Cold stress improves the production of artemisinin depending on the increase of endogenous jasmonate

Wanhong Liu[1,3], Huanyan Wang[2], Yupei Chen[2], Shunqin Zhu[2], Min Chen[4], Xiaozhong Lan[5], Guoping Chen[1*], Zhihua Liao[2*]

[1]Key Laboratory of Biorheological Science and Technology, Ministry of Education, College of Bioengineering, Chongqing University, Chongqing 400044, People's Republic of China

[2]Key Laboratory of Eco-environments in Three Gorges Reservoir Region (Ministry of Education), SWU-TAAHC Medicinal Plant Joint R&D Centre, School of Life Sciences, Southwest University, Chongqing 400715, People's Republic of China

[3]School of Chemistry and Chemical Engineering, Chongqing University of Science and Technology, Chongqing 401331; People's Republic of China

[4]SWU-TAAHC Medicinal Plant Joint R&D Centre, College of Pharmaceutical Sciences, Southwest University, Chongqing 400715, People's Republic of China

[5]TAAHC-SWU Medicinal Plant Joint R&D Centre, Agricultural and Animal Husbandry College, Tibet University, Nyingchi of Tibet 860000, China

[*]Correspondence: Guoping Chen (chenguoping@cqu.edu.cn), Zhihua Liao (zhliao@swu.edu.cn)

Abstract

Previous publications reported that the artemisinin level was increased in *Artemisia annua* following a night-frost period. However, the molecular mechanism was not clear. In this study, we found that exogenous jasmonate effectively enhanced the freezing tolerance of *A. annua*. The jasmonate biosynthetic genes (*LOX1*, *LOX2*, *AOC* and *JAR1*) were induced by cold stress, leading to an increase in endogenous jasmonate in cold-treated *A. annua*. Increased endogenous jasmonate enhanced the expression of three jasmonate-responsive transcription factors, ERF1, ERF2, and ORA, all of which were reported to transcriptionally activate the expression of artemisinin biosynthetic genes, such as *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*. Furthermore, the expression levels of the four artemisinin biosynthetic genes were also significantly increased under cold stress. Consequently, the levels of artemisinin and related secondary metabolites, such as dihydroartemisinic acid, artemisinin B and

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/bab.1493.

artemisinic acid, were increased in *A. annua* under cold stress. Our study points to a molecular mechanism in which the production of artemisinin is regulated by cold stress in *A. annua*.

Key words: *Artemisia annua* L., Cold stress, endogenous jasmonate, artemisinin biosynthesis

Abbreviations:

ADS Amorpha-4,11-diene synthase

ALDH1 Aldehyde dehydrogenase 1

AOC Allene oxide cyclase

bHLH basic helix-loop-helix

CYP71AV1 Cytochrome P450 monooxygenase

DBR2 Artemisinic aldehyde Δ11(13) reductase

DW Dry weight

ELISA Enzyme-linked immunosorbent assay

ERF1 Ethylene response factor 1

ERF2 Ethylene response factor 2

FW Fresh weight

JA Jasmonate

JAR1 JASMONATE RESISTANT 1

LOX Lipoxygenase

ORA Octadecanoid-responsive AP2/ERF

qPCR Quantitative PCR

TFs Transcription factors

1. Introduction

Artemisinin, an endoperoxide sesquiterpene lactone, is effective in the treatment of malaria, a severely infectious disease leading to millions of deaths every year [1]. Artemisinin is isolated from the Chinese traditional herb *Artemisia annua*, which contains artemisinin at a low concentration of 0.01–0.8% of dry weight [2]. To improve the production of artemisinin, several strategies have been used, such as

chemical synthesis, biosynthesis of artemisinic acid in microbes, and transgenic plants through overexpressing genes encoding the limiting enzymes or depression of enzymes in the competitive pathway [3]. In addition, a few groups reported that the production of artemisinin was regulated by exogenous factors, such as plant hormones [4-7], salt stress [8], radiation stress [9], sugar [10] and biotic stress [11]. Therefore, studies on the regulatory mechanism underline the increase in artemisinin content in *A. annua* in response to exogenous factors and suggest an important direction in the field of artemisinin metabolic engineering.

Low temperature is one of the most harmful abiotic stresses and can increase the content of cryo-protective compounds to enhance cold tolerance in plants [12]. Previous studies reported that low temperature stress can effectively promote the accumulation of secondary metabolites. The anthocyanin content of blood orange fruit stored at 4ºC sharply increased throughout the entire storage period [13]. The production of anthocyanin was also increased by cold stress in apple trees, and the molecular mechanism was revealed. The cold-induced transcription factor *MdbHLH3* interacted physically and specifically with *MdMYB1*, which could activate the expression of the anthocyanin biosynthesis genes *MdDFR* and *MdUFGT* in apple trees [14]. The contents of putrescine and spermidine were significantly increased in wheat after cold treatment, which improved the expression level of the polyamine biosynthetic pathway genes encoding arginine decarboxylase and ornithine decarboxylase [15]. The level of artemisinin was increased in *A. annua* after a night-frost period [16]. However, the exact molecular mechanism of cold stress response is still unclear in *A. annua*.

Jasmonate (JA), a vital signaling molecule derived from lipids, plays an important role in diverse plant stress responses [17]. Exogenous MeJA treatment effectively alleviates the chilling injury in tropical plants and is widely used for the storage of fruits such as mango, tomato and papaya [18-20]. Endogenous JA content was increased, and several genes encoding critical enzymes involved in JA biosynthesis were upregulated by low temperature stress in *Arabidopsis* [21]. As a powerful molecule, JA also affects the accumulation of secondary metabolites in plants [22, 23]. For example, the content of total tanshinones was notably increased 3.10-fold in *Salvia miltiorrhiza* hairy roots with MeJA treatment [24]. It is well known that MeJA had been used to enhance artemisinin production in whole *A. annua* plants [4, 25-27]. Recently, several transcription factors (TFs), including AaERF1, AaERF2 and AaORA, from *A. annua* were functionally characterized, and all of them were confirmed to positively regulate artemisinin biosynthetic genes [28, 29]. *AaERF1* and *AaERF2* were highly expressed in inflorescences where the artemisinin level was higher than that in any other organ, while the two TFs were strongly induced by JA. Transient expression of either *AaERF1* or *AaERF2* in tobacco induced the promoter activities of *ADS* or *CYP71AV1*, which encode critical enzymes in artemisinin biosynthesis [28]. Overexpression of *AaORA* in *A. annua*, another positive regulator involved in regulating artemisinin biosynthesis, significantly increased the transcript levels of artemisinin biosynthesis pathway genes and increased the content of artemisinin and its precursor dihydroartemisinic acid [29]. These findings on artemisinin biosynthesis at the molecular level will facilitate the unveiling of the

mechanism by which cold stress enhances artemisinin production.

To better understand how low temperature stress enhances artemisinin production, we investigated the effect of exogenous MeJA treatment on reducing the chill injury in *A. annua*. Using a time-course, we analyzed the contents of endogenous JA, artemisinin and related metabolites under the cold stress and the expression levels of JA biosynthetic genes, TFs regulating artemisinin biosynthesis and artemisinin biosynthetic genes in *A. annua*. The results revealed that artemisinin content was enhanced under cold stress and depended on an increase in endogenous JA.

2. Materials and methods

2.1. Plant materials and treatments

A. annua seeds were harvested from the botanical garden of Southwest University (Chongqing, China) in November of 2014. The seeds were sowed in pots and generated into plantlets at 25ºC under a daily 16 h light and 8 h dark photoperiod at 80–100 mE $m^{-2}s^{-2}$. Tap water was irrigated twice a week until measurement. Two-month-old plantlets in the illumination incubator at 4ºC from 1 h to 24 h, using 0 h as the control, were used as samples for the assays of endogenous JA and target gene expression. These samples were collected and immediately frozen in liquid nitrogen and stored at -80ºC for further use. Artemisinin and related secondary metabolites were isolated from the two-month-old plantlets in the illumination incubator at 4ºC from 12 h to 48 h, and 0 h samples were used as the control. Three biological replicates were performed in this study.

2.2. Freezing tolerance assays

To investigate the role of exogenous MeJA in low temperature stress, a freezing tolerance assay was performed as described with some modifications [21]. *A. annua* seeds germinated into very young seedlings in 7 d after inoculation on Murashige and Skoog (MS) agar medium [30] at 25ºC. Subsequently, the very young seedlings were immediately transferred to MS medium with or without 3 or 5 μM MeJA. All the seedlings grew at 25ºC for 15 d. Then, these petri dishes of plants (22-day old) were placed in a freezing chamber (in darkness) set at -7ºC for 24 h. After the freezing treatment, the seedlings were incubated in an illumination incubator at 25ºC. The survival rates of the seedlings were scored visually after 7 d. The experiments were biologically repeated 3 times.

2.3. Determination of endogenous JA level in *A. annua*

A plant JA assay kit (TSZELISA, Lexington, USA) was used for the determination of JA contents in *A. annua* according to the protocol. Briefly, approximately 1 g fresh leaves from 4° C-treated plants (from 1 h to 48 h, 0 h as control) was ground to a very fine powder in liquid nitrogen. All powders were transferred into a phosphate buffered saline (PBS) (pH 7.4) solution and centrifuged (3000 rpm, 20

min). The supernatant was diluted 100 times by dilution buffer and then subjected to an ELISA assay. The prepared samples were added to a 96-well ELISA plate, and then the 96-well plate was covered with a film and incubated at 37ºC for 30 min. Next, the 96-well ELISA plate was washed 5 times with washing buffer and abandoned waste liquid. A 50 μl volume of enzyme labeled reagent was added to the well and incubated in 37ºC for 30 min. The reaction residues were washed 5 times with washing buffer. Chromomeric agents A and B were then added and mixed in the wells, followed by incubation in the dark at 37ºC for 10 min. Then, the reaction was stopped using 50 μl termination buffer. The OD450 values were measured by a microplate reader, and the supernatant contained JA. Finally, the JA content was calculated based on a standard curve.

2.4. RNA extraction and gene expression analysis by quantitative real-time PCR

An RNAsimple Total RNA Kit (Tiangen Biotech, Beijing, China) was used for total RNA extraction according to the manual. Contaminated DNA was digested using DNase I (TaKaRa, Dalian, China) in all the samples. The quality and quantity of RNA was assayed using a Nano spectrophotometer (ND-100, Thermo scientific, city, country). In total, 2 μg RNA was reverse transcribed using a GoScript™ Reverse Transcription System (Promega, USA) for first strand cDNA synthesis according to the protocol. The RT procedures were carried out at 42ºC for 60 min and 70ºC for 15 min for reverse transcriptase inactivation. All cDNA samples were diluted 50 times with RNase-free water, and then 8 μL cDNA (16 ng) was used as a template. qPCR was performed on a iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) in 96-well plates using GoTaq® qPCR Master Mix (Promega, USA) according to the protocol. The internal control genes corresponded to *actin* and *EF1a*, which were previously reported to be stable reference genes under low temperature stress [31]. The specific primers for target genes are shown in Table 1. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative fold changes in gene expression [32].

2.5. Detection of artemisinin and related metabolites

Following previous reports [33, 34], the extraction method for artemisinin and related metabolites is described briefly as follows: The leaves were collected at 12 h, 24 h and 48 h from *A. annua* under low temperature stress and dried at 50ºC, using non-treatment as the control. Then, the powdered dried samples were extracted with 20 mL petroleum ether and treated in an ultrasonic bath for 1 h. The petroleum solvent was collected and dried in a rotavapor at 50ºC. Finally, the residue was re-dissolved in 5 mL of methanol. A volume of 1 mL of the solution was filtered through a 0.22 μm nylon membrane filter. The filtered solutions were used for high-performance liquid chromatography (HPLC) analysis by Shimadzu LC-6AD HPLC system (SPD-M20A PDA detector) with a C18 column (Phenomenex, CA, USA) at a wavelength of 209 nm. The injection volume was 20 μL for each sample. The mobile phase was isocratic and composed of 50% acetonitrile and 50% of a 0.1% acetic acid aqueous solution (pH 3.2) with a flow rate of 1.0 mL min^{-1} at 30ºC [34, 35]. Control samples of artemisinin were purchased from Sigma (Sigma-Aldrich, USA); dihydroartemisinic acid, artemisinic acid, and arteannuin B were purchased from AiKang Biological Co., Ltd (Changsha, China). There were three biological replicates in the

experiments.

2.6. Statistical analysis

Three biological replicates were used in the present study. The survival rates were analyzed by one-way analysis of variance (ANOVA) using SPSS 16.0 software (SPSS Inc., Arlington, VA, USA). The expression levels of all the target genes and the contents of artemisinin and related metabolites were analyzed with a *t*-test.

3. Results

3.1. Exogenous MeJA enhanced the freezing tolerance of *A. annua*

In the presence of MeJA (3 and 5 μM), the roots of *A. annua* seedlings were slightly shorter and accumulated more anthocyanins than those without MeJA treatment (Figure 1A, 1B, 1C). The phenotypes of *A. annua* under MeJA treatment were consistent with those reported for *A. thaliana* [36]. The survival rates of 3 and 5 μM MeJA-treated plants were 57.13% and 86.67%, respectively, which were significantly higher than those of control plants with a survival rate of 34.29% (Figure 1D). The higher concentration of MeJA leading to higher survival rates of *A. annua* suggested that the effect of MeJA might be dosage dependent. These results showed that exogenous MeJA effectively enhanced the freezing tolerance of *A. annua* and suggest that JA signaling is positively involved in the freezing tolerance of *A. annua*.

3.2. Cold stress upregulated the expression levels of JA biosynthetic genes and increased endogenous JA production in *A. annua*

To provide further evidence that JA is the positive regulator responding to the cold stress in *A. annua*, the expression levels of JA biosynthetic genes and the productions of endogenous JA were assayed in cold-treated plants. We examined the expression levels of four genes involved in JA biosynthetic pathway, *LOX1*, *LOX2*, *AOC* and *JAR1*. At 6 h after low temperature treatment, the relative expression level of *LOX1* was 8-fold higher than that in control plants (Fig. 2B). *LOX2*, another member of the *LOX* gene family, showed a higher expression level following treatment with cold stress from 3 h to 6 h (Fig. 2C). The expression level of *AOC* slightly decreased at the beginning of cold treatment and then gradually increased; it reached the peak level at 12 h after cold stress (Fig. 2D). The expression of *JAR1* increased significantly from 1 to 24 h when plants were treated with a low temperature, and the highest expression level was observed at 6 h of treatment (Fig. 2E). It could be concluded that cold stress upregulated the expression of JA biosynthetic genes. The increase in JA biosynthetic gene expression might lead to the increased content of endogenous JA, which was confirmed by the following experiments.

To explore the variation in the endogenous JA level in *A. annua* under cold stress, we measured JA production in plants following cold treatment. Endogenous JA production was induced by low temperature and reached a peak level in the plants treated for 6 h. Subsequently, the level of JA

drastically decreased and was restored to normal within 12 to 48 hours (Fig. 2A).

3.3. Cold stress upregulated the expression levels of TFs that positively regulate artemisinin biosynthetic genes

In the present study, qPCR was used to detect the expression levels of three transcription factors, ERF1, ERF2 and ORA, which were confirmed to regulate artemisinin biosynthesis *via* JA signaling pathways [28, 29]. The time course was from 0 h to 24 h, while the samples at 0 h were used as a control. *ERF1* was strongly induced in the cold-treated plants from 1 h to 12 h, and its expression was more decreased than the control at 24 h (Fig. 3A). The expression level of *ERF2*, a gene homologous to *ERF1*, increased significantly from 3 h to 6 h. The expression of ERF2 in the other samples, including those from the 1 h, 12 h and 24 h time points, was more decreased than in the control (Fig. 3B). The highest expression level of *ORA* appeared at 6 h, and there were no significant differences in gene expression at the other time points (Fig. 3C). The three TFs showed significant increases in expression level at some time point, suggesting similar expression patterns.

3.4. The relative expression levels of the artemisinin biosynthetic genes were promoted under cold stress

Four genes involved in the artemisinin biosynthetic pathway, *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*, were tested by qPCR in cold-treated plants. The time course was from 0 h to 24 h, and the samples at 0 h were used as the control. Generally, the expression levels of all four genes decreased at the beginning of cold treatment. The expression of both *ADS* and *ALDH1* reached a peak level at 6 h, which was the time point with the highest endogenous JA production (Fig. 4A, 4D). The expression levels of *CYP71AV1* and *DBR2* were significantly increased at 12 h and 24 h after cold treatment, respectively (Fig. 4B, 4C). The relative expression levels of the four genes were induced at different periods in cold-treated plants, suggesting that the four genes might be regulated by different transcription factors.

3.5. Artemisinin and related metabolites increased under cold stress

Artemisinin and related metabolites were analyzed in plants treated at 4ºC by HPLC, and non-treated samples were used as the control. The artemisinin content increased significantly in plants after treatment at 24 h and 48 h. In the plants treated for 24 h, artemisinin content ranged from 2.09 to 3.05 mg g^{-1} DW, which was 1.34–1.96-fold higher compared with the artemisinin content of 1.56 mg g^{-1} DW in the control. In the plants treated for 48 h, artemisinin content ranged from 4.59 to 4.88 mg g^{-1} DW, which was 2.95–3.13-fold higher compared with that of the control (Fig. 5A). Dihydroartemisinic acid (DHAA), as a direct precursor to artemisinin, was also detected. The HPLC results showed that DHAA content increased significantly in cold-treated plants at 12 h compared with that in the control (Fig. 5B). The contents of arteannuin B and artemisinic acid, metabolites in the competitively branch pathway of artemisinin biosynthesis, were also tested by HPLC. Arteannuin B content, ranging from 0.031 to 0.051

mg g^{-1} DW, significantly increased in the treated plants at 48 h (Fig. 5C). The content of artemisinic acid, as a precursor to arteannuin B, increased significantly in cold-treated plants at 12 h (Fig. 5D). These results demonstrated that cold stress enhanced the productions of artemisinin and related metabolites in *A. annua*.

4. Discussion

Low temperature is one of the most harmful environmental factors and limits plant growth, development and production. Plant hormones play important roles in regulating responses to cold stress [37]. A pervious study demonstrated that ethylene signaling negatively regulates cold and freezing tolerance in *Arabidopsis* [38]. Conversely, JA, as a positive regulator, enhanced the cold and freezing tolerance of *A. thaliana* [21]. In our study, exogenous JA significantly improved the survival rate of *A. annua* seedlings under cold stress (Figure 1). These results showed that cold tolerance was positively regulated by the JA signaling pathway in *A. annua*.

Furthermore, JA biosynthetic pathway genes, such as *LOX1*/*2*, *AOC*, and *JAR1*, were rapidly elevated in *A. thaliana* and *Oryza sativa* plants when exposed to cold stress [21, 39]. The endogenous JA levels increased in *AaAOC*-overexpressing transgenic *A. annua* and increased artemisinin levels [40]. In our study, the expression levels of JA biosynthetic pathway genes were also elevated in *A. annua* treated with cold stress. The increased endogenous JA promoted intracellular signals, which improved artemisinin biosynthesis. Similar phenomena were found in Taxus cells. Endogenous salicylic acid enhanced the accumulation of taxuyunnanine C in suspension cultures of *Taxus chinensis* [41]. It is worth noting that the expression of *JAR1* was strongly induced by cold stress (Figure 2E) from 1 h to 24 h (the entire cold treatment period), while upstream genes, including *LOX1*, *LOX2* and *AOC*, only had higher expression levels at some time points. This result suggested that more JA-Ile molecules were continuously formed through JA as a substrate catalyzed by JAR1 during the entire cold treatment period. In fact, JA-Ile, as a product of JAR1, was the most potent molecule in JA signaling [42]. The conversion of JA to JA-Ile facilitated cold tolerance in *A. annua*. These results explained the phenomenon that endogenous JA content decreased sharply after 6 h of cold treatment. Although the mechanism of JA biosynthetic genes responding to cold stress is still unclear, our data showed that endogenous JA was significantly increased in cold-stressed plants and reached a peak level at 6 h (Figure 2A), suggesting that JA signaling pathways are triggered in cold treated plants.

To date, several transcription factors have been confirmed to positively regulate artemisinin biosynthetic genes *via* JA signaling pathways. Two JA-responsive AP2 family transcription factors, ERF1 and ERF2, were strongly induced by exogenous JA. Both ERF1 and ERF2 improved the promoter activities of *ADS* and *CYP71AV1*, which are the key genes involved in artemisinin biosynthesis [28]. ORA, a trichome-specific AP2/ERF transcription factor, was proved to positively regulate the expression of artemisinin biosynthetic genes such as *ADS*, *CYP71AV1*, and *DBR2*.[29]. In our study, the expression levels of ERF1/2 and ORA were induced by cold stress (Figure 3). This increase was caused by the increase in endogenous JA in *A. annua* under cold stress. Additionally, ERF1/2 and ORA may be regulated

by other cold-responsive transcription factors. The previous studies showed that JA signaling and cold-response signaling pathways share several common components, such as SFR6/MED16 [43, 44]. As a result, the higher expression levels of the TFs mentioned above would definitively cause higher expression levels of artemisinin biosynthetic genes. The present study showed that the expression levels of artemisinin biosynthetic genes, including *ADS*, *CYP71AV1*, *DBR2* and *ALDH1*, were induced in *A. annua* under cold stress. This result could be explained by higher expression levels of JA-responsive transcription factors such as ERF1/2 and ORA under cold stress.

It is worth noting that the gene expression profiles suggested that the high expression levels appeared at some time point. The expression levels of JA biosynthetic genes, including *LOX1/2* and *JAR1*, peaked at 6 h (Figure 2B, 2C, 2E), which might be the reason for the highest level of JA in 6 h cold-treated plants. Two transcription factors, ERF1 and ORA, showed expression that peaked at 6 h due to the highest JA being present at 6 h (Figure 3A, 3C). The expression of *ADS* and *CYP71AV1* appeared to be early and transiently downregulated at the beginning of cold stress, which could been explained by the fact that plants start to undergo substantial changes in metabolism under cold stress, and an initial inhibition of metabolism due to reduced enzymatic activities occurs under cold [45]. The expression levels of artemisinin biosynthesis-related genes peaked at 6 h, 12 h and 24 h (Figure 4), which indicated that these genes were regulated by multiple different TFs in cold-treated *A. annua*. We speculate that a particular sequence occurred in the response to cold stress in the order of JA biosynthetic genes, JA level, TF expression, artemisinin biosynthetic genes, and artemisinin level.

In this study, the contents of artemisinin and related metabolites were measured in *A. annua* under cold stress. Artemisinin production increased in cold-treated plants (Figure 5A), which was consistent with results from previous studies [16]. The production of Arteannuin B (Figure 5C), the branch point metabolite of the artemisinin biosynthetic pathway, increased in cold-treated plants at 48 h because artemisinin and arteannuin B have the common precursor artemisinic aldehyde, which was generated from artemisinic alcohol by CYP71AV1. The influence of low temperature on artemisinin biosynthesis was first reported in Vietnamese *A. annua* plants after a night-frost period [16]. However, the molecular mechanism by which cold stress increases artemisinin content is not clear. Early studies showed that the expression levels of ADS and CYP71AV1 were induced by cold stress and inhibited by lanthanum chloride or the Ca^{2+} chelating agent EGTA, which indicated that the Ca^{2+}-CaM signal transduction pathway might be involved in the mechanism by which cold stress induces *ADS* and *CYP71AV1* gene expression [46]. Based on the data in this study, we propose a simplified model to explain how cold stress enhances artemisinin biosynthesis in *A. annua* (illustrated in figure 6). Exogenous JA enhances the freezing tolerance of *A. annua*. Cold stress increases the expression of genes involved in JA biosynthesis, and consequently, the endogenous JA level increases in *A. annua* under cold stress. The expression levels of JA-responsive transcription factors are induced by endogenous JA, and the TFs led to the up-regulation of artemisinin biosynthesis genes. Finally, the higher expression levels of artemisinin biosynthetic genes eventually increased the biosynthesis of artemisinin and related metabolites.

5. Conclusions

In this study, the expression levels of *A. annua* JA biosynthetic genes were upregulated under stress, leading to an enhancement of JA biosynthesis. The JA-responsive transcription factors positively regulating artemisinin biosynthesis were also upregulated at the transcriptional level. Consequently, the expression levels of artemisinin biosynthetic genes were increased. Finally, artemisinin biosynthesis was improved under cold stress. These results indicated that cold stress promoted artemisinin biosynthesis due to increased endogenous JA.

6. Acknowledgments

This work was financially supported by the NSFC project (31070266; 31200223), the Program for New Century Excellent Talents in University (NCET-12-0930), the National 863 Hi-Tech Plans (2011AA100605; 2011AA100607) and the Fundamental and Frontier Research Project of Chongqing (cstc2014jcyjA10005). The authors declare no conflicts of interest.

7. References

[1] Brown, G. D. (2010). Molecules 15, 7603-7698.

[2] Weathers, P. J., Elkholy, S., Wobbe, K. K. (2006). In Vitro Cell Dev.-Pl. 42, 309-317.

[3] Tang, K. X., Shen, Q., Yan, T. X., Fu, X. Q. (2014). Plant cell Rep. 33, 605-615.

[4] Xiang, L. E, Zhu, S. Q., Zhao, T. F., Zhang, M., Liu, W. H., Chen, M., Lan, X. Z, Liao, Z. H. (2015). Plant Growth Regul. 75, 435-441.

[5] Guo, X. X., Yang, X. Q., Yang, R. Y., Zeng, Q. P. (2010). Plant Sci. 178, 390-397.

[6] Banyai, W., Mii, M., Supaibulwatana, K. (2011). Plant Growth Regul. 63, 45-54.

[7] Jing, F. Y., Zhang, L., Li, M. Y., Tang, Y. L., Wang, Y. L., Wang, Y. Y., Wang, Q., Pan, Q. F., Wang, G. F., Tang, K. X. (2009). Biologia 64, 319-323.

[8] Irfanqureshi, M., Israr, M., Abdin, M., Iqbal, M. (2005). Enviro. Exp. Bot. 53, 185-193.

[9] Rai, R., Meena, R. P., Smita, S. S., Shukla, A., Rai, S. K., Pandey-Rai, S. (2011). J. Photochem. Photobiol. B 105, 216-225.

[10] Arsenault, P. R., Vail, D. R., Wobbe, K. K., Weathers, P. J. (2010). Molecules 15, 2302-2318.

[11] Kapoor, R., Chaudhary, V., Bhatnagar, A. K. (2007). Mycorrhiza 17, 581-587.

[12] Janska, A., Marsik, P., Zelenkova, S., Ovesna, J. (2010). Plant Biol. (Stuttg) 12, 395-405.

[13] Crifò, T., Puglisi, I., Petrone, G., Recupero, G. R., Piero, A. R. L. (2011). Gene 476, 1-9.

[14] Xie, X. B., Li, S., Zhang, R. F., Zhao, J., Chen, Y. C., Zhao, Q., Yao, Y. X., You, C. X., Zhang, X. S., Hao, Y. J. (2012). Plant Cell Environ. 35, 1884-1897.

[15] Kovacs, Z., Simon-Sarkadi, L., Szucs, A., Kocsy, G. (2010). Amino acids 38, 623-631.

[16] Wallaart, T. E., Pras, N., Beekman, A. C., Quax, W. J. (2000). Planta Med. 66, 57-62.

[17] Dar, T. A., Uddin, M., Khan, M. M. A., Hakeem, K. R., Jaleel, H. (2015). Environ. Exp. Bot. 115, 49-57.

[18] González-Aguilar, G. A., Fortiz, J., Cruz, R., Baez, R., Wang, C. Y. (2000). J. Agric. Food Chem. 48, 515-519.

[19] Ding, C.K., Wang, C. Y., Gross, K. C., Smith, D. L. (2001). Plant Sci. 161, 1153-1159.

[20] González-Aguilar, G. A., Buta, J. G., Wang, C. Y. (2003). Postharvest Biol. Tech. 28, 361-370.

[21] Hu, Y. R., Jiang, L. Q., Wang, F., Yu, D. Q. (2013). Plant cell 25, 2907-2924.

[22] Qian, Z. G., Zhao, Z. J., Xu, Y. F., Qian, X. H., Zhong, J. J. (2004). Biotechnol. Bioeng. 86, 809-816.

[23] Hu, F. X., Huang, J. H., Xu, Y. F., Qian, X. H., Zhong, J. J. (2006). Biotechnol. Bioeng. 94, 1064-1071.

[24] Hao, X. L., Shi, M., Cui, L. J., Xu, C., Zhang, Y. J., Kai, G. Y. (2015). Biotechnol. Appl. Bioc. 62, 24-31.

[25] Wang, H. H., Ma, C. F., Li, Z. Q., Ma, L. Q., Wang, H., Ye, H. C., Xu, G. W., Liu, B. Y. (2010). Ind. Crop Prod. 31, 214-218.

[26] Caretto, S., Quarta, A., Durante, M., Nisi, R., De Paolis, A., Blando, F., Mita, G. (2011). Plant Biology 13, 51-58.

[27] Supaibulwattana, K., Kuntawunginn, W., Cha-um, S., Kirdmanee, C. (2011). Plant Omics 4, 75-81.

[28] Yu, Z. X., Li, J. X., Yang, C. Q., Hu, W. L., Wang, L. J., Chen, X. Y. (2012). Mol. plant 5, 353-365.

[29] Lu, X., Zhang, L., Zhang, F. Y., Jiang, W. M., Shen, Q., Zhang, L. D., Lv, Z. Y., Wang, G. F., Tang, K. X. (2013). New Phytol. 198, 1191-1202.

[30] Murashige, T., Skoog, F. (1962). Physiolo. Plantarum 15, 473-497.

[31] Liu, W. H., Zhao, T. F., Wang, H. Y., Zeng, J. L., Xiang, L., Zhu, S. Q., Chen, M., Lan, X. Z., Liu, X. Q., Liao, Z. H. (2015). Plant Cell Tiss. Org. 121, 141-152.

[32] Livak, K. J., Schmittgen, T. D. (2001). Methods 25, 402-408.

[33] Yuan, Y., Liu, W. H., Zhang, Q. Z., Xiang, L. E., Liu, X. Q., Chen, M., Lin, Z., Wang, Q., Liao, Z. H. (2015).

Biotechnol. Appl. Bioc. 62, 17-23.

[34] Ferreira, J. F., Gonzalez, J. M. (2009). Phytochem. Analysis 20, 91-97.

[35] Briars, R., Paniwnyk, L. (2013). Ind. Crop Prod. 42, 595-600.

[36] Staswick, P. E., Su, W., Howell, S. H. (1992). P. Natl. Acad. Sci. USA 89, 6837-6840.

[37] Kazan, K. (2015). Trends Plant Sci. 20, 219-229.

[38] Shi, Y. T., Tian, S. W., Hou, L. Y., Huang, X. Z., Zhang, X. Y., Guo, H. W., Yang, S. H. (2012). Plant Cell 24, 2578-2595.

[39] Du, H., Liu, H. B., Xiong, L. Z. (2013). Front. Plant Sci. 4, 397.

[40] Lu, X., Zhang, F. Y., Shen, Q., Jiang, W. M., Pan, Q. F., Lv, Z. Y., Yan, T. X., Fu, X. Q., Wang, Y. L., Qian, H. M., Tang, K. X. (2014). PloS one 9, e91741

[41] Zhou, X., Zhong, J. J. (2011). Biotechnol. Bioeng. 108, 216-221.

[42] Sheard, L. B., Tan, X., Mao, H., Withers, J., Ben-Nissan, G., Hinds, T. R., Kobayashi, Y., Hsu, F. F., Sharon, M., Browse, J., He, S. Y., Rizo, J., Howe, G. A., Zheng, N. (2010). Nature 468, 400-405.

[43] Knight, H., Mugford, S. G., Ulker, B., Gao, D., Thorlby, G., Knight, M. R. (2009). Plant J. 58, 97-108.

[44] Wathugala, D. L., Hemsley, P. A., Moffat, C. S., Cremelie, P., Knight, M. R., Knight, H. (2012). New Phytol. 195, 217-230.

[45] Lee, B. H., Henderson, D. A., Zhu, J. K. (2005). Plant Cell 17, 3155-3175.

[46] Zeng, Q. P., Zhao, C., Yin, L. L., Yang, R. Y., Zeng, X. M., Huang, Y., Feng, L. L., Yang, X. Q. (2008). Sci. China Series C 51, 232-244.

Table 1 The primers used for the real-time quantitative PCR

Gene name	Primer sequences (5′-3′)	Purpose
LOX1	GTGGAAGGCTCAGTTTGGCAGTT	The expression analysis of genes involved in JA biosynthetic pathway by qPCR
	GCAGCAATCACAAATGGCTCAAC	
LOX2	CCACCAAGAATGCGTCGCCTAAT	
	GGACAATGTATCCACCACTGCCATA	
AOC	GGGCACTGGGATATTTGCTGGA	
	AACCGCAGGCGAAGGAGTCA	
JAR1	TTGTTGACGCGGGCTATGTGAG	
	ACTGGTTCATTGCGGACCCTTG	
ERF1	ATGAGTGAAGCCGTTAGTGGTTAAG	The expression analysis of artemisinin biosynthesis relative transcription factors by qPCR
	TTCTAACCTCACATATTTTTCACATTACTC	
ERF2	AAACCGCTATTGAAGCCGCCAA	
	TCATCTTCCCTCACCGCTACCATC	
ORA	ATTTCCAACTAAACACGGTTGAGCCT	
	GGATCTTGAAGTGTTGCATATAATGAAAGT	
ADS	AATGGGCAAATGAGGGACAC	
	TTTCAAGGCTCGATGAACTATG	
CYP71AV1	CACCCTCCACTACCCTTG	The expression analysis of genes involved in artemisinin biosynthetic pathway by qPCR
	GACACATCCTTCTCCCAGC	
DBR2	CTTGGGTTACAAGCTGTGGCTCAAG	
	ATATAATCAAAACTAGAGGAGTGACC	
ALDH1	CATCGGAGTAGTTGGTCACAT	
	TAATCACGCCATCAGGAACACCAG	
ACTIN	CCAGGCTGTTCAGTCTCTGTAT	The reference genes used in qPCR analysis
	CGCTCGGTAAGGATCTTCATCA	
EF1α	GACCTACTCTCCTTGAAG	
	GTTCCAATACCACCAATC	

Fig. 1. Effect of exogenous MeJA on plant freezing tolerance. Freezing phenotypes of 3-weeks-old wild-type seedlings grown on MS medium or MS medium containing 3 or 5 μM MeJA. (A) 0 μM MeJA, (B) 3 μM MeJA, (C) 5 μM MeJA . (D) Survival rates of plants after exposure to freezing temperatures. Error bars show SD from three replicates. The different letters indicate significant differences ($P<0.05$). Experiments were performed three times.

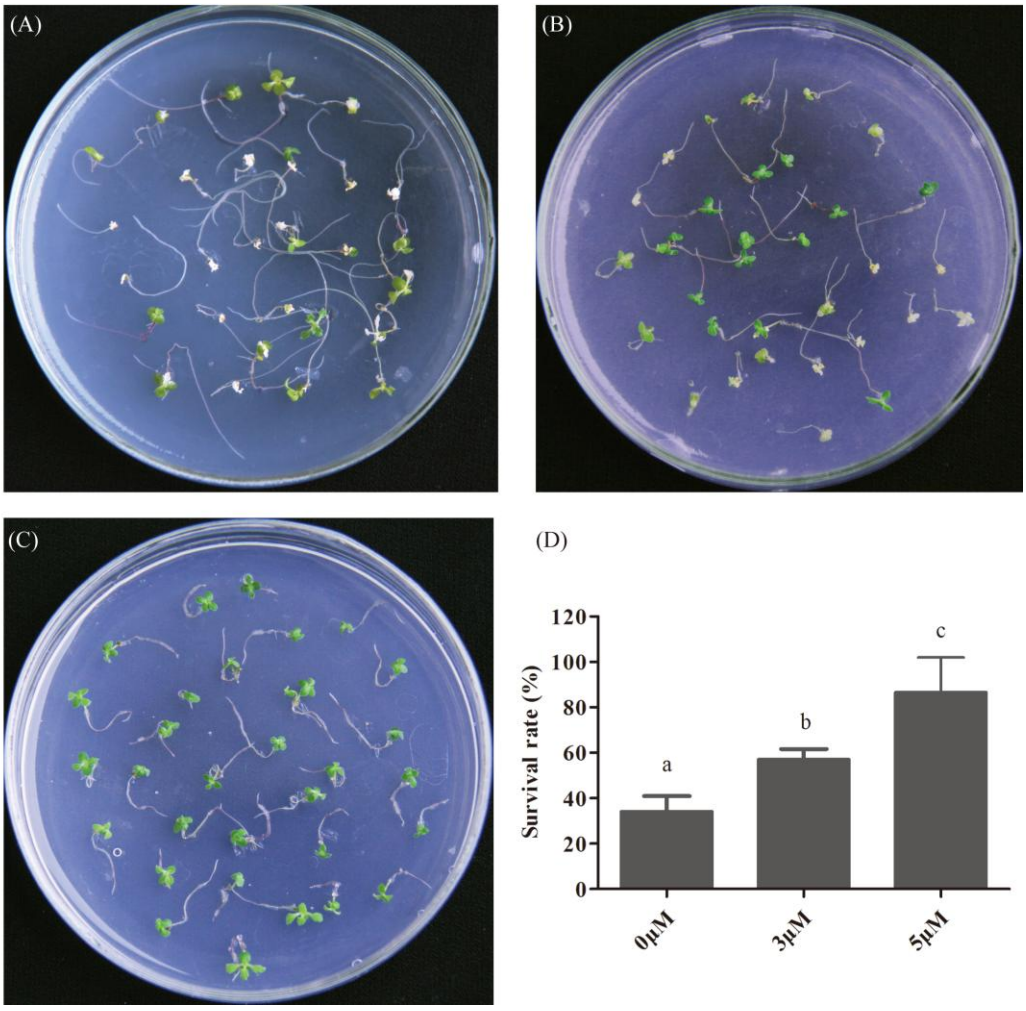

Fig. 2. The production of endogenous jasmonate and relative genes expression in *A. annua* under cold stress. (A) The level of endogenous jasmonate in *A. annua* leaves stressed by low temperature. The expression levels of (B) *LOX1*, (C) *LOX2*, (D) *AOC*, (E) *JAR1* were compared between control and cold stress at 0, 1, 3, 6, 12 and 24 h. Error bar mean ± SD. The statistical significance of gene expression difference was analyzed by one sample *t*-test. The markers *, **, and *** represent significant differences at P<0.05, 0.01, and 0.001, respectively. The population size was n = 3.

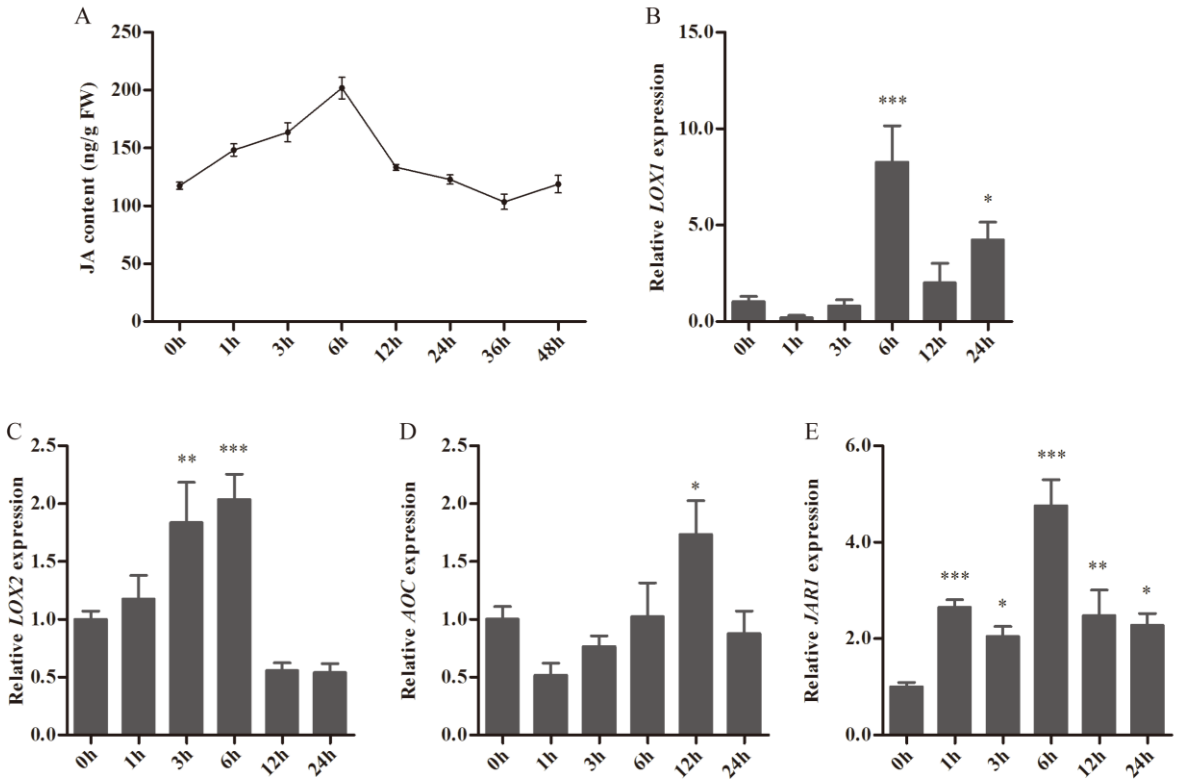

Fig. 3. The relative expression levels of transcription factors in *A. annua* under cold stress during 24 h. The expression levels of (A) *ERF1*, (B) *ERF2*, (C) *ORA*, were compared between control and cold stress at 0, 1, 3, 6, 12 and 24 h. Error bar mean ± SD. The statistical significance of gene expression difference was analyzed by one sample *t*-test. The markers * and *** represent significant differences at P<0.05, 0.01, and 0.001, respectively. The population size was n = 3.

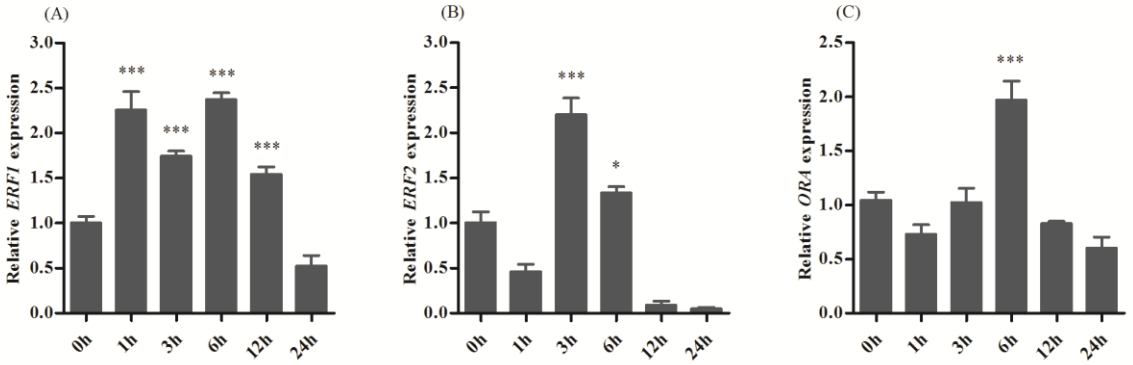

Fig. 4. The relative expression levels of key genes involved in artemisinin biosynthesis pathway in *A. annua* under cold stress during 24 h. The expression levels of (A) *ADS*, (B) *CYP71AV1*, (C) *DBR2*, (D) *ALDH1*, were compared between control and cold stress at 0, 1, 3, 6, 12 and 24 h. Error bar mean ± SD. The statistical significance of gene expression difference was analyzed by one sample *t*-test. The markers *** represent significant differences at P<0.001, respectively. The population size was n = 3.

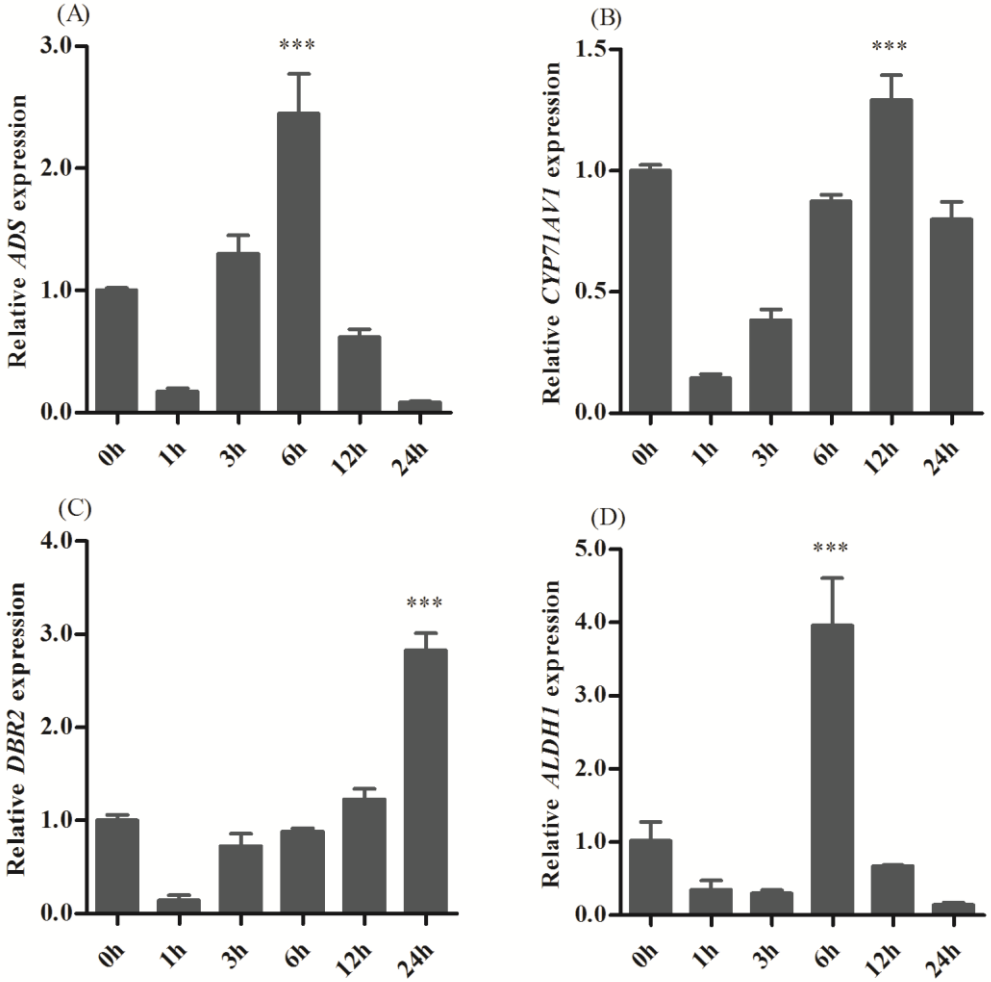

Fig. 5. The contents of artemisinin and its relative metabolites in *A. annua*. (A) the content of artemisinin; (B) the content of dihydroartemisinic acid; (C) the content of arteannuin B; (D) the content of artemisinic acid; the markers * and *** represent significant differences at P<0.05, 0.01, and 0.001, respectively. The population size was n = 3.

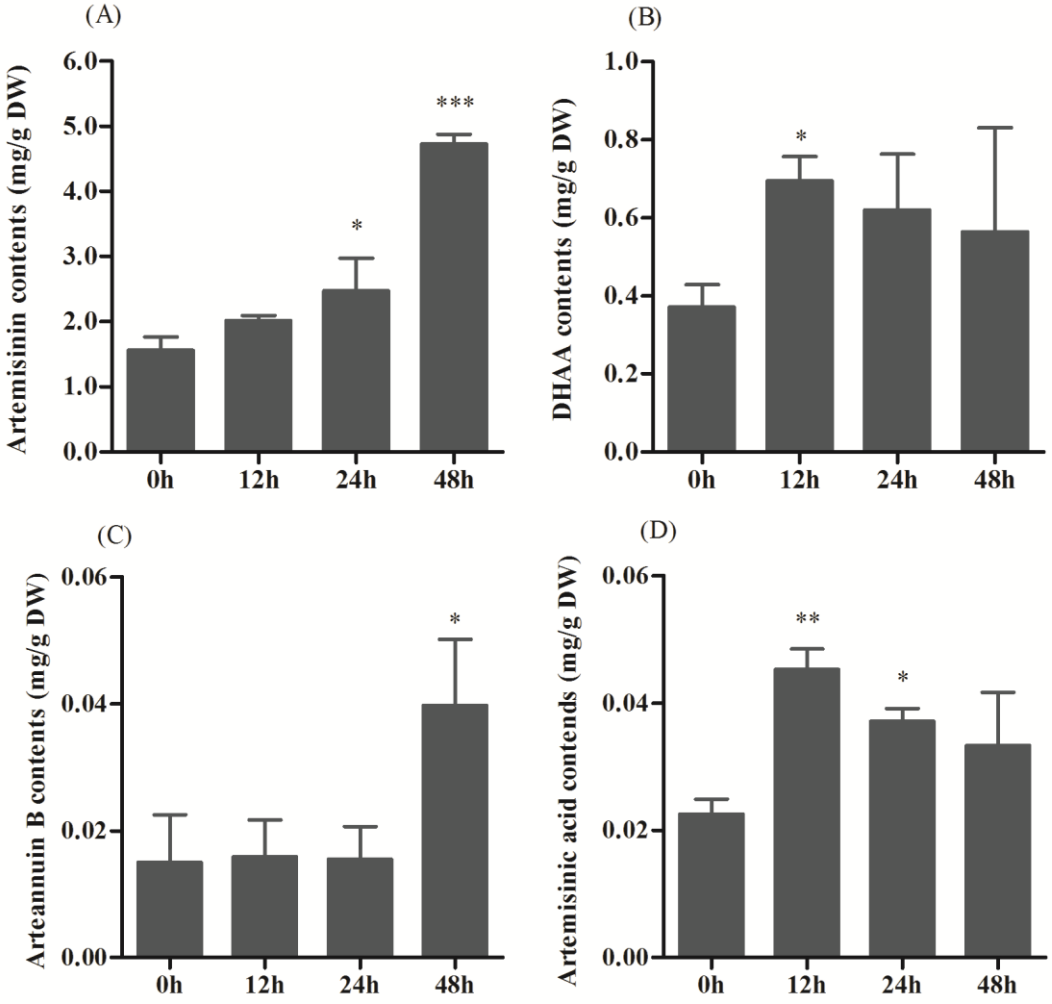

Fig. 6. The model of molecular mechanism of cold stress responses in *A. annua*. The upregulated expression genes were marked with symbol "↑".