* subsp. *formosana* plants used in this study are a tetraploid cultivar (TS97) purchased from Taiwan Sugar Corporation. Plants of 4–5 leaves (~14 cm in length) grew ex vitro for 10 months in 6 inch pots with sphagnum moss in a 25°C growth chamber with 12 h light and 12 h dark, 75% relative humidity and 90 μmol m^{-2} s^{-1} of illumination provided by cool-white fluorescent lights, and watered weekly with half-strength Hoagland solution. Inflorescences induction was carried out by exposing plants to 23/18°C for 2 weeks. Plants having inflorescences of 3–5 open flowers and 2–5 floral buds are defined as reproductive (R) stage plants. The vegetative (V) stage plants are those constantly grown at 25°C, and do not have an inflorescence. To analyze the cold stress effect, half of the plants (~14 cm) were transferred to a 4°C growth chamber. Leaves, roots and flowers were harvested at different times after exposure to cold and immediately frozen in liquid nitrogen for RNA extraction or immersed in water for ion leakage measurements. To examine abiotic stress, the youngest fully developed leaves were detached from V stage plants which had 4–5 leaves of approximately 7 cm in length and grew ex vitro for 3 months in 2 inch pots. The incised leaves were immersed in distilled water, 0.05% ethanol, 1 mM or 0.1 mM ABA (in 0.05% alcohol), 300 mM sodium chloride or 10% PEG 6000, or air-dried for 4 h at 25°C.

Transgenic *A. thaliana* ecotype Columbia (Col-0) plants were generated by the floral dipping method using *Agrobacterium* GV3101 (Clough and Bent 1998). Seeds of the transformants were selected on half-strength Murashige and Skoog (MS) medium containing 3% sucrose, 20 μg ml^{-1} hygromycin and 250 μg ml^{-1} carbenicillin. For abiotic stresses, 2-week-old transgenic Arabidopsis plants were transferred to 4°C, dehydration (air-dried) or a half-strength MS plate containing saline (200 mM), ABA (0.1 mM) and mannitol (200 mM) for 6 h.

Electrolyte leakage measurement and photosynthesis measurement

Membrane damage was determined by measuring electrolyte leakage with a conductivity meter (SC-170; Suntex) using leaf or flower discs from more than three different orchid plants at the indicated time points. After treatment, leaf discs were punched with a sharp cork borer and incubated in 20 ml of deionized water at 25°C for 2 h with shaking at 100 r.p.m. before conductivity measurement. The solution conductivity was measured as the initial conductivity (C_0). Samples were then frozen for 48 h. After returning to room temperature with shaking at 100 r.p.m., the conductivity was measured as C_t. Electrolyte leakage was calculated as C_0/C_t. Electrolyte leakage analysis of Arabidopsis plants was applied as described by Lee et al. (1999) with minor modifications. Fully developed leaves of 5-week-old plants were placed in 15 ml falcon tubes with 100 μl of deionized water then immersed in a 0°C bath or placed at 25°C. The temperature of the bath was decreased to −8°C with a 1°C decrement per 30 min, then the tubes were placed on ice for 18 h. A 5 ml aliquot of deionized water was added to each tube and the tubes were incubated at 25°C for 30 min with shaking at 100 r.p.m. before C_0 was measured. After autoclaving, samples were returned to room temperature with shaking at 100 r.p.m. and C_t was measured.

For photosynthetic measurements, plants were grown at 4 or 25°C and leaves were adapted to the dark for 40 min prior to measurements. Chl fluorescence was recorded with a pulse-amplitude modulated fluorometer (PAM-2100; Heinz Walz) on intact plants at room temperature. The minimal fluorescence yield (F_o) and maximal fluorescence yield (F_m) were measured. Photosynthetic activities were assessed based on the maximum Chl fluorescence ratio (F_v/F_m), which determines the maximum quantum efficiency of PSII primary photochemistry.

RNA isolation, cDNA synthesis and semi-quantitative/quantitative RT–PCR

Total cellular RNA of orchid plants was extracted as previously described (Chen et al. 2007), Arabidopsis RNA was extracted using TRIzol reagent (Invitrogen) and used for first-strand cDNA synthesis with the M-MLV (H−) Reverse Transcriptase kit (Promega). Each semi-quantitative RT–PCR containing single-strand cDNA (synthesized from 100 ng of total RNA) was performed with 0.5 U μl^{-1} *Taq* DNA polymerase, 2.5 mM dNTP and a primer pair for the target gene (5 μM each, **Supplementary Table S1**). Thermocycling conditions were 94°C for 5 min followed by 25–28 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, with a final polymerization step at 72°C for 5 min. The amplified product was resolved on a 2% (w/v) agarose gel, visualized by ethidium bromide staining and then quantified with ImageJ software (Schneider et al. 2012). The qRT–PCR was performed according to the method described in Chen et al. (2007).

Genomic DNA extraction

Plant genomic DNA was extracted according to the method of Rogers and Bendich (1985), and then treated with RNase A (0.1 mg ml^{-1}) in digestion solution (300 mM Tris–HCl pH 7.5, 300 mM sodium chloride, 60 mM magnesium chloride) at 37°C for 2 h. An equal volume of phenol and chloroform/isoamyl alcohol (24 : 1) was used to remove the enzyme. After precipitation, the genomic DNA was dissolved in sterile water and stored at −20°C prior to use.

Gene isolation and bioinformatic analysis

To isolate the *P. aphrodite CBF*, *DHN* and *ICE* genes, homologous sequences from monocots in the NCBI database were aligned with the ClustalW/X program (Larkin et al. 2007). The degenerate primers (**Supplementary Table S1**) were designed according to the conserved domain for RT–PCR amplification of a partial cDNA fragment. The gene-specific primers (GSPs; **Supplementary Table S1**) were used to obtain complete cDNA sequence using the SMART™ RACE cDNA Amplification Kit (BD Bioscience Clontech) and genomic DNA was obtained by consecutive and overlapping genomic walking using the GenomeWalker™ Universal Kit (Clontech) (**Supplementary Table S1**). A partial cDNA of actin was isolated from a *P. aphrodite* leaf cDNA library, and the cDNA was completed using primers PaACTP1 and P2 (**Supplementary Table S1**) according to the *Phalaenopsis* sp. 'True Lady' actin-like protein (ACT2) mRNA (AF246715), and designated *PaACT1*. The *PaACT1* cDNA which contains 1,134 nucleotides was used as an internal control for quantitative comparison of mRNA. *PaACT1* shares 99.7% identity with *PATC157348* (Su et al. 2013) and 99.5% identity with AF246715.

To validate the TSS location of *PaCBF1*, 5′-RACE experiments were performed with total RNA extracted from cold-treated plant using a SMART™ RACE cDNA Amplification Kit (BD Bioscience Clontech) (**Supplementary Table S1**). The TSS was also predicted using TSSP (Softberry). The *cis*-acting regulatory elements of the promoter were predicted by PLACE (Higo et al. 1999).

Alignments of protein sequences were carried out by the ClustalW/X program (Larkin et al. 2007) and trees were generated with 1,000 bootstrap trials using the Neighbor–Joining method and visualized by MEGA5 (Tamura et al. 2011). The SUMOylation site was predicted using the SUMOsp program (Ren et al. 2009). The NLS was predicted by the programs PSORT (Nakai and

Kanehisa 1992), cNLS Mapper (Kosugi et al. 2009), NLStradamus (Nguyen Ba et al. 2009) and LOCtree (Nair and Rost 2005). Protein sequences were assigned to existing domains and family classification using the Pfam database (Punta et al. 2012). The websites of software programs used in this study are listed in **Supplementary Table S7**.

PEG-mediated protoplast transformation

Transformation was performed according to the method described in Yoo et al. (2007). For transactivation analysis, the following plasmids were added: 16 μg of effector plasmid (*35S::PaICE1*), 2 μg of internal control plasmids (*35S::RLuc*) and 8 μg of GUS reporter plasmids containing four times repeated MYC or GCC box in tandem (**Supplementary Table S1**) and a basal transcription vector containing a CaMV 35S minimal promoter (−1 to −51) and a TATA-box. The transformed protoplasts were incubated at 25°C in the dark for 16 h. The protoplasts were harvested by centrifugation at 100×*g* for 5 min and the supernatant was removed. The transformed protoplasts were stored at −70°C prior to analysis.

Quantification of RLuc and GUS activity in transformants

The *Renilla* luciferase (RLuc) activity was determined using the *Renilla* Luciferase® Assay System (Promega) according to the manufacturer's instructions. The frozen protoplast pellets were resuspended in 100 μl of Lysis Buffer and vortexed vigorously to rupture the protoplasts. Cell debris were removed by centrifugation at 14,000× *g* for 5 min at 4°C and the lysate kept on ice prior to analysis. The RLuc activity assay was carried out in a 96-well black plate (PerkinElmer) and the activity was measured using a Wallac Victor3 mutilabel plate reader (PerkinElmer). The RLuc activity was used as the internal control for GUS activity. To measure GUS activity, 50 μl of lysate of each sample and an equal volume of GUS assay buffer [2 mM 4-methylumbelliferyl glucuronide (4-MUG), 50 mM sodium phosphate pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100] was mixed and incubated for 1 h at 37°C in a 96-well black plate. An aliquot of 200 μl of 0.2 M sodium carbonate was added to each sample to stop the enzyme reaction. GUS activity was measured using a Wallac Victor3 mutilabel plate reader with emission and excitation filters set at 465 and 360 nm, respectively.

Two-week-old T_4 generation transgenic Arabidopsis plants carrying *p1311::GUS* were transferred to half-strength MS agar plates supplemented with 200 mM sodium chloride, 0.1 mM ABA and 200 mM mannitol for 6 h. For the cold stress treatment, plants were subjected to 4°C for 1, 2, 4, 6 and 8 h. For the drought treatment, transgenic Arabidopsis plants were removed from the medium plates and exposed to air for 6 h. Samples were ground in liquid nitrogen and vortexed with GUS assay buffer without 4-MUG. The supernatant was collected after centrifugation at 14,000×*g* for 5 min at 4°C and analyzed as protoplast samples. The quantity of total protein was used as the internal control for GUS activity. Protein concentration was determined using the Protein Assay Dye Reagent Concentrate (Bio-Rad) and the absorbance at 595 nm was measured with a Wallac Victor3 multilabel plate reader.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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