Phytohormones Regulate the Non-Redundant Response of ω -3 Fatty Acid Desaturases to Low Temperatures in *Chorispora Bungeana*

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Abstract

To explore the contribution 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 a *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 non-green tissues. Low temperatures (4, 0 and -4 °C) resulted in significant increases in trienoic fatty acids (TAs, mainly C18:3), which were consistent with the non-redundant expression of *CbFAD3* and *CbFAD8* in suspension-cultured cells, and the coordination of *CbFAD7* and *CbFAD8* in leaves. Furthermore, the contribution of *CbFAD8* increased as temperature decrease in the two tissues. Our data revealed that jasmonic acid and brassinosteroids participated in the cold-responsive expression of these genes in both tissues, and 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 contribution of ω -3 CbFADs to maintain appropriate level of TAs under low temperatures, which help *C. bungeana* survive in cold environments.

Key Message

This is the first evidence that jasmonic acid and brassinosteroids participate in the non-redundant response of ω -3 fatty acid desaturases to low temperatures in *Chorispora bungeana*

Introduction

Low temperature is one of the major environmental stresses influencing plant performance and distribution. To withstand this stress, plants have developed various protective mechanisms that prevent and reduce the negative impacts (Iba 2002; Khan et al. 2017). Cell membranes, serving as a boundary and an 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). Membrane integrity and function, affected by temperature variation, can be maintained through the modification of fatty acids (Mikami and Murata 2003; Shi et al. 2008). In fact, the content of TAs, represented mainly by C18:3, changes to a certain extent in response to low temperatures, thus help maintaining membrane fluidity and structure (Matsuda et al. 2005; Upchurch 2008; Karabudak et al. 2014).

The production of TAs is catalyzed 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 most important factors for plant cold response (Iba and Kodama 1994), the expressions of ω -3 FAD genes have been researched in various plants. The first finding was come from maize leaves, which

showed an increase in *ZmFAD8* mRNA accompanied by a decrease in *ZmFAD7* mRNA under 5°C exposure (Berberich et al. 1998). Then, the cold-responsive expression of each plastidial and microsomal ω -3 FAD gene was studied in some other plants, such as birch (Martz et al. 2006), *Descurainia sophia* (Tang et al. 2007), purslane (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). All these researches were performed in plant leaves exposure to chilling temperatures (2 to 16 °C), and found an increased *FAD8* mRNA accompanied by a decreased *FAD7* or *FAD3* mRNA. However, little attention has been paid to the typical cold-tolerant plants (i.e. cryophytes) as well as severe low temperatures (e.g. subzero temperatures). Therefore, it is unclear the difference between cryophytes and the other plant species in the contribution of ω -3 FADs to cold stress response.

Besides that, though some phytohormones, for example abscisic acid (ABA), salicylic acid (SA), jasmonic 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), whether these hormones participate in the cold-responsive expressions of ω -3 FAD genes doesn't attract enough attention. There was only one direct evidence confirmed that JA partially participates in the chilling-induced regulation of ω -3 FAD genes in *Arabidopsis* (Shi et al. 2011). Recently, a study from *Arabidopsis* leaves indicated that AtFAD7 protein levels decreased upon ABA treatment, while AtFAD8 protein levels increased upon cold or JA exposure (Soria-García et al. 2019). However, the new findings haven't directly proved the regulation of ABA and JA on the cold response of AtFAD7 and AtFAD8. So far, the phytohormones that transmitted the low temperature signal to ω -3 FAD genes and induced their expression are still ambiguous. As a result, research is far from having an integrated view of how ω -3 FADs in typical cold-tolerant plants respond to low temperatures and through which mechanism this role is actually executed.

C. bungeana is a perennial cruciferous cryophyte, having close genetic relationship with *Arabidopsis* (Zhao et al. 2012). It inhabits periglacial regions (about 3800–3900 m), experiencing low temperature and freeze-thaw cycle all year round. Our previous studies found that *C. bungeana* possesses 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), which help it surviving in the extreme cold. Moreover, we found that the cold tolerance of *C. bungeana* suspension-cultured cells was closely related to the marked increased C18:3 (Shi et al. 2008), i.e. the product of ω -3 CbFADs. However, the contribution of each ω -3 CbFADs to the cold-induced C18:3 accumulations as well as the cold tolerance are unknown in *C. bungeana*.

In this work, we cloned two plastidial ω -3 FAD genes from *C. bungeana* and studied the cold-responsive expression of different ω -3 CbFAD genes to understand how they respond to low temperatures. Meanwhile, the relationship between the cold-responsive gene expression and the phytohormones, including ABA, SA, JA, BRs and GA3, were confirmed through the exogenous addition, the inhibitory

effects and the concentration detection of corresponding hormones. The experiments were performed on *C. bungeana* cell suspension cultures as well as on the leaves of regenerated plants to extend the analysis to a tissue system. The data suggest the existence of phytohormone regulation on ω -3 CbFADs in *C. bungeana* affecting specific isoforms in both plastid and microsome to maintain appropriate level of TAs under low temperatures and contribute to cold tolerance.

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 °C, 0 °C or -4 °C for 24 h, respectively. For low-temperature germination, *Arabidopsis* seeds planted on MS medium were exposed to 15 °C for 4 weeks. For exogenous hormone treatments, the cell suspensions and regenerated plants were moved to 1/2 MS medium with 100 μ M JA, 0.5 μ M BRs, 100 μ M SA, 100 μ M ABA, or 100 μ M GA₃ for 24 h, respectively. For phytohormone inhibitions, the cell suspensions and regenerated plants were moved to 1/2 MS medium with 10 μ M sodium diethyldithiocarbamate trihydrate (DIECA), 10 μ M propiconazole (Pcz), 10 μ M fluricbne (Flu), or 10 μ M 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 the 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 RNA were assessed by UV spectrophotometry and agarose gel electrophoresis. Then, the qualified total RNAs were employed in reverse transcription reaction by using PrimeScript™ Reverse Transcriptase (Takara, Japan) and Oligo(dT)₁₅ primer (Takara, Japan) following the manufacturer's instruction. Reverse-transcribed cDNA samples were stored at -20 °C 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 (P17 and 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 and Fig. S1), respectively. 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 and 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 °C for 30 sec, and 40 cycles of 95 °C for 5 s and 58 °C for 34 s. This was followed by 15 s at 95 °C, 60 s at 60 °C and 15 s at 95 °C (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:

$$F = \frac{(E_{\text{target}})^{\Delta C_{\text{t target}}(\text{control-sample})}}{(E_{\text{housekeeping}})^{\Delta C_{\text{t housekeeping}}(\text{control-sample})}}$$

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

Extraction and analysis of total fatty acids

The cell suspensions and the plant leaves of *C. bungeana* as well as the leaves of *Arabidopsis* (2 g each) were grinded with liquid N₂, respectively. Lipids and total fatty acids of each sample were prepared and analyzed as our previous reports (Shi et al. 2008; Shi et al. 2011). 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

The *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*

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 sequences showed remarkable similarity to other known plant FAD8 sequences and the highest identity (84%) to the *Arabidopsis* AtFAD8(NM120640). These data suggest 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 HXXXXXHVIHH, 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 determine 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 markedly higher than that in *fad7fad8* mutants; moreover, the C18:3 content in F7 lines was much higher than that in F8 lines, which may indicate a stronger conversion effect of CbFAD7 on C18:3 (Fig. 2a). Being exposed to 15 °C, 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 plastidial ω -3 FAD genes.

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

The expression profiles of plastidial and microsomal ω -3 CbFAD genes were analyzed in the cell suspensions and the regenerated plants of *C. bungeana*. Both *CbFAD7* and *CbFAD8* were highly expressed in leaves and lowly expressed 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* in suspension-cultured cells was just lower than that in leaves, while the expression level of *CbFAD8* in cultured cells was as low as that in roots. *CbFAD3* were highly expressed in non-green tissues, e.g. suspension-cultured cells and roots, and lowly expressed in stems and leaves (Fig 3a), showing the tissue-specific expression pattern with the characteristic feature of microsomal ω -3 FAD genes.

Given that microsomal and plastidic ω -3 CbFAD genes mostly expressed in suspension-cultured cells and plant leaves, respectively, the cold-responsive expression of these genes were detected in the two tissues. In cell suspensions, the expression of *CbFAD3* rapidly increased at 4 (6.3-fold) and 0 °C (5.7-fold), and decreased at -4 °C (Fig. 3b). Conversely, the expression of *CbFAD8* was almost no change at 4 °C, but sharply increased at 0 (13.1-fold) and -4 °C (27.7-fold) (Fig. 3b). In fact, 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). Being compared with cell suspensions, the expression pattern of *CbFAD3* and *CbFAD7* in plant leaves interchanged, the former decreased at all tested low temperatures, the latter rapidly increased at 4 (4.1-fold) and 0 °C (2.6-fold) (Fig. 3b). Like that observed in cultured cells, the up-regulation of leaf *CbFAD8* was only induced by severe low temperatures, with the increase of 3.4-fold at 0 °C and 6.7-fold at -4 °C (Fig. 3b). In this case, the cold-induced increase in *CbFAD7* and *CbFAD8* mRNA 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 respond to different low temperatures.

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

The expression of ω -3 CbFAD genes respond to various exogenous hormones were studied in the cell suspensions and the plant leaves of *C. bungeana*. In suspension-cultured cells, the expression of *CbFAD3* was up-regulated by ABA, SA and BRs, but down-regulated by JA and GA3; among these regulations, the BRs-induced up-regulation (peaked at being treated for 3 h, 6.2-fold) was similar to those induced by chilling temperatures, and the JA-resulted down-regulation was in accord with that reduced by subzero temperatures (Fig. 3a and 4a). Although the expression of *CbFAD7* was increased by ABA, SA and GA3, the decreased expression regulated by BRs and JA were consistent with the cold-reduced ones (Fig. 3a and 4a). The increase in *CbFAD8* mRNA was induced by all tested hormones except GA3, however, only the JA- and the BRs-induced increases (9.3-fold and 8.1-fold, respectively) peaked at being treated for 3 h and resembled those induced by severe low temperatures (Fig. 3a and 4a). Unlike that be found in cell suspensions, the expression of *CbFAD3* in leaves was down-regulated by all tested hormones with different dynamics, each of which approached the cold-reduced ones in varied degrees (Fig. 3b and 4b). Meanwhile, the expression of leaf *CbFAD7* was increased by BRs, JA and GA3, but reduced by ABA; among these changes, the increases induced by BRs and GA3 (1.6-fold and 1.9-fold, respectively) peaked at being treated for 3 h and were like those induced by chilling temperatures (Fig. 3b and 4b). Similarly, the expression of leaf *CbFAD8* was also induced by three hormones (ABA, BRs and JA) and reduced by one (GA3), however, only the increases induced by ABA and JA (4.3-fold and 3.6-fold, respectively) peaked at being treated for 6 h and resembled those induced by severe low temperatures (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 cultured cells and plant leaves, while ABA and GA3 may only participate in the cold-responsive gene expressions in leaves.

To further confirm the participations of these hormones, the corresponding inhibitors were used. Data showed that in cell suspensions (Fig. 5a), the cold-induced expression of *CbFAD3* was completely inhibited by Pcz (a synthetic inhibitor of BRs), but the inhibition could be partly eliminated by DIECA (a synthetic inhibitor of JA, 45.1%); the cold-reduced expression of *CbFAD7* was mainly eliminated by DIECA (65.8%) and partially eliminated by Pcz (32.9%), but completely eliminated by the synergism of them; in contrast, the cold-induced expression of *CbFAD8* gene was mostly inhibited by either DIECA (79.9%) or Pcz (78.9%), but completely inhibited by the cooperation of them. In plant leaves (Fig. 5b), the cold-reduced expression of *CbFAD3* was completely eliminated by the coordination 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%); the cold-induced expression of *CbFAD7* can be mainly inhibited by Pcz (66.0%) and partially inhibited by Pac (33.9%), but completely inhibited by the collaboration of them; likewise, the cold-induced expression of *CbFAD8* can be mostly inhibited by DIECA (73.1%) and partially inhibited by Flu (29.9%), but completely inhibited by the combined effect of them.

All these results suggested that in *C. bungeana* cultured cells, the cold-responsive expression of *CbFAD3* may be induced by BRs and reduced by JA; the synergism of JA and BRs may decrease *CbFAD7* mRNA and increase *CbFAD8* mRNA in response to low temperatures, however, the over-high concentration of BRs can in turn inhibit the expression of *CbFAD8* (Fig. 3b, 6b and S2). In *C. bungeana* leaves, the cold-reduced expression of *CbFAD3* was regulated by the cooperation of BRs, JA, GA3 and ABA; while the cold-induced expressions of *CbFAD7* and *CbFAD8* were regulated by the coordination of BRs and GA3 as well as JA and ABA, respectively.

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

To provide more evidence to the hormone-regulated cold response, the content changes of related phytohormones were detected in the cell suspensions and the plant leaves of *C. bungeana* at different low temperatures. Being treated at 0 °C, the levels of all tested phytohormones presented a rapid and two-peaks increase in both tissues (Fig. 6a and 7a). In cultured cells, the level of BRs peaked at 1 (1.7-fold) and 3 h (1.5-fold), while that of JA peaked at 1.5 (2.6-fold) and 3 h (2.4-fold). In plant leaves, the content changes of BRs and JA were the same as or similar to those in cultured cells, except the peak times of JA increase (2 and 4 h) were a little later; at the same time, the level of GA3 peaked at 0.5 (1.8-fold) and 2.5 h (2.3-fold), and that of ABA peaked at 1 (1.6-fold) and 3 h (1.9-fold). Overall, the cold-induced hormone increases, especially the first peaks, were before the cold-responsive expressions of corresponding ω -3 *CbFAD* genes (Fig. 3b, 6a and 7a); furthermore, the content increases in synergistic pairs, such as JA and ABA, or BRs and GA3, presented a peak stagger trend to avoid redundant effect at the same temperature (Fig. 6a and 7a).

Actually, all tested low temperatures could induce the content increases in these phytohormones, but the increment of the same phytohormone was varied (Fig. 6b and 7b). In suspension-cultured cells (Fig. 6b), the level of BRs highly increased at 4 °C (2.6-fold) and slightly increased at 0 °C (1.7-fold); as for JA, the highest content increment was found at -4 °C (3.1-fold), and the increments at 4 and 0 °C were the same

(2.6-fold). The cold-induced hormone increases in leaves were more regular than those in cultured cells (Fig. 7b): the content increment of BRs decreased as temperature decrease, conversely, that of JA increased; meanwhile, the level of GA3 showed the highest and the lowest increases at -4 (4.2-fold) and 0 °C (1.8-fold), respectively, while those of ABA presented in an opposite way (1.9-fold at 0 °C and 1.2-fold at -4 °C). On the whole, the increment changes in these phytohormones, were consistent with the dynamic expression of corresponding ω -3 CbFAD genes at different low temperatures; furthermore, the content increment of antagonistic pairs, for example GA3 and ABA, showed a reverse tendency in response to temperature variation, which was 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 expressions of ω -3 CbFAD genes may affect the level of TAs, the C18:3 and the C16:3 contents of total lipids were detected in the cell suspensions and the plant leaves of *C. bungeana* under different low temperatures. Because of lacking chloroplast, no C16:3 was detected in suspension-cultured cells (Fig. 8a). Data showed that the level of C18:3 was obviously induced by all tested low temperatures and reached the maximum at being treated for 12 h in both tissues; the C18:3 content in cultured cells increased from about 20.6% to 46.2-55.0% of total fatty acids, and that in leaves increased from about 46.3% to 58.6-60.7% of total fatty acids (Fig. 8). Similarly, the C16:3 content of 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 °C) or 24 h (-4 °C) (Fig. 8b). In general, the marked increases in TAs were in accordance with but lagging behind the cold-responsive expressions of ω -3 CbFAD genes (Fig. 3 and 8).

To further confirm that the phytohormones affect the cold-induced level of TAs through regulating ω -3 CbFAD genes, the C18:3 and the C16:3 contents of total lipids were also detected in the cell suspensions and the plant leaves of *C. bungeana* under hormone-inhibitor treatments. In suspension-cultured cells, the cold-induced increase in C18:3 content was completely inhibited by the synergism of DIECA and Pcz, but partially inhibited by either of them (Fig. 8a); In plant leaves, the cold-induced increases in C18:3 and C16:3 contents were all 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 expressions of ω -3 CbFAD genes (Fig. 5 and 8), suggesting that with the help of ABA and GA3 in leaves, JA and BRs participated in maintaining appropriate level of TAs in *C. bungeana* through regulating these genes under low temperatures.

Discussion

This work analyzed the behavior of the microsomal and plastidial ω -3 FADs in *C. bungeana* at the level of both 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. Obviously, low temperatures resulted in significant transcriptional changes on each ω -3 FAD, notably the sharp increased *CbFAD8* mRNA occurred in both suspension-cultured cells and the leaves from regenerated plants (Fig. 3b). 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 (McConn et al. 1994). From then on, the increased expression of *FAD8* were found in many plant species at various chilling temperatures (4-10 °C) (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), excepting a few different observations (Román et al. 2012; Guan et al. 2014; Zhang et al. 2018). In this work, the increased *CbFAD8* mRNA were not induced by the chilling temperature (4 °C), but by severe low temperatures (0 and -4 °C), and the increments increased as temperature decreased in both tested tissues. The results from *C. bungeana* not only support the previous view that FAD8 was a cold-specific desaturase, but also indicate that CbFAD8 is the major ω -3 desaturase contributed to the synthesis of TAs in response to severe low temperatures, particularly subzero temperatures. 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* under subfreezing temperatures may be essential for plant cold tolerance.

As for CbFAD3 and CbFAD7, the cold-responsive transcriptional changes presented a tissue-specific profile (Fig. 3b). During low temperature exposure, the *CbFAD3* mRNA increased in suspension-cultured cells and decreased in plant leaves, while the expressions of *CbFAD7* in the two tissues were just the opposite. Previous researches showed that even in the same type of tissue under a similar temperature, the expression profiles 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 consistent with ours but the others not. Although we don't know why these enzymes provide different contributions in distinct plant species, it is not surprising that the contributions of CbFAD3 and CbFAD7 in *C. bungeana* were proportional to their transcript abundance in distinct tissues (Fig.3).

In fact, the cold-induced increments of *CbFAD3* or *CbFAD7* mRNA decreased as temperature decreased, and formed a non-redundant complementation with the gradually increased *CbFAD8* mRNA in distinct tissues (Fig. 3b). In *C. bungeana* cell suspensions, the non-redundant coordination of *CbFAD3* and *CbFAD8* indicate the distinct TA needs under different low temperatures, for FAD3 affecting total TA levels and FAD8 affecting plastid TA levels (Zhang et al. 2005; Penfield 2008). The non-redundant cooperation of *FAD7* and *FAD8* was also found in *Arabidopsis* leaves at different temperatures (8, 22 and 30 °C; Román et al. 2015). Though both FAD7 and FAD8 affect the synthesis of TAs in chloroplastic lipids, the lipid specificity of them are different (Soria-García et al. 2019): AtFAD7 prefer galactolipids, which are the major chloroplast lipids with higher TA content; while AtFAD8 prefer phosphatidylglycerol (Román et al. 2015), which has specific role in the stability of photosynthetic complexes (Wada and Murata 2007). Hence, the non-redundant cooperation of *CbFAD7* and *CbFAD8* in *C. bungeana* leaves may due to the distinct needs of photosynthetic activity and stability under different low temperatures. 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 (Fig. 5 and 8) and phytohormone detection (Fig. 6 and 7), we confirmed that the cold-responsive non-redundant expression of ω -3 CbFAD genes were regulated by certain phytohormones, notably JA and BRs. JA is mainly responsible for the induction of *FAD8* under freezing/subfreezing temperatures, with the association of BRs in cell suspensions, and ABA in plant leaves; BRs is in charge of the induction of *CbFAD3* or *CbFAD7* under chilling/freezing temperatures, with or without the help of GA3 in distinct tissues. Moreover, both JA and BRs took part in the down-regulation of corresponding ω -3 CbFAD gene in response to cold stress, with or without the participation of ABA and GA3 in different tissues. These results 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. Among these interactive hormones, ABA may function synergistically with JA signaling to modulate cold-responsive genes (Hu et al. 2017; Kim et al. 2018; Wang et al. 2020); while the concentration of BRs decides it in synergism or antagonism with JA during stress defense (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). The interactions of these phytohormones with JA all consist with the observations from *C. bungeana*. A recent study showed that in *Arabidopsis* leaves, AtFAD8 protein levels increased upon cold or JA exposure, but did not respond to ABA (Soria-García et al. 2019). All the findings indicate that though the interactive hormones 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 all plant species.

BRs are another important pythohormone took part in the cold-responsive expressions of ω -3 FAD genes in *C. bungeana*. 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). The findings provide support to our observation that the cooperation of BRs and GA3 participated in the cold-responsive expressions of *CbFAD3* and *CbFAD7* in *C. bungeana* leaves. Besides, 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 cold-responsive down-regulation of leaf *CbFAD3*. However, because there was no report about the function of BRs on the cold-responsive expressions of ω -3 FAD genes, we cannot predict that BRs also regulates the cold induction of *FAD3* or *FAD7* in other plant species before further research.

It is worth to note that the interactions between different pythohormones on ω -3 CbFAD genes suggest each of the genes having the ability in response to multiple hormones (Fig. 4-8; Table 1). A similar phenomenon from *Arabidopsis* showed that 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 was first found in the promoter of *AtFAD7* in *Arabidopsis* roots, which was required by JA-responsive expression (Nishiuchi

et al. 1999). After that, no hormone-responsive element was confirmed in the promoter of ω -3 FAD genes. Until recently, the multiple ABA responsive elements (ABREs) were found in the promoter of microalgae ω -3 FAD gene (Norlina et al. 2020). However, the data is still too limited to have an integrated understanding.

As another necessary factor for the hormone regulation on ω -3 FAD genes, TFs have been studied at recent years. On the one hand, some TFs for regulating the expression of ω -3 FAD genes were verified: the expression of *FAD3* is up-regulated by bZIP67 in *Arabidopsis* seeds (Mendes et al. 2013), but down-regulated by MaMYB4 in banana fruits (Song et al. 2019) and by HD in soybean (Jo et al. 2021); WIPK is involved in wound-responsive expression of *AtFAD7* gene in transgenic tobaccos (Kadoma et al. 2000). On the other hand, it is confirmed in *Arabidopsis* that 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 (Banerjee and Roychoudhury 2017; Ramirez and Poppenberger 2020). Although these relevant information pieces are incomplete, 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 non-redundant enzyme cooperation to maintain 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.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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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
<i>CbFAD3</i>	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
<i>CbFAD7</i>	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
<i>CbFAD8</i>	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, $n=5\times3$. 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
<i>CbFAD3</i>	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***
<i>CbFAD7</i>	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**
<i>CbFAD8</i>	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, $n=5\times3$. Significant positive correlation ($R > 0.50$, $P < 0.05$) is indicated in bold. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Supplementary Figures And Tables

Supplementary Figures and Tables are not available with this version.

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 $\pm$ 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 cell suspensions and plant leaves. 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 $\pm$ SE of three replicates.

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 plant leaves. 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 $\pm$ SE of three replicates.

Figure 5

Effects of hormone inhibitors on 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 plant leaves. 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 $\pm$ 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 °C (b) Levels of BRs and JA at different low temperatures (4, 0 and -4 °C). 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 $\pm$ SE of five replicates.

Figure 7

Pytohormone analysis in the plant leaves of *C. bungeana* at low temperatures. (a) Changes in the level of BRs, JA, GA3 and ABA at 0 °C. (b) Levels of BRs, JA, GA3 and ABA at different low temperatures (4, 0 and -4 °C). 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 $\pm$ 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 plant leaves. 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 $\pm$ SE of five replicates.