Phytohormones Regulate the Non-redundant Response of ω-3 Fatty Acid Desaturases to Low Temperatures in Chorispora bungeana

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Abstract

Key message **The cold-induced expression of ω-3 *CbFAD* genes were mainly regulated by jasmonie acid and brassinosteroid, and the accumulation of TAs below 0 ℃ was due to the contribution of CbFAD8**

To explore the contributions of ω-3 fatty acid desaturases (FADs) to cold stress response in a special cryophyte, *Chorispora bungeana* (*C. bungeana*), two plastidial ω-3 *FAD* genes (*CbFAD7* and *CbFAD8*) were cloned and verified in an *Arabidopsis fad7fad8* mutant, before being compared with the microsomal ω-3 *FAD* gene (*CbFAD3*) on expression profile. Though these genes were expressed in all tested tissues of *C. bungeana*, *CbFAD7* and *CbFAD8* have the highest expression in leaves, while *CbFAD3* was mostly expressed in suspension-cultured cells. Low temperatures (4, 0 and -4 ℃) resulted in significant increases in trienoic fatty acids (TAs, mainly C18:3), which were consisted with the cooperation of *CbFAD3* and *CbFAD8* in cultured cells, and the coordination of *CbFAD7* and *CbFAD8* in leaves. Furthermore, the cold induction of *CbFAD8* in the two tissues were increased with decreasing temperature and independently contributed to TAs accumulation at -4 ℃. A series of experiments revealed that jasmonie acid and brassinosteroids participated in the cold-responsive expression of ω-3 *CbFAD* genes in both *C. bungeana* cells and leaves, while the phytohormone regulation in leaves was more complicated with the participation of abscisic acid and gibberellin. These results point to the hormone-regulated non-redundant contributions of ω-3 CbFADs to maintain appropriate level of TAs under low temperatures, which helps *C. bungeana* survive in cold environments.

Introduction

Low temperature is one of the major environmental stresses influencing the distribution of plant species. To withstand this stress, plants have developed adapted mechanisms, which are rather complex and include the regulation of cell components as well as metabolic changes (Iba 2002; Khan et al. 2017). Cell membranes, serving as the boundary and active interface between cells/organelles and their environment, are the major targets of low temperature acclimation (Iba 2002; Shi et al. 2008; Khan et al. 2017). Though the structural and functional integrity of cell membranes are usually affected by low temperatures, the membrane integrity can be maintained through fatty acid modification (Mikami and Murata 2003; Shi et al. 2008). In fact, the content of trienoic fatty acids (TAs), represented mainly by C18:3, change to a certain extent in response to low temperatures, thus maintaining membrane fluidity and integrity (Matsuda et al. 2005; Upchurch 2008; Karabudak et al. 2014).

The synthesis of TAs is performed by ω-3 FADs through adding an unsaturated double bond to the ω-3 position of dienoic fatty acids (Iba 2002). It is known that ω-3 FADs are one kind of acyl-lipid desaturases, which could be classified into two types according to cellular localization: The plastid-type desaturase (FAD7 and FAD8) is localized in plastid membranes (Iba et al. 1993; Gibson et al. 1994), and the microsome-type desaturase (FAD3) is localized in the endoplasmic reticulum (Arondel et al. 1992). As one of the important factors for cold response (Iba and Kodama 1994), the expression of ω-3 *FAD* genes have been widely researched in plants. The first finding from maize leaf showed an increase in *ZmFAD8*

mRNA accompanied by a decrease in *ZmFAD7* mRNA under 5℃ exposure (Berberich et al. 1998). Later, relevant studies have been carried out in various plant species, such as birch (Martz et al. 2006), *Descurainia sophia* (Tang et al. 2007), purslanen (Teixeira et al. 2010), soybean (Román et al. 2012), *Arabidopsis* (Shi et al. 2011; Román et al. 2015), safflower (Guan et al. 2014), *Gossypium raimondii* (Liu et al. 2015), *Medicago truncatula* (Zhang et al. 2018) and rice (E et al. 2019). However, most of the studies focused on the common plants or corps undergoing chilling temperatures (2 to 16 ℃), little attention has been paid to the cryophytes (typical cold-tolerant plants) surviving the extreme cold conditions. Therefore, it is unclear whether the contributions of ω-3 FADs to cold stress response are difference between cryophytes and the other plant species.

Besides that, though some phytohormones, for example abscisic acid (ABA), salicylic acid (SA), jasmonie acid (JA), brassinosteroids (BRs) and gibberellin (GA) were thought to be the signal molecules involved in plant cold response (Shi et al. 2015; Verma et al. 2016; Eremina et al. 2016; Khan et al. 2017; Ku et al. 2018), we still poorly understand whether these hormones participate in the cold response of ω-3 *FAD* genes. There was only one direct evidence confirmed that JA partially participated in the chilling-induced expression of ω-3 *FAD* genes in *Arabidopsis* (Shi et al. 2011). Recently, a study from *Arabidopsis* leaf indicated that AtFAD7 protein levels decreased upon ABA treatment, while AtFAD8 protein levels increased upon cold or JA exposure (Soria-García et al. 2019). Unfortunately, the new findings have not directly proved the influence of ABA and JA on the cold response of AtFAD7 and AtFAD8. So far, the hormones that transmit low temperature signals to ω-3 *FAD* genes and regulate their expression are ambiguous. Perhaps the research on cryophytes will help us get more information.

C. bungeana is a perennial cruciferous cryophyte, having close genetic relationship with *Arabidopsis* (Zhao et al. 2012). It inhabits periglacial areas (about 3800-3900 m), where experience the bitter cold in winter and the freeze-thaw cycles in summer. To survive in the extreme environment, *C. bungeana* has adapted certain physiological and molecular mechanisms (Fu et al. 2006; Zhang et al. 2006; Shi et al. 2008; Di et al. 2009; Wu et al. 2009; Zhang et al. 2009; Zhao et al. 2012; Yue et al. 2019) instead of special morphological characteristics (Ayiturhan et al. 1998). Using cell suspension cultures, we found that the cold tolerance of *C. bungeana* was closely related to the accumulation of C18:3 (Shi et al. 2008), however, the contribution of each ω-3 CbFAD on this progress is unknown.

Plant cell suspension cultures are widely used as a versatile experimental system to analyze complex plant physiological processes in a more simplified system compared to the organism *in toto* (Sello et al. 2017; Cortese et al. 2021). In our lab, *C. bungeana* suspension-cultured cells are often used to study the physiological and molecular mechanisms of cold tolerance, and have provided much valuable information (Guo et al. 2006; Shi et al. 2008; Liu et al. 2010; Liu et al. 2011). In view of the low yield of wild *C. bungeana* and the sterility of cultivated *C. bungeana*, the regenerated plants of *C. bungeana* are another useful experimental system to meet the research needs on tissue or organism level (Fu et al. 2006; Zhang et al. 2006; Zhao et al. 2012; Yue et al. 2019). Considering that *CbFAD3* (microsomal) as well as *CbFAD7* and *CbFAD8* (plastidial) were mostly expressed in suspension-cultured cells and the leaves of regenerated plants, respectively, the experiments of this work were performed on the two

materials to extend the analysis from cellular level to tissue level, which can not only continue our previous researches (using suspension-cultured cells) but also can be compared with the conventional studies (using plant leaves). Additionally, the common phenomenon from the different analysis systems can be identified as the core adaption mechanism of *C. bungeana*.

Materials And Methods

Plant material and experiment treatments

The suspension-cultured cells and the regenerated plants of *C. bungeana* were prepared as described by Shi et al. (2008) and Fu et al. (2006), respectively. Regenerated plants having 3-5 cm long roots were used for experiments. *Arabidopsis seeds of Col-0 (WT), fad7fad8 mutant (N8036, NASC, UK), and complemented fad7fad8 mutants (F7 and F8) were cultivated as our previous procedure (Shi et al. 2011).*

For cold treatments, *C. bungeana* cell suspensions and regenerated plants were exposed to 4 ℃, 0 ℃ or -4 ℃ for 24 h, respectively. *For low-temperature germination, Arabidopsis seeds planted on MS medium were exposed to 15* ℃ for 4 weeks. For exogenous hormone treatments, the cell suspensions and regenerated plants were moved to 1/2 MS medium with 100 μM JA, 0.5 μM BRs, 100 μM SA, 100 μM ABA, or 100μM GA_3 for 24 h, respectively. For phytohormone inhibitions, the cell suspensions and regenerated plants were moved to 1/2 MS medium with 10 μM sodium diethyldithiocarbamate trihydrate (DIECA), 10 μM propiconazole (Pcz), 10 μM fluricbne (Flu), or 10μM paclobutrazol (Pac) for 24 h, respectively. To ensure the hormone/inhibitor application on leaves, the over ground part of each plant was sprayed with corresponding solution after moving, and the excess liquid was blotted by cotton balls. The *C. bungeana* cells and leaves were collected at different time spots according to the experimental design in each treatment, while the leaves of different *Arabidopsis* lines were collected from 5 weeks old plants under normal growth conditions. All the collected samples were stored at -80 °C until use.

RNA isolation and cDNA synthesis

Total RNAs of *C. bungeana* were isolated from suspension-cultured cells or the different tissues of regenerated plants (0.1 g each) according to the standard procedure of Plant RNA Kit (Omega, USA). After being treated with RQ1RNase-free DNase (Promega, USA), the purity and integrity of total RNAs were assessed by UV spectrophotometry and agarose gel electrophoresis. Then, the qualified total RNAs were employed in reverse transcription reaction by using PrimeScript^{TM} Reverse Transcriptase (Takara, Japan) and Oligo$(dT)_{15}$ primer (Takara, Japan) following the manufacturer's instruction. Reverse-transcribed cDNA samples were stored at -20 ℃ until further use.

Cloning and bioinformatics analysis of *CbFAD7* and *CbFAD8*

A 732-bp fragment of *CbFAD7* and a 982-bp fragment of *CbFAD8* were cloned from *C. bungeana* by using degenerate primers (P1 and P2 for *CbFAD7*; P3 and P4 for *CbFAD8*; Table S1) designed basing on the

conserved domain database from tobacco, *Brassica napus* and *Arabidopsis*. Amplification of 5' and 3' ends of *CbFAD7* and *CbFAD8* were accomplished using specific primers (P5-P8 for *CbFAD7*; P9-P12 for *CbFAD8*; Table S1) and SMARTer™ RACE cDNA Amplification Kit (Clontech, Japan). Full-length cDNA of *CbFAD7* and *CbFAD8* were gotten by using specific primers (P13 and P14 for *CbFAD7*; P15 and P16 for *CbFAD8*; Table S1). PCR products were cloned into the pMD-18T vector (Takara, Japan) and sequenced by GENEWIZ Inc. (Suzhou, China). Then these sequences were analyzed by DNAman 5.2.9, MEGA 6.06 and ClustalX 1.83 software. The prediction of transmembrane domain and transit peptide were analyzed by the online server program TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and TargetP (http://www.cbs.dtu.dk/services/TargetP/), respectively.

Complementation of *CbFAD7* and *CbFAD8* in *Arabidopsis* mutant

The coding region of *CbFAD7* amplified using specific primers (P17and P18; Table S1), were cloned within the XbaI-SacI site of the binary vector pBI121 to replace the GUS gene and construct the recombinant plasmid, pBI121-*CbFAD7*, under the control of CaMV 35S promoter. In the same way, the recombinant plasmid pBI121-*CbFAD8* was constructed with the coding region of *CbFAD8* amplified using specific primers (P19 and P20; Table S1). Then, the two recombinant plasmids and the *Arabidopsis* seeds of *fad7fad8* mutant were sent to Shanghai Weidi Biotechnology Co., Ltd (Shanghai, China) to get the complemented mutants, F7 and F8. The transgenic *Arabidopsis* plants were generated through the floral dip by *Agrobacterium*-mediated transformation. Positive transgenic lines of F7 and F8 exhibiting 3:1 segregation ratio were identified by PCR using specific primers (P17 and P18 for *CbFAD7*; P19 and P20 for *CbFAD8*; Table S1; Fig. S1). The homozygous lines were obtained by backcrossing or self-pollination, and the expression level of *CbFAD7* and *CbFAD8* were verified by qRT-PCR using specific primers (P23 and P24 for *CbFAD7*; P25 and P26 for *CbFAD8*; Table S1; Fig. S1), respectively. IPT2 (AT2G27760) was taken as the house keeping gene (Cheng et al. 2017) using primers P29 and P30 (Table S1). Three independent homozygous T3 transgenic lines of F7 and F8 showing higher expression levels were used in the experiments, respectively.

Quantitative real-time PCR

The SYBR Green I (Takara, Japan) assay and the Real-Time PCR System (Mx3000P, Agilent Stratagene, USA) were used for detecting the expression of *CbFAD3*, *CbFAD7* and *CbFAD8* in *C. bungeana*. The housekeeping gene, *CbACT* (AY825362), was used as a control for the stable expression (Di et al. 2009; Wu et al. 2009; Zhang et al. 2009; Shi et al. 2018). The amplification specificity of the primers (P21 and P22 for *CbFAD3*; P23 and P24 for *CbFAD7*; P25 and P26 for *CbFAD8*; P27 and P28 for *CbACT*; Table S1) were checked by gel electrophoresis before real-time PCR. The amplification condition was as follow: 95 ℃ for 30 sec, and 40 cycles of 95 ℃ for 5 s and 58 ℃ for 34 s. This was followed by 15 s at 95 ℃, 60 s at 60 ℃ and 15 s at 95 ℃ (determination of melting curve). PCR data were obtained from three independent biological samples for each experiment. The relative gene expression (F) was normalized against the housekeeping gene according to the formula:

$$F=\frac{(E_{target})^{\Delta Ct_{target}(control-sample)}}{(E_{housekeeping})^{\Delta Ct_{housekeeping}(control-sample)}},$$

which was regarded as a high accuracy and reproducibility mathematical model (Pfaffl 2001).

Extraction and analysis of total fatty acids

C. bungeana cell suspensions and leaves as well as *Arabidopsis* leaves (2 g each) were grinded with liquid N2, respectively. The lipids and the total fatty acids of each sample were prepared and analyzed as our previous reports (Shi et al. 2008; Shi et al. 2011). The fatty acid methyl esters of each sample were analyzed by GC-MS (6890N-5975C, Agilent, USA) fitted with a capillary column (Agilent DB-FFAP, 30 m×0.25 mm×0.5 μm) according to our previous *procedure* (Shi et al. 2018). Fatty acid data were obtained from five independent biological samples for each experiment.

Extraction and analysis of phytohormones

C. bungeana suspension-cultured cells and leaves (0.5 g each) were sent to Genepioneer Biotechnologies (Nanjing, China) to detect the concentration of JA, BRs, ABA and GA3, respectively. Phytohormones were extracted through grinding and organic solvent extraction, and then analyzed using Plant JA ELISA Kit (Sinobestbio, China), Plant BRs ELISA Kit (Sinobestbio, China), Plant ABA ELISA Kit (Sinobestbio, China) and Plant GA3 ELISA Kit (Sinobestbio, China), respectively. During the double-antibody sandwich ELISA, the absorbance (OD value) was detected by microplate reader (Infinite F50, Tecan, SWIT), and the concentration was calculated through standard curve method. Phytohormone data were from five biological replicates for each experiment.

Statistical analysis

Statistical analysis was performed using the method of one-way ANOVA, followed by Duncan's multiple range test at the $P<0.05$ or $P<0.01$ levels.

Results

cDNA isolation and sequence analysis of *CbFAD7* and *CbFAD8* from *C. bungeana*

According to genomic data analysis (data not shown), each *FAD7* and FAD8 gene has one single copy in the *Chorispora* genome. Based on the nucleotide sequences of known plastidial ω-3 *FAD* genes, two pairs of degenerate primers were designed and used for getting conserved sequences. After RACE PCR reactions and full length verification, two full length cDNA of 1805 and 1563 bp were obtained and designated as *CbFAD7* (KY069282) and *CbFAD8* (KY069283), respectively. *CbFAD7* contains an ORF

encoding a predicted protein (CbFAD7) of 439 aa, which corresponds to a calculated molecular mass of 50.2 kDa and a pI of 7.89. The deduced CbFAD7 sequence displayed significant similarity to other known plant FAD7 sequences, exhibiting the highest identity (85%) to *Brassica napus* BnFAD7 (FJ985690). *CbFAD8* contains an ORF encoding a predicted protein (CbFAD8) of 397 aa, which corresponds to a calculated molecular mass of 45.6 kDa and a pI of 8.92. The deduced CbFAD8 sequence showed remarkable similarity to other known plant FAD8 sequences and the highest identity (84%) to the *Arabidopsis* AtFAD8 (NM120640). These data indicate that *CbFAD7* and *CbFAD8* cloned from *C. bungeana* encode the plastidial ω-3 FADs.

Analysis of the deduced CbFAD7 and CbFAD8 sequences with targeting prediction tools, showed the 50 and 60 aa N-terminal transit peptides with the characteristic features of chloroplast targeting peptide (Arrowed in Fig. 1a), respectively, predicting a subcellular localization in chloroplasts. Alignment of the two deduced protein sequences found there were three conserved histidine clusters (HDGCH, HXXXXXHRTHH and HHXXXXHVIHH, asterisked in Fig. 1a) and four transmembrane domains (TMD, underlined in Fig. 1a), suggesting that the predicted proteins of *CbFAD7* and *CbFAD8* are chloroplast membrane-bound ω-3 FADs.

To elucidate the phylogenetic relationship, the predicted proteins of *CbFAD7*, *CbFAD8* and *CbFAD3* were included in a dendrogram representing the known plant ω-3 FADs for comparison. CbFAD7 (ARL62096) and CbFAD8 (ARL62097) were positioned in the group corresponding to plastidial ω-3 FADs, whereas CbFAD3 (AKN35208) was grouped with those of microsomal location (Fig. 1b).

The functionality of *CbFAD7* and *CbFAD8* were verified in *Arabidopsis* mutant

To verify the functionality of *CbFAD7* and *CbFAD8*, the ORF of the two genes were expressed in double *fad7fad8* mutants under the CaMV 35S promoter of pBI121 vector, respectively. The fatty acids of leaf lipids showed that though the C18:3 contents in the complemented mutants F7 and F8 were still lower than that in WT plants, they were apparently higher than that in *fad7fad8* mutants. Moreover, the C18:3 content in F7 lines was much higher than that in F8 lines, which may imply a stronger conversion effect of CbFAD7 on C18:3 (Fig. 2a). Being exposed to 15 ℃, the germination rates of F7 and F8 seeds were significantly higher than that of *fad7fad8* mutant seeds, and close to that of WT seeds (Fig. 2b). These data confirmed that both *CbFAD7* and *CbFAD8* were functional plastical ω-3 *FAD* genes.

The tissue-specific and cold-responsive expressions of ω-3 *CbFAD* genes in *C. bungeana*

The expression profiles of plastical and microsomal ω-3 *CbFAD* genes were analyzed in the suspension-cultured cells and the regenerated plants of *C. bungeana*. *CbFAD7* and *CbFAD8* have the highest expression in leaves and the lowest expression in roots (Fig. 3a), showing the tissue-specific expression pattern with the characteristic feature of plastidial ω-3 *FAD* genes. However, the expression level of *CbFAD7* and *CbFAD8* in suspension-cultured cells were dissimilar, the former was just lower than its expression level in leaves, and the latter was as low as its expression level in roots. Besides, *CbFAD3* were

mostly expressed in suspension-cultured cells, and lowly expressed in stems (Fig 3a), exhibiting the tissue-specific expression pattern with the characteristic feature of microsomal ω-3 *FAD* genes.

Given that the microsomal and the plastidial ω-3 *CbFAD* genes have the highest expression in suspension-cultured cells and plant leaves, respectively, the cold-responsive expression of them were detected in the two materials. In suspension-cultured cells, the expression of *CbFAD3* was rapidly increased at 4 (6.3-fold) and 0 ℃ (5.7-fold), and decreased at -4 ℃；meanwhile, the expression of *CbFAD8* had almost no change at 4 ℃, but was sharply increased at 0 (13.1-fold) and -4 ℃ (27.7-fold) (Fig. 3b). The increases in *CbFAD3* and *CbFAD8* mRNA all peaked at being treated for 3 h, and were accompanied by a decrease in *CbFAD7* mRNA at different low temperatures (Fig. 3b). Similar to that found in cultured cells, the induction of leaf *CbFAD8* only appeared at 0 (3.4-fold) and -4 ℃(6.7-fold) (Fig. 3b). However, the expression patterns of *CbFAD3* and *CbFAD7* in leaves were just the reverse of what presented in cells, the decrease in *CbFAD3* mRNA was occurred at all tested low temperatures, while the rapid increase in *CbFAD7* mRNA was found at 4 (4.1-fold) and 0 ℃ (2.6-fold) (Fig. 3b). Moreover, the cold-induced expression of *CbFAD7* and *CbFAD8* peaked at being treated for 3 and 6 h, respectively. On the whole, the expression of ω-3 *CbFAD* genes presented a non-redundant pattern in response to different low temperatures.

The hormone- and inhibitor-responsive expressions of ω-3 *CbFAD* genes in *C. bungeana*

To be in line with the cold-responsive experiments, the hormone-responsive experiments on ω-3 *CbFAD* genes were also studied in suspension-cultured cells and the leaves from regenerated plants. In suspension-cultured cells, the expression of *CbFAD3* was up-regulated by ABA, SA and BRs, but down-regulated by JA and GA3; the changes resulted from JA and BRs (6.2-fold, peaked at being treated for 3 h) were close to those brought by different low temperatures (Fig. 3a and 4a). Though the expression of *CbFAD7* was induced by ABA, SA and GA3, the inhibitions caused by JA and BRs were similar to the cold-inhibited expressions (Fig. 3a and 4a). All of the tested hormones could induce the expression of cell *CbFAD8* except GA3, however, only the increases induced by JA (9.3-fold) and BRs (8.1-fold) resembled the cold-induced ones (peaked at being treated for 3 h; Fig. 3a and 4a). Unlike that found in cultured cells, the expression of leaf *CbFAD3* was down-regulated not only by JA and GA3, but also by ABA and BRs, and each of the down-regulation approached the cold-inhibited expressions in some degree. Although the positive effect of GA3 on *CbFAD7* was the same in leaves, the effects of ABA, JA and BRs were just reversed, and the increases induced by BRs (1.6-fold) and GA3 (1.9-fold) were in accord with the changes induced by chilling temperatures (peaked at being treated for 3 h). At the meantime, the active role of JA and BRs in *CbFAD8* induction were unchanged in leaves, however, the role of ABA and GA3 were exchanged, and the increases induced by JA (4.3-fold) and ABA (3.6-fold) were consistent with those induced by cold temperatures (peaked at being treated for 6 h; Fig. 3b and 4b). The Pearson correlations between the cold- and the hormone-responsive expressions of ω-3 *CbFAD* genes (Table 1 and 2) indicated that JA and BRs may participate in the cold-responsive expressions of these genes in both suspension-cultured cells and plant leaves, while ABA and GA3 may only take part in the cold-induced regulation in leaves.

To further confirm the participation of these hormones, the corresponding inhibitors were used. Data showed that in suspension-cultured cells (Fig. 5a), the chilling-induced increase in *CbFAD3* mRNA was completely inhibited by Pcz (a synthetic inhibitor of BRs), but the inhibition could be partly relieved by DIECA (a synthetic inhibitor of JA); the cold-inhibited expression of *CbFAD7* was partially eliminated by Pcz (32.9%) or DIECA (65.8%), but was completely eliminated by the combination of them; on the contrary, the cold-induced expression of *CbFAD8* was mostly inhibited by either Pcz (78.9%) or DIECA (79.9%), but was completely inhibited by the cooperation of them. In *C. bungeana* leaves (Fig. 5b), the decrease in *CbFAD3* mRNA caused by low temperatures was completely eliminated by the synergism of Pcz, DIECA, Pac (a synthetic inhibitor of GA3) and Flu (a synthetic inhibitor of ABA), and the synergistic effect of Pcz and DIECA play a major role (79.0%); Although the chilling induction of *CbFAD7* could be suppressed by Pcz (66.0%) or Pac (33.9%) to different extent, the complete suppression need the joint action of them; likewise, the cold induction of *CbFAD8* was incompletely inhibited by either DIECA (73.1%) or Flu (29.9%), but was thoroughly inhibited by the combined effect of them.

Together, these results (Fig. 3b, 6b and S2) suggested that JA and BRs may cause the opposite changes in *CbFAD3* mRNA, while the synergism of them may be responsible for the down-regulation of *CbFAD7* as well as the up-regulation of *CbFAD8* in suspension-cultured cells; however, the high concentration of BRs, in turn, can inhibit the expression of cell *CbFAD8*. By contrast, the hormone-regulation in *C. bungeana* leaves were more sophisticated: in this case, JA and BRs may result in the decrease in *CbFAD3* mRNA with the help of ABA and GA3, and the combined effect of BRs and GA3 may active the chilling induction of *CbFAD7*, while the joint action of JA and ABA may trigger the cold induction of *CbFAD8*.

The level of related phytohormones in *C. bungeana* during low-temperature treatments

To provide more evidence to the hormone-regulated cold response, the level of related phytohormones were detected in suspension-cultured cells and the leaves from regenerated plants at different low temperatures. Being treated at 0 ℃, the level of tested pyhtohormones all presented a rapid and two-peaks increase in both analysis systems, however, the peak time and the peak value of them were various (Fig. 6a and 7a). In cultured cells, the level of BRs peaked at being treated for 1 (1.7-fold) and 3 h (1.5-fold), while that of JA peaked at being treated for 1.5 (2.6-fold) and 3 h (2.4-fold). Though the accumulations of BRs and JA in leaves were very similar to those in cultured cells, the peak values of them were slightly lower and the peak time of JA (2 and 4 h) was a little later. In comparison, the change trends of GA3 (1.8- and 2.4-fold) and ABA (1.6- and 2.0-fold) were closer to each other, however, the peak time of GA3 (0.5 and 2.5 h) was half an hour earlier than that of ABA. Overall, the cold-induced increases in phytohormones, especially the first peak, preceded the cold-responsive regulation of corresponding ω-3 *CbFAD* genes (Fig. 3b, 6a and 7a); furthermore, the level of synergistic hormone pairs, such as JA and ABA, or BRs and GA3, reached the peak level at staggered hours to avoid redundant effect (Fig. 6a and 7a).

Although the level of tested phytohormones could be induced by different low temperatures, the increments were varied with temperature (Fig. 6b and 7b). In suspension-cultured cells, the increment of

BRs at 4 ℃ (2.6-fold) was higher than that at 0 (1.7-fold) and -4 ℃ (2.1-fold); as for JA, the highest increment occurred at -4 ℃ (3.1-fold), and the increments at 4 and 0 ℃ were the same (2.6-fold). By contrast, the cold-induced increases in phytohormones were more regular in *C. bungeana* leaves (Fig. 7b): when the treated temperature was decreased from 0 ℃ to -4 ℃, the increment of BRs was decreased too, but that of JA was increased; at the same time, the increments of GA3 and ABA at 0 (1.8- and 1.9-fold, respectively) and -4 ℃ (4.2- and 1.2-fold, respectively) were presented in the opposite way. On the whole, the changes in hormone increase were consistent with the dynamic expressions of corresponding ω-3 *CbFAD* genes at different low temperatures; moreover, the increments of antagonistic hormone pairs, for example GA3 and ABA, showed a reverse trend in response to temperature variation, which may be due to the trade-offs between plant growth and cold stress response (Fig. 3b, 6b and 7b).

The level of TAs in *C. bungeana* during low-temperature and hormone-inhibitor treatments

Considering that the cold-responsive regulation on ω-3 *CbFAD* genes may affect the accumulation of TAs, the content of C18:3 and C16:3 were tested in the total lipids of suspension-cultured cells and the leaves from regenerated plants under different low temperatures. In the absence of chloroplasts, no C16:3 was detected in the lipids from cultured cells (Fig. 8a). Data showed that the accumulation of C18:3 was obviously induced by low temperatures and reached the maximum at being treated for 12 h in both analysis systems: the content of C18:3 in cell lipids increased from about 20.6% to 46.2-55.0% of total fatty acids, while that in leaf lipids increased from about 46.3% to 58.6-60.7% of total fatty acids (Fig. 8). Similarly, the content of C16:3 in leaf lipids increased from about 2.8% to 4.3-5.2% of total fatty acids, and reached the maximum at being treated for 12 (4 and 0 ℃) or 24 h (-4 ℃) (Fig. 8b). In general, the marked increases in TAs were in accord with but lagging behind the cold-induced expression of ω-3 *CbFAD* genes (Fig. 3 and 8).

To further confirm that the phytohormones affect the cold-induced accumulation of TAs through gene regulation, the hormone-inhibitor treatments were performed. In suspension-cultured cells, the cold-induced accumulation of C18:3 was completely inhibited by the synergism of DIECA and Pcz, but partially inhibited by either of them (Fig. 8a). In *C. bungeana* leaves, the cold-induced accumulation of C18:3 and C16:3 were both completely inhibited by the synergistic effect of DIECA, Pcz, Pac and Flu, but partially inhibited by either of them (Fig. 8b). These results consisted with the inhibitor-responsive expression of ω-3 *CbFAD* genes (Fig. 5 and 8), revealing that with or without the help of ABA and GA3 in distinct tissues, JA and BRs participated in maintaining appropriate level of TAs in *C. bungeana* through regulating ω-3 *CbFAD* genes under low temperatures.

Discussion

This work analyzed the behavior of plastidial and microsomal ω-3 FADs in *C. bungeana* at the level of gene expression and fatty acid content, to determine how low temperature affected the contribution of each ω-3 FAD to the synthesis of TAs, especially C18:3. From the result, it can be seen that low temperatures resulted in significant transcriptional changes on each ω-3 CbFAD. Among these changes,

the sharply increased *CbFAD8* mRNA induced only by severe low temperatures (notably the subzero temperature) was observed both in the suspension-cultured cells and in the leaves from regenerated plants (Fig. 3b). Considering the difference between the two analysis systems, this common phenomenon shows the critical role of CbFAD8 in the synthesis of TAs under freezing and subfreezing temperatures, which was different with the way most plant species do. In most plant species, the level of *FAD8* transcript was increased in response to chilling temperatures ranging from 4 ℃ to 15 ℃ (Berberich et al. 1998; Martz et al. 2006; Wang et al. 2006; Tang et al. 2007; Teixeira et al. 2010; Shi et al. 2011; Liu et al. 2015; Román et al. 2015). FAD8 was originally identified as a cold-specific desaturase by phenotypic analysis of a *fad3/fad7* double mutant from *Arabidopsis* that was capable of producing TAs only at low temperatures (15 ℃; McConn et al. 1994). Given that FAD8 plays an important role in the biosynthesis of plastid TAs (Zhang et al. 2005; Penfield 2008) required for the correct biogenesis and maintenance of chloroplasts (Routaboul et al. 2000) as well as the recovery from photo inhibition at low temperatures (Vijayan and Browse 2002; Tovuu et al. 2016), the induction of *FAD8* shows a common choice of plants, i. e. protecting plastids, especially chloroplasts. In *C. bungeana*, the induction of *CbFAD8* occurred at or below 0 ℃ may be due to its original lipid unsaturation of plastid membrane brought by FAD8 is higher than that in most other plants, which can help the plastids to get through chilling temperatures. This may also explain why the cold resistance of *C. bungeana* is much higher than that of normal plants. Of course, it still needs further researches.

In response to chilling temperatures, the expression of *CbFAD3* and *CbFAD7* presented a tissue-specific profile (Fig. 3b). The *CbFAD3* mRNA was increased in suspension-cultured cells and decreased in plant leaves, while the expression profile of *CbFAD7* was just the opposite in the two tissues. Combined with previous studies, we found that even in the same tissue under similar temperature conditions, the cold-induced expression of *FAD3* and *FAD7* were varied with plant species (Berberich et al. 1998; Martz et al. 2006; Tang et al. 2007; Shi et al. 2011; Zhang et al. 2011; Román et al. 2012; Karabudak et al. 2014; Ma et al. 2014; Yurchenko et al. 2014), which means some observations are in accord with ours but the others are not. Although we don't know why the contributions of the microsomal and plastidial desaturases differ among plant species, it is not surprising that the contributions of CbFAD3 and CbFAD7 in *C. bungeana* were proportional to their transcript abundance in corresponding tissues (Fig. 3). Furthermore, this expression profile suggests that CbFAD3 would be more important in *C. bungeana* cultured cells while CbFAD7 might contribute to TAs production in *C. bungeana* leaves under chilling temperatures.

When the temperature was further decreased, the cold-induced increase in *CbFAD3* or *CbFAD7* mRNA was reduced and even turned into a decrease at -4 ℃, which form a non-redundant complementation with the gradual increase in *CbFAD8* mRNA (Fig. 3b). The coordination of *CbFAD3* and *CbFAD8* indicates the distinct TA needs of *C. bungeana* cells at different low temperatures, for FAD3 affecting total TA levels and FAD8 affecting plastid TA levels (Zhang et al. 2005; Penfield 2008). The cooperation of *FAD7* and *FAD8* was not only found in *C. bungeana* leaves, but also found in *Arabidopsis* leaves at different temperatures (8, 22 and 30 ℃; Román et al. 2015). Although both FAD7 and FAD8 affect the synthesis of TAs in chloroplastic lipids, the lipid specificity of them are not the same (Soria-García et al. 2019): AtFAD7 prefers galactolipids, which are the major chloroplast lipids with higher TA content; AtFAD8 likes

phosphatidylglycerol (Román et al. 2015), which has a specific role in the stability of photosynthetic complexes (Wada and Murata 2007). Hence, the trade-off between *CbFAD7* and *CbFAD8* in leaves reflects the strategy to protect photosynthetic activity and stability with temperature variation. Nevertheless, the non-redundant expression of ω-3 *CbFAD* genes maintained appropriate level of TAs, especially C18:3, in response to low temperatures (Fig. 8a).

Through a series of verification, including exogenous hormone application (Fig. 4), correlation analysis (Table 1), inhibitor treatments (Figs. 5 and 8) and phytohormone detection (Figs. 6 and 7), we confirmed that the cold-responsive expression of ω-3 *CbFAD* genes were regulated by certain phytohormones, notably JA and BRs. JA was mainly responsible for *FAD8* induction under freezing/subfreezing temperatures, with the tissue-specific assistance of BRs or ABA; BRs was in charge of the induction of *CbFAD3* or *CbFAD7* under chilling temperatures, with or without the help of GA3 in distinct tissues. Moreover, both JA and BRs took part in the cold-responsive inhibition of corresponding ω-3 *CbFAD* expression, with or without the participation of ABA and GA3 according to tissue specificity. These data not only provide new insights into our previous findings that JA partially mediates the chilling-induced transcription of ω-3 *FAD* genes in *Arabidopsis* (Shi et al. 2012), but also agree with that JA may act as a core signal by interacting with other phytohormones to regulate the balance between plant growth and stress response (Yang et al. 2019). As we know, ABA may interact synergistically with JA signaling to regulate the expression of cold-responsive genes (Hu et al. 2017; Kim et al. 2018; Wang et al. 2020); BRs acts in synergism or antagonism with JA, depending on BRs' concentration in response to stress (Yang et al. 2019); GA usually inhibits JA signaling, but in some cases, the synergistic effect of them is also exist (Hu et al. 2017; Yang et al. 2019; Wang et al. 2020). In this study, the interactions between JA and the other tested hormones are in line with the well-known findings. Notably, the joint action of JA and ABA regulated the cold-induced expression of leaf *CbFAD8*, and the combined effect of JA and BRs triggered the induction of *CbFAD8* in cultured cells. Our previous study on *Arabidopsis* (Shi et al. 2011), which confirmed the participation of JA in the chilling-induced expression of ω-3 *FAD* genes, also implied the existence of JA's partner. Besides, a recent study reported that in *Arabidopsis* leaves, AtFAD8 protein levels were increased upon cold or JA exposure, but did not respond to ABA (Soria-García et al. 2019). All these data prove the fact that though the partner of JA signaling may vary with tissues and plant species, JA did regulate the cold induction of *FAD8* in both *C. bungeana* (cryophyte) and *Arabidopsis* (modal plant), which may be common in most plant species.

In *C. bungeana*, BRs is another important pyhtohormone involved in the low-temperature regulation on ω-3 *FAD* genes. It is confirmed that BRs can improve frost tolerance by promoting GA biosynthesis and interplaying with GA at the signaling level (Ramirez and Poppenberger 2020), which supports the observation that the cold response of *CbFAD3* and *CbFAD7* in *C. bungeana* leaves was mediated by the cooperation of BRs and GA3 (Fig. 5b). Recent studies also demonstrated that BRs may participate in drought or cold stress acclimation by three interconnected mechanisms, one of which is in communication with ABA signaling (Bulgakov and Avramenko 2020). This may explain the synergistic effect of BRs and ABA on the down-regulation of leaf *CbFAD3* during low temperature exposure (Fig. 5b). To date, there were no reports about the participation of BRs in the cold-responsive expression of ω-3 *FAD*

genes, so we cannot predict that BRs also regulates the cold induction of *FAD3* or *FAD7* in other plant species before further research.

It is worth noting that the interaction between different phytohormones demonstrates that each ω-3 *CbFAD* gene can respond to multiple hormones (Fig. 4–8; Table 1). A similar phenomenon was found in *Arabidopsis*, which showed the expression of *AtFAD3* was regulated through the synergistic and antagonistic interactions of auxin, cytokinin and ABA during plant development (Matsuda et al. 2001). These results reflect the existence of various promoter cis-elements combined with different transcription factors (TFs) from corresponding hormone signaling. A G-box-like motif required by JA-responsive expression was first found in the promoter of *AtFAD7* from *Arabidopsis* roots (Nishiuchi et al. 1999). After that, the SA- and ABA-responsive elements were found in the promoter of cabbage *FAD8* (Liu et al. 2014). Recently, the analysis on *AmFAD7* and *AmFAD8* in *Ammopiptanthus mongolicus* confirmed that both of the promoters contain the elements for the response to ABA, JA, GA and MeJA (Xue 2019). Moreover, the multiple ABA-responsive elements (ABREs) were found in the promoter of microalgae ω-3 *FAD* genes (Norlina et al. 2020). All the findings support the idea that ω-3 *FAD* genes can be directly regulated by more than one hormone.

As another necessary factor for the hormone regulation, TFs have been studied at recent years. On the one hand, some TFs related to ω-3 *FAD* expression were verified: for example, the expression of *FAD3* was up-regulated by bZIP67 in *Arabidopsis* seeds (Mendes et al. 2013), but down-regulated by MaMYB4 in banana fruits (Song et al. 2019) or by HD in soybean (Jo et al. 2021); WIPK was involved in wound-responsive expression of *AtFAD7* gene in transgenic tobaccos (Kadoma et al. 2000). On the other hand, it is confirmed that in *Arabidopsis*, some TFs (such as WRKYs, MYCs, bHLHs and ICEs) occurred at the downstream of cold-induced JA signaling (Hu et al. 2017; Wang et al. 2020), while some others (for example bZIPs) belong to the cold-responsive BR and/or ABA signaling pathways (Banerrjee and Roychoudhury 2017; Ramirez and Poppenberger 2020).

Though all the relevant information are not quite complete, if we piece them together, it is not difficult to speculate that through the common or distinct TFs combined with corresponding promoter elements, JA and BRs as well as ABA and GA3 achieve the synergistic or antagonistic regulation on ω-3 *CbFAD* genes, which result in the non-redundant cooperation on maintaining appropriate level of TAs and then help *C. bungeana* survive in cold environments.

Declarations

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Author contribution statement

YS and LA conceived and designed research; YS and XY conducted experiments; SY and ZZ contributed to material preparation; YS analyzed data and wrote the manuscript; XY and LA provided suggestions and revised the manuscript. All authors read and approved the manuscript.

Data availability

The DNA and protein sequences generated during and/or analysed during the current study are available in the NCBI Genbank repository (https://www.ncbi.nlm.nih.gov/nuccore/or https://www.ncbi.nlm.nih.gov/protein/). The other datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1

Pearson correlation coefficients (two-tailed) between the cold-responsive and the hormone-regulated expressions of ω-3 CbFAD genes in *C. bungeana* cells.

Gene name	Temperature (°C)	SA	BRs	JA	ABA	GA3
CbFAD3	4	-0.26	**0.99*** **	0.07	0.04	-0.55
	0	-0.35	**0.97*** **	-0.07	-0.16	-0.67
	-4	-0.94	0.35	**0.51***	-0.09	0.10
CbFAD7	4	-0.02	**0.78****	**0.94****	0.22	-0.65
	0	0.23	**0.91*** **	**0.72****	-0.14	-0.64
	-4	-0.61	0.48	**0.82*** **	-0.23	-0.78
CbFAD8	4	-0.73	**0.72****	**0.71****	-0.75	-0.34
	0	-0.16	**0.99*** **	**0.97*** **	-0.26	-0.60
	-4	0.02	**0.91*** **	**0.87*** **	0.00	-0.83

Three replications for each sample, n=5×3. Significant positive correlation (R > 0.50, P <0.05) is indicated in bold. * P<0.05, ** P<0.01, *** P<0.001.

Table 2

Pearson correlation coefficients (two-tailed) between the cold-responsive and the hormone-regulated expressions of ω-3 CbFAD genes in *C. bungeana* leaves.

Gene name	Temperature (°C)	BRs	JA	ABA	GA3
CbFAD3	4	**0.85*** **	**0.60***	**0.90*** **	**0.78****
	0	0.23	**0.99*** **	**0.86*** **	**0.71****
	-4	0.22	**0.95*** **	**0.91*** **	**0.82*** **
CbFAD7	4	**0.94*** **	-0.24	0.47	**0.86*** **
	0	**0.93*** **	-0.29	0.49	**0.95*** **
	-4	**0.90*** **	-0.77	0.46	**0.74****
CbFAD8	4	-0.76	0.36	0.39	**0.69***
	0	-0.21	**0.97*** **	**0.97*** **	-0.11
	-4	-0.08	**0.98*** **	**0.98*** **	-0.29

Three replications for each sample, n=5×3. Significant positive correlation (R > 0.50, P <0.05) is indicated in bold. * P<0.05, ** P<0.01, *** P<0.001.

Figures

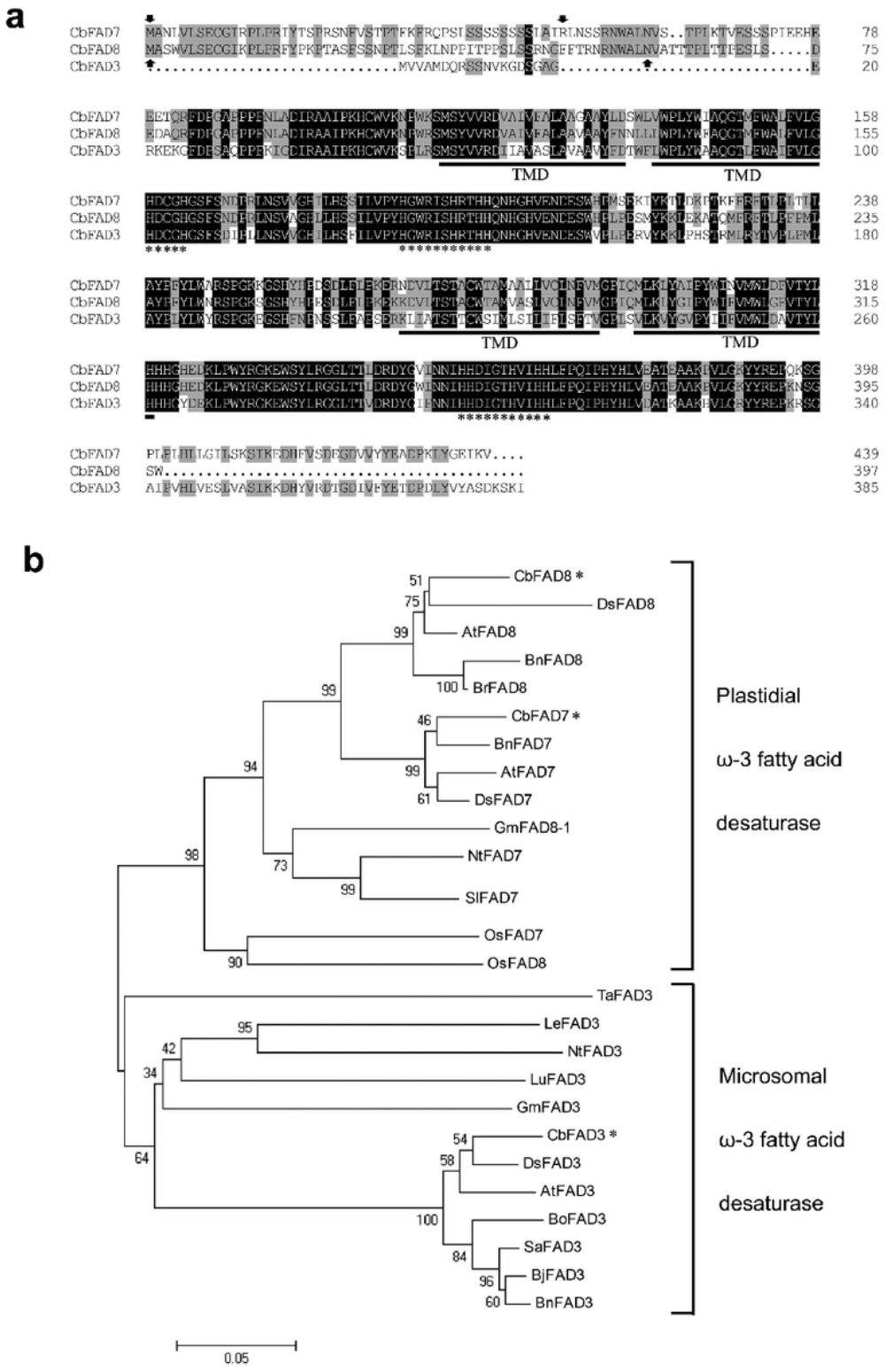

Figure 1

Analysis of the deduced amino acid sequences of *C. bungeana* ω-3 FADs. (a) Sequence comparison of CbFAD3, CbFAD7 and CbFAD8. Identical and similar residues are shown on a background of black and gray, respectively. The sequences of the putative chloroplast transit peptides of CbFAD7 and CbFAD8 are arrowed. The three conserved histidine clusters are indicated by asterisks, and the four transmembrane domains (TMD) are underlined. (b) Phylogenetic tree analysis of CbFAD3, CbFAD7 and CbFAD8. The

positions of ω-3 CbFADs are asterisked. The accession number of different ω-3 FADs included in this analysis: *Arabidopsis thaliana* AtFAD3 (NP180559), *Brassica juncea* BjFAD3 (ADJ58019), *Brassica napus* BnFAD3 (NP001302640), *Brassica oleracea* BoFAD3 (AGH20189), *Chorispora bungeana* CbFAD3 (KM591203), *Chorispora bungeana* CbFAD7 (KY069282), *Chorispora bungeana* CbFAD8 (KY069283), *Descurainia sophia* DsFAD3 (ABK91879), *Glycine max* GmFAD3 (NP001237507), *Lycopersicon esculentum* LeFAD3 (ABX24525), *Linum usitatissimum* LuFAD3 (AFJ53089), *Nicotiana tabacum* NtFAD3 (P48626), *Sinapis alba* SaFAD3 (AHA05997), *Triticum aestivum* TaFAD3 (BAA28358), *Arabidopsis thaliana* AtFAD7 (P46310), *Brassica napus* BnFAD7 (ACS26170), *Descurainia sophia* DsFAD7 (ABS86961), *Nicotiana tabacum* NtFAD7 (D79979), *Solanum lycopersicum* SlFAD7 (NP001234592), *Oryza sativa* OsFAD7 (BAE79783), *Arabidopsis thaliana* AtFAD8 (P48622), *Brassica napus* BnFAD8 (NP001302644), *Brassica rapa* BrFAD8 (AAW78909), *Descurainia sophia* DsFAD8 (ABK91881), *Glycine max* GmFAD8-1 (NP001238609), *Oryza sativa* OsFAD8 (BAE79784).

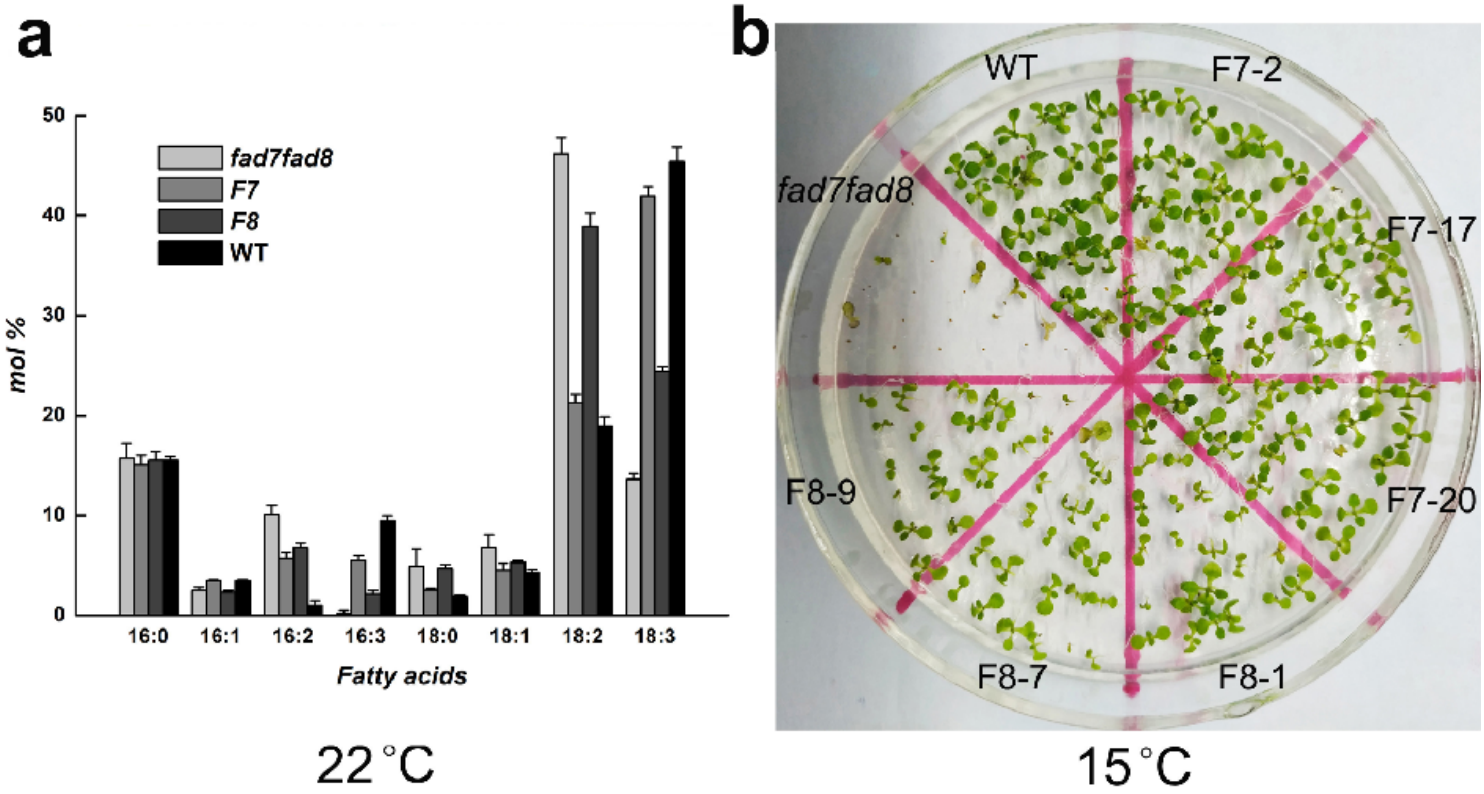

Figure 2

Functionality of *CbFAD7* and *CbFAD8* were verified in *Arabidopsis* mutant. (a) Comparison of total leaf fatty acids between *Arabidopsis* lines under normal conditions. (b) Comparison of low-temperature germination between *Arabidopsis* lines. WT means wild type (Col-0); *fad7fad8* means double *fad7fad8* mutant; F7 means *CbFAD7*-complemented mutant; F8 means *CbFAD8*-complemented mutant. Each value represents the mean ± SE of five replicates.

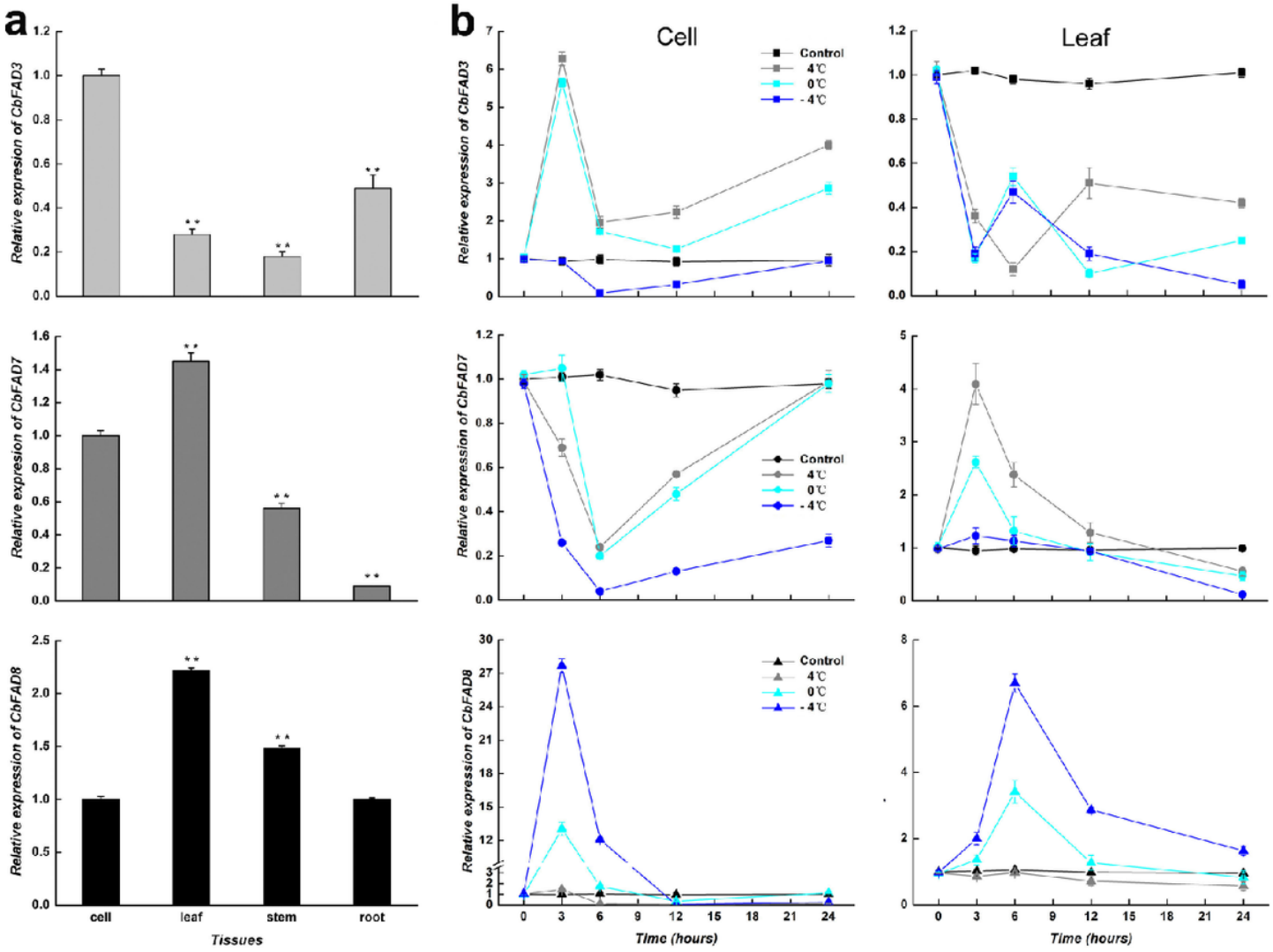

Figure 3

Expression patterns of ω-3 *CbFAD* genes in *C. bungeana.* (a) Tissue-specific expressions of *CbFAD3*, *CbFAD7* and *CbFAD8*. Data were presented as relative expression ratios being compared with the expression levels of corresponding genes in suspension-cultured cells, which were set at a value of 1. (b) Cold-responsive expressions of *CbFAD3*, *CbFAD7* and *CbFAD8* in suspension-cultured cells and the leaves from regenerated plants. Data were presented as relative expression ratios being compared with the expression levels of corresponding genes before treatment (0 h), which were set at a value of 1. Each value represents the mean ± SE of three replicates. The relative expressions of *CbFAD3*, *CbFAD7* and *CbFAD8* are indicated by square, circle and triangle, respectively.

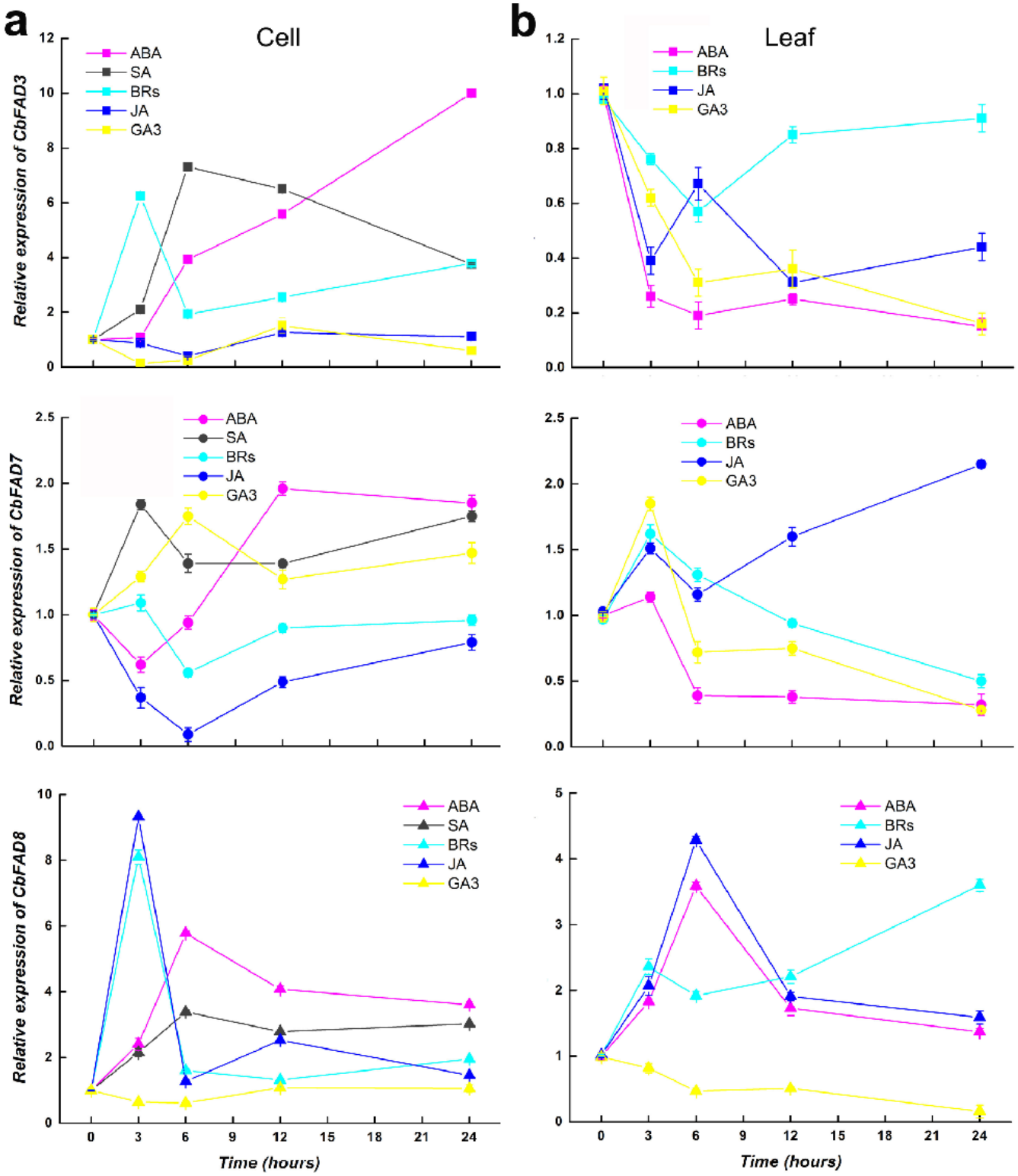

Figure 4

Hormone-responsive expressions of ω-3 *CbFAD* genes in *C. bungeana.* (a) Expression patterns of *CbFAD3*, *CbFAD7* and *CbFAD8* in suspension-cultured cells. (b) Expression patterns of *CbFAD3*, *CbFAD7* and *CbFAD8* in the leaves from regenerated plants. Data were presented as relative expression ratios being compared with the expression levels of corresponding genes before treatment (0 h), which were set at a

value of 1. Each value represents the mean ± SE of three replicates. The relative expressions of *CbFAD3*, *CbFAD7* and *CbFAD8* are indicated by square, circle and triangle, respectively.

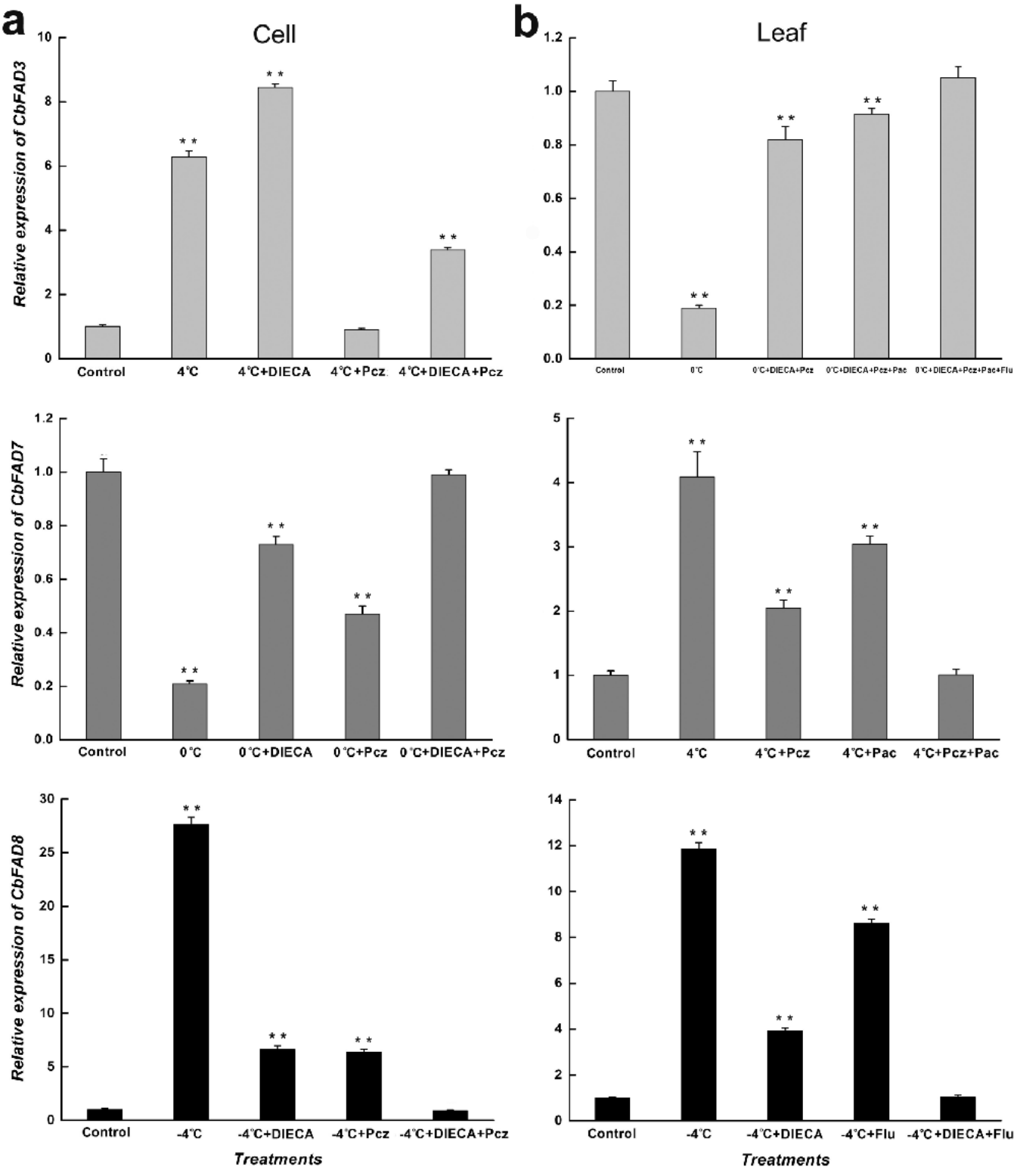

Figure 5

Hormone inhibitors affected the cold-responsive expressions of ω-3 *CbFAD* genes in *C. bungeana.* (a) Expression levels of *CbFAD3*, *CbFAD7* and *CbFAD8* in suspension-cultured cells. The expressions of

CbFAD3 and *CbFAD8* were detected at being treated for 3 h, while that of *CbFAD7* was detected at being treated for 6 h. (b) Expression levels of *CbFAD3*, *CbFAD7* and *CbFAD8* in the leaves from regenerated plants. The expressions of *CbFAD3* and *CbFAD7* were detected at being treated for 3 h, while that of *CbFAD8* was detected at being treated for 6 h. Data were presented as relative expression ratios being compared with the expression levels of corresponding genes under normal conditions (Control), which were set at a value of 1. Each value represents the mean ± SE of three replicates.

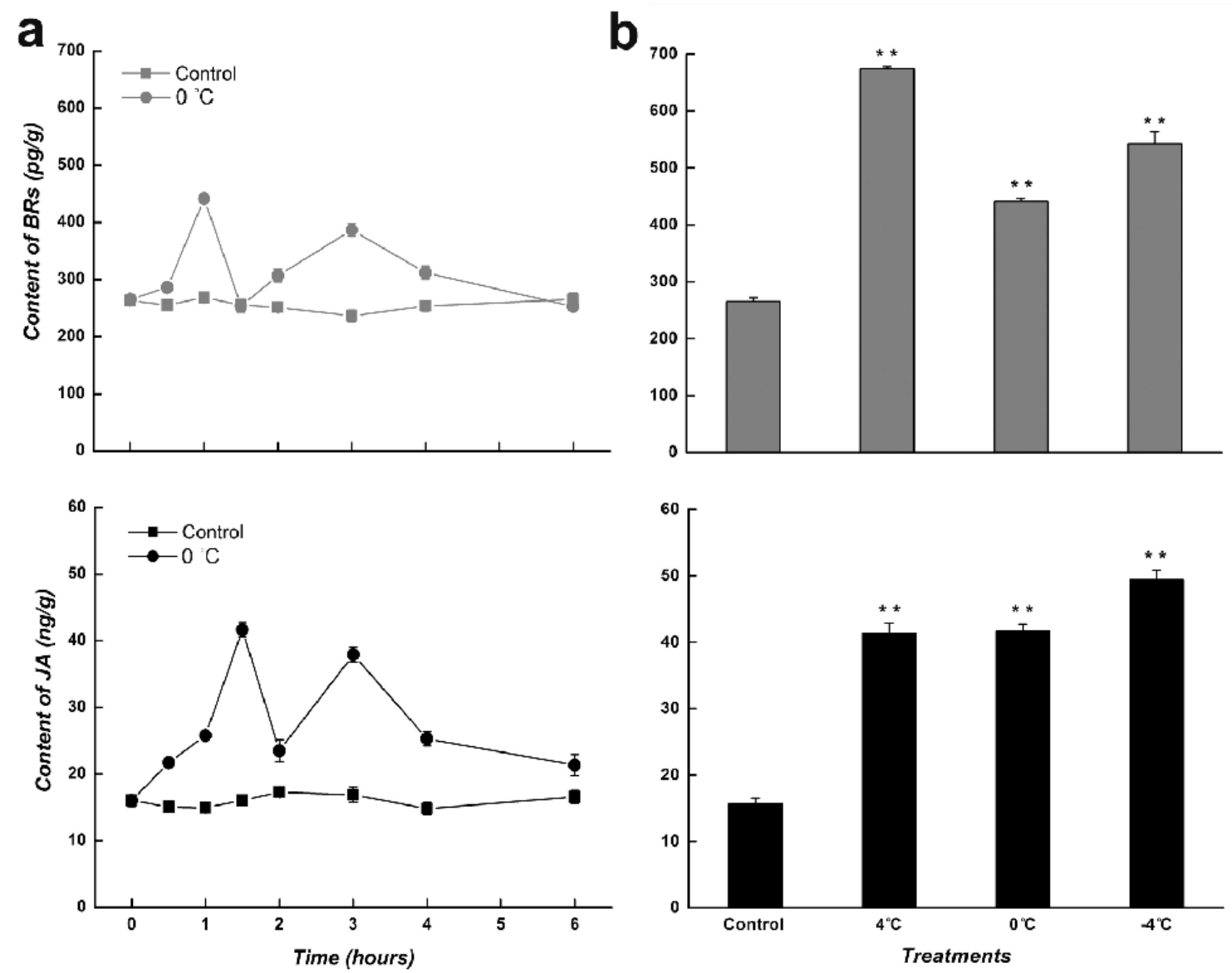

Figure 6

Pytohormone analysis in the suspension-cultured cells of *C. bungeana* at low temperatures. (a) Changes in the level of BRs and JA at 0 ℃ (b) Levels of BRs and JA at different low temperatures (4, 0 and -4 ℃). The data of BRs was detected at being treated for 1 h, and that of JA was detected at being treated for 1.5 h. The corresponding data at the same time point under normal conditions was taken as the control. Each value represents the mean ± SE of five replicates.

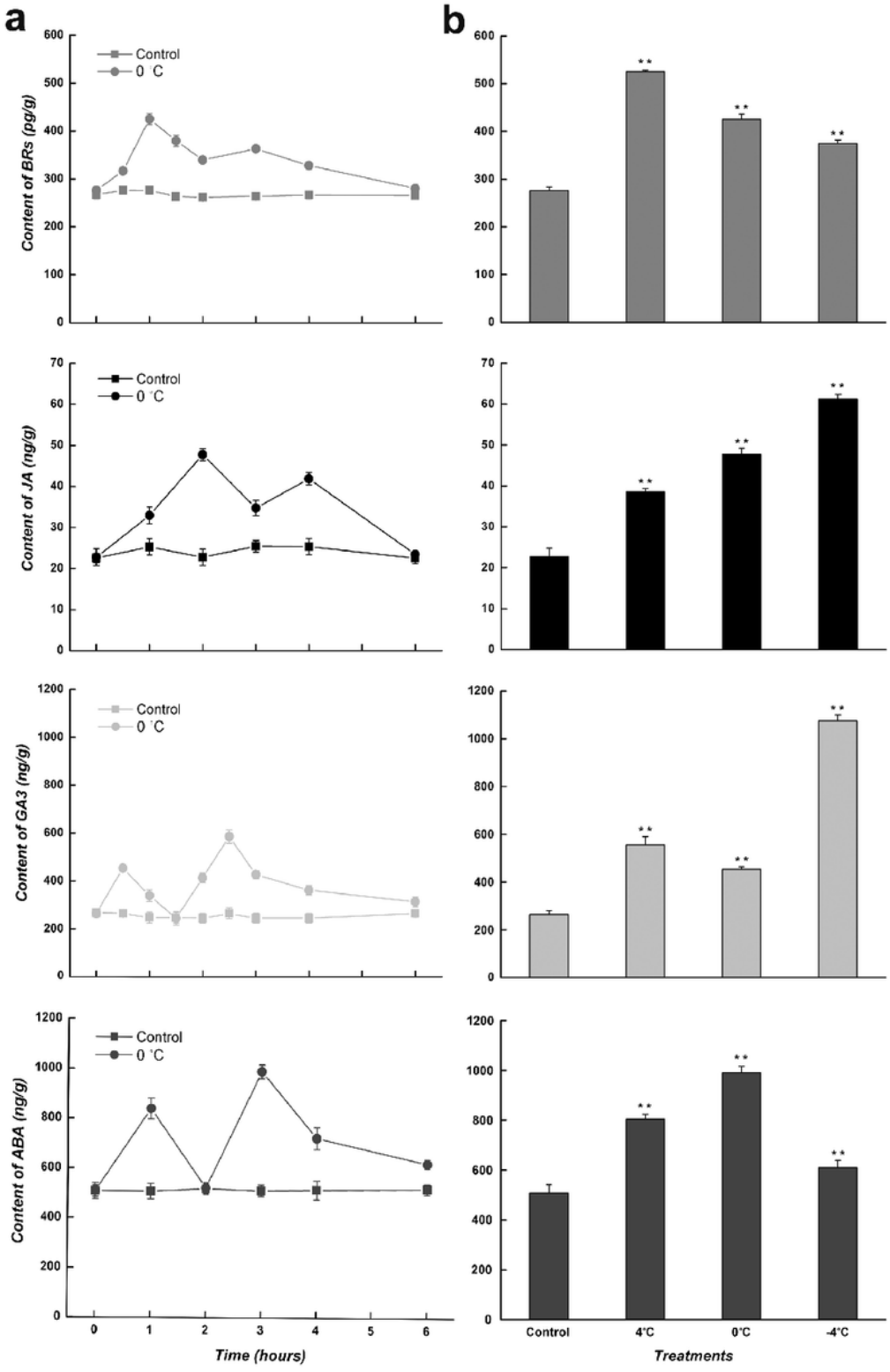

Figure 7

Pytohormone analysis in the leaves from *C. bungeana* regenerated plants at low temperatures. (a) Changes in the level of BRs, JA, GA3 and ABA at 0 ℃. (b) Levels of BRs, JA, GA3 and ABA at different low temperatures (4, 0 and -4 ℃). The data of BRs and ABA were detected at being treated for 1 h, and those of JA and GA3 were detected at being treated for 2 and 0.5 h, respectively. The corresponding data at the

same time point under normal conditions was taken as the control. Each value represents the mean ± SE of five replicates.

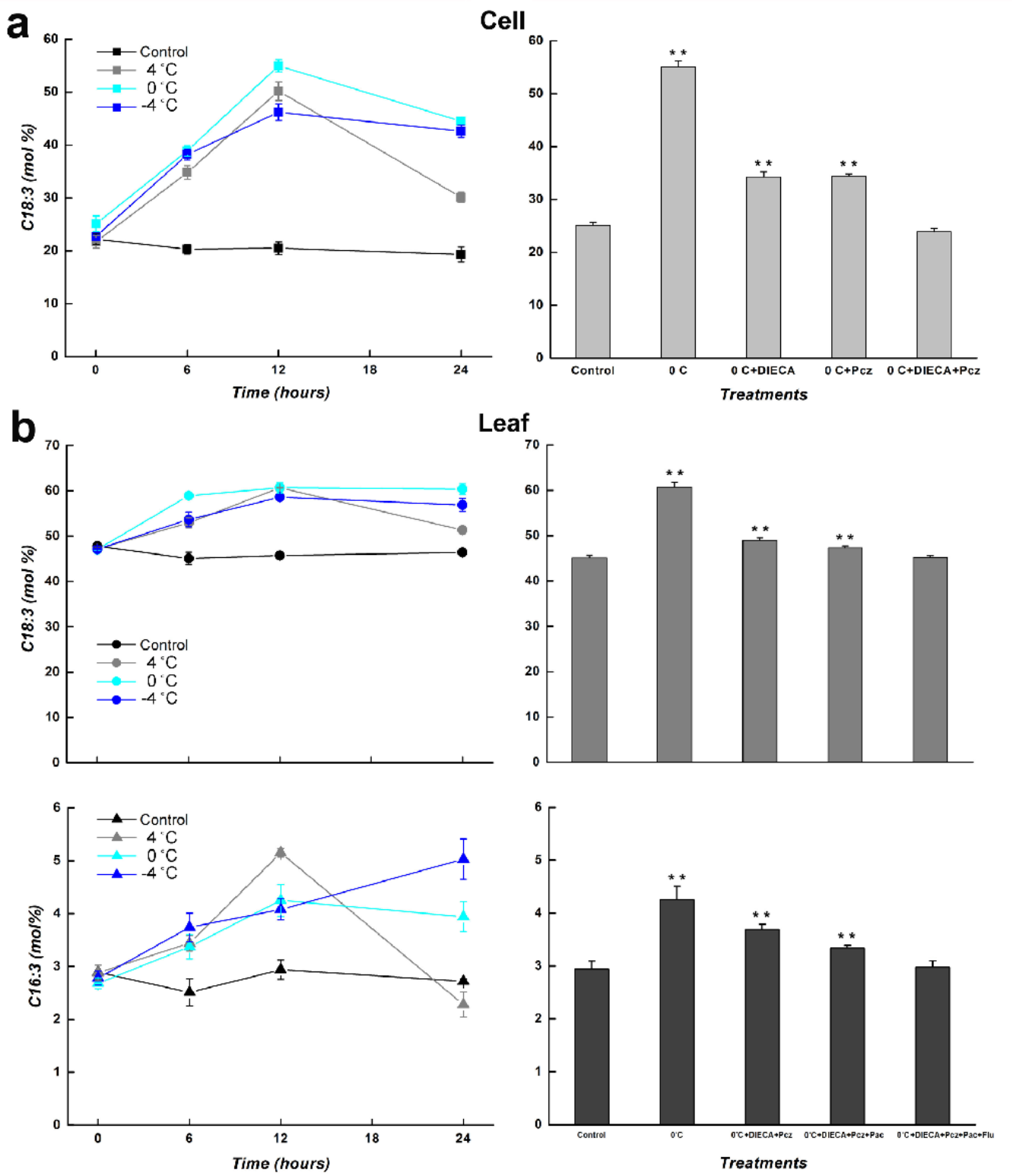

Figure 8

Levels of TAs in *C. bungeana* under low-temperature and hormone-inhibition treatments. (a) Levels of C18:3 in the total lipids of suspension-cultured cells. (b) Levels of C18:3 and C16:3 in the total lipids of

the leaves from regenerated plants. The data of inhibition treatments were detected at being treated for 12 h. The corresponding data at the same time point under normal conditions was taken as the control. Each value represents the mean ± SE of five replicates. The content of C18:3 in cell lipids and leave lipids as well as the content of C16:3 in leave lipids are indicated by square, circle and triangle, respectively.

Supplementary Files

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