Co-regulation Role of Endogenous Hormones and Transcriptomics Profiling Under Cold Stress in Tetrastigma hemsleyanum

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Abstract
Tetrastigma hemsleyanum Diels et Gilg is a valuable medicinal herb. Chilling sensitivity is the major limiting factor of the artificial cultivation of the plant. Some key genes, such as ZEP and NCED genes of ABA biosynthesis, GA2ox, GA3ox, and GA20ox genes of GA biosynthesis, ACO genes of ET biosynthesis pathway were screened to be crucial in cold response. The response of ABA and ABA/GA1+3 to cold stress was prior to that of GA1+3, ZR, ABA/IAA, and ABA/ZR. The increasing changes in ABA/GA1+3 turned to a steep decline with the extension of stress time, which might be one factor contributing to cold sensitivity of T. hemsleyanum. The cold tolerance of T. hemsleyanum would be repressed by GA3 but enhanced by ABA when ABA orGA3 was used alone. Both the ABA-mediated promotion and GA-mediated repression of cold tolerance could be attenuated using a combination of the two phytohormones within 6 h. When the biosynthesis of ABA and GA were inhibited by FLU and PAC, respectively, the effects of GA and ABA treatment were reversed partially. In summary, we presented the first study of global hormone-regulated transcripts expression patterns in T. hemsleyanum. This study suggested that GA and ABA could work antagonistically to balance the cold stress responses. PAC, a GA biogenesis inhibitor, as well as exogenous ABA, might be potential plant growth regulators that can promote cold tolerance of T. hemsleyanum. The study also provided valuable hints in revealing the potential candidate genes that regulated cold tolerance of T. hemsleyanum.

Keywords Tetrastigma hemsleyanum · Transcriptome · Cold stress · Phytohormone

Abbreviations
ABA Abscisic acid
GA Gibberellic acid
CK Cytokinin
ZR Zeatin riboside
PAC Paclorbutrazol
ET Ethylene
JA Jasmonic acid
BR Brassinosteroid
FLU Fluridone
AAO3 Abscisic-aldehyde oxidase
CESA Cellulose synthase A
ZEP Zeaxanthin epoxidase
EUPL E3 ubiquitin-protein ligase
ELP ETO1-like protein
LOX2S Lipoygenase
AOC Allene oxide cyclase
4CL 4-Coumarate–CoA ligase
EKES Ent-kauar-16-ene synthase
ATH1 Homeobox protein ATH1
ACDD Acyl-CoA dehydrogenase
KCT 3-Ketoacyl-CoA thiolase

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Introduction

_Tetrastigma hemsleyanum_ Diels et Gilg is a perennial herb, and it contains abundant flavonoids and polysaccharides that are beneficial to human health (Ru et al. 2019; Peng et al. 2016). Due to over exploitation, wild resources are on the verge of extinction in recent years. However, it is a cold-sensitive species, with the optimum growing temperatures ranging from 20 to 30 °C. Cold susceptibility has limited the artificial cultivation of _T. hemsleyanum_. A complete understanding of the gene expression profile of _T. hemsleyanum_ under cold stress is imperative, which could be an important theoretical basis to study cold tolerance mechanisms for improving the cold resistance.

Studies have been reported to provide evidence of global changes in gene expression in response to cold stress (Fu et al. 2016; Gao et al. 2019), which highlighted the significance of transcriptional regulation in plant stress adaptation. Stress tolerance has been reported to be accompanied by changes of hormonal-related genes expression and subsequently physiochemical modifications in some plants (Jakubowicz et al. 2020). Plants could survive from stress by utilizing hormones to coordinate growth. These hormone-related genes could act in the upstream of stress response and trigger hormone signal pathway.

Materials and Methods

Plant Materials and Cold Stress Treatments

Healthy plantlets of _T. hemsleyanum_ were obtained and cultured as the same as our previous report (Peng et al. 2015). According to the botanical characteristics of the plants (Ji et al. 2021), the specimens were identified and authenticated by Prof. Xin Peng, Zhejiang Pharmaceutical College. Based on our preliminary results of physiological parameters, the stress treatment process was optimized (Peng et al. 2019). For both the control and stress tests, 4-week-old seedlings were placed in a low-temperature growth chamber (DGZE-350D, Hangzhou Levn Co., Ltd., China) under a 16/8 h photoperiod (day/night). Plants under cold stress treatment were kept at 0 °C, whereas the controls were kept at conditions identical to cold stress groups except the different temperatures at 25 °C. After 0 h, 12 h, 24 h, and 48 h of cold stress treatment, the young leaves of 10 individual 4 week-old seedlings were collected for RNA extraction and qPCR analysis. Three biological replicates were performed.
RNA Extraction, Quality Determination, and De Novo Assembly

Total RNA was extracted with Trizol reagent (Invitrogen, CA, USA), according to the instructions. RNA quality was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The clustering was performed on a cBot cluster generation system and sequenced by Illumina HiSeq2500. De novo assembly was performed by the software Trinity (Grabherr et al. 2011). The clean data were mapped to the assembled transcripts by Bowtie 2 (Langmead and Salzberg 2012).

Transcriptome Functional Annotation, RPKM Estimation and DEG Analysis

The candidate-coding regions assembled by Trinity were identified by TransDecoder (https://transdecoder.github.io/). The unigene FPKM values were counted by HT Kilobase Million Mapped Reads. The levels of differential gene expression were estimated by DESeq (v1.16). ‘q ≤ 0.05 and \(|\log_2\text{ratio}| ≥ 1\)’ were set as the threshold to screen the differentially expressed genes (DEGs). GO enrichment and KEGG analysis were performed.

GO and KEGG Enrichment Analysis of DEGs

The Gene Ontology (GO, https://geneontology.org/) enrichment of DEGs was implemented by the hypergeometric test, and GO terms with q < 0.05 were considered to be significantly enriched. Kyoto Encyclopedia of Genes and Genomes (KEGG https://www.kegg.jp/) analysis of the DEGs was performed to identify the associated biochemical and signal transduction pathways. The KEGG enrichment of DEGs was implemented by the hypergeometric test, in which P value was adjusted by multiple comparisons as q value. KEGG terms with q < 0.05 were considered to be significantly enriched.

Validation of RNA-seq Data by qRT-PCR

The expression patterns of candidate unigenes were examined by qPCR using a SteponePlus real-time PCR system (Applied Biosystems, CA, USA). The primers are listed in Table 1. Each reaction system contained 1 μl of cDNA, 5 μl of 2x QuantiFast SYBR Green PCR Mix (Qiagen, Hilden, Germany), and 1 μl of forward and reverse primers in a final volume of 10 μl. The PCR reactions were performed under the following conditions: 95 °C for 3 min, followed by 45 cycles of 95 °C for 15 s, and 60 °C for 45 s. The GAPDH gene was used as the reference gene. Dissociation curve analysis was performed to verify the amplification specificity. Relative expression levels of genes were calculated by $2^{−\Delta\Delta Ct}$. Three technical replicates were used for each biological sample.

Determination of Phytohormones Concentration by ELISA

The double-antibody sandwich ELISA was performed as our previously described (Peng et al. 2015) with slight modifications for the phytohormones quantification. Briefly, 50 mg of fresh leaves was ground, then subjected to extraction (80% methanol containing 10 mg/l butylhydroxytoluene v/v) for 18 h at 4 °C, and then centrifuged at 4000 rpm for 15 min. The supernatant was passed through C18 solid-phase extraction cartridge (Waters Corporation, Millford, MA, USA) and then was dried by pure N2. Endogenous phytohormones

| Gene ID  | Unigene name | Tm   | Primer F (5′–3′) | Primer R (5′–3′) |
|----------|--------------|------|-----------------|-----------------|
| c81504_g1 | ZEP_4        | 55.8 | TACAGTTTATGGCCGAC | TTTCTACTCGTTTCCATCC |
| c102056_g1 | AAO3_2       | 53.7 | AACGGGTAGTTGGTGGTGGTG | GAAGGTGAGTGTTGGAGAC |
| c66096_g3 | NCE D_3      | 56.9 | CACCTCTGCTTCACCTTC | TTTCTCCTGACATTCCCTTA |
| c94964_g1 | AAO1_3       | 54.8 | TAGATCTCCAACACGACAGCAG | GGAAGGGAGATAGAAAGAG |
| c72906_g1 | CRMP LOG1    | 55.0 | AGACAGTTTGGAGAGTGAAGG | TTATCTATGAGAGGAGAG |
| c73984_g2 | CRMP LOG7    | 51.2 | AAAACTTCCTCTTCACCTTC | GCCTACACTTCCTCACCACAT |
| c80750_g2 | CRMP LOG8    | 56.5 | CTTCTCTTGGTGATTGAG | AGTTATGGGTCTGGGTTC |
| c100822_g2 | ACO_1       | 55.3 | CTCACAAACAAATGCTCTTC | TTTCTCTGATTCTCCACCCAG |
| c90679_g2 | ACO_4        | 52.5 | ACTTTTGTGTCACTCCTCTAT | GTCGACACTAATCCCTCCT |
| c99260_g1 | ACS_1        | 56.0 | TTTTTGACGGGTATGGTGGAG | GATTGAGGAGTTGGTGGAG |
| c93517_g2 | GA20ox_2     | 52.0 | ATGGGTTTTGTTTTCAGAGTC | AAAGGGAGTTTGGTGGAG |
| c92581_g1 | GA20ox_4     | 56.4 | TGGGGGAGTTGGTGGAGGG | GCGTTGAGAGCAGGGTGG |
| c90130_g1 | KCT          | 55.8 | CTTCTTCTCGCTTTCCTGT | ACAACCCAACTGTGCTACCCCC |
| c52785_g1 | AOC           | 56.0 | CAGATCCCCGCGTCGAT | GCGCCCAACGAGCAGCCAG |
levels were determined by the phytohormones-testing kit (MLBIO, Shanghai El Biotechnology Co., Ltd. China). The concentrations of ABA, IAA, ZR and GA were measured by the imark microplate reader (Bio-Rad Co. Ltd., Hercules, USA) at 450 nm.

Hormone and Inhibitor Treatments

GA and/or ABA treatments were employed in the in vivo experiments for the evaluation of hormonal mediation in cold response. The leaves of ten seedlings were included for each treatment. In vivo treatments were carried out by spraying with 5 ml 20 µM GA3 or 100 µM ABA at 6 h, and then sampled after 48 h. Seedlings sprayed with 0.1% ethanol in deionized water were sampled as a control. The inhibitors of GA and ABA biosynthesis, paclobutrazol (PAC), and fluridone (FLU) were also sprayed in this study. In the experiment protocol described above, 10 µM PAC or 0.1 µM FLU was sprayed with or without the phytohormones.

The experiment using a combination of GA and ABA was further performed to evaluate the interaction between the effects of GA and ABA, in which, after spraying with ABA for 2, 6, 12, or 24 h, GA was sprayed and then was assayed at 48 h. Conversely, when GA was the first hormone to spray, ABA was then added during the in vivo experiment. Three biological replicates were performed at the same time. Each biological replicate consisted of a pool of leaves from ten seedlings.

Physiological Parameters' Determination

MDA content was measured as our previous report (Peng et al. 2019). MDA content (µmol/g) = (C_{MDA} \times V_r \times 10^{-3} \times V_t) \times (W \times V_s)^{-1}. C_{MDA} = 6.45(A_{532} - A_{600}) - 0.56A_{450}, (µmol/l); V_r: reaction volume (ml); V_t: total volume of total extractive enzyme (ml), V_s: tested enzyme volume (ml); and W: the weight of leaves (g).

Electrolyte leakage was determined as our previous report (Peng et al. 2019). The fresh leaves (1 g) were incubated with 15 ml deionized water at 25 °C for 24 h. The electrolyte leakage was expressed as percent (EL%) = EC_1/EC_2 \times 100%.

Soluble protein content was measured by the Coomassie Brilliant Blue G-250 staining. Soluble protein content (mg/g) = C \times V_t \times (V_s \times W \times 1000)^{-1}; C: protein quantity determined from the standard curve (µg); V_t: sample solution volume (ml); V_s: total volume of the extracted solution (ml); and W: the weight of samples (g).

Statistical Analysis

All data are presented as mean ± SD. Statistical analyses were performed using the SPSS 21.0 (SPSS, Chicago, IL, USA). The difference among the multiple groups was analyzed by one-way analysis of variance followed by LSD multiple comparison tests. P < 0.05 was considered to be significant.

Results

Our previous results showed that the 0 °C was the optimum treatment temperature (Peng et al. 2019). The physiological and biochemical parameters remained essentially stable during 0 h and 8 h, significantly changed from 12 to 48 h, and remained stable after 48 h. So RNA-seq was employed to investigate the changes in gene expression under 0 °C exposure for four time points (0, 12, 24, and 48 h).

Overview of Transcriptome Sequencing

Four T. hemsleyanum cDNA libraries were constructed. After removing low-quality reads and adapter sequences, 45,346,334; 46,460,402; 44,804,246; and 44,801,200 clean reads generated for the 0, 12, 24, and 48 h treatment group libraries, respectively. We obtained 106,275 unigenes annotated in Nr, NT, BLASTX, and BLASTP with an average length of 676 bp and N50 of 1121 bp (Fig. 1). Among the unigenes, 18,082 unigenes had hits in all four databases with informative annotations. 53,511 unigenes had hits that significantly matched against the Nt database (50.35% of all unigenes). According to Nt annotations, 41,743 unigenes were matched to Vitis vinifera, followed by Zea mays. The E value distribution of the Nr blast results indicated that 81.91% of the matched sequences (43,831) had strong...
homologies. Nr, BLASTX, and BLASTP were also used to align the unigenes, 43,827; 28,775; and 19,598 high-score annotate unigenes were matched, respectively.

**GO Enrichment Analysis of DEGs**

In the 12-, 24-, and 48-h cold treatment samples, 8205 (72.4%), 14,400 (83.5%), and 15,402 (85.1%) unigenes were annotated in GO, respectively. In the 12-h treatment group (Fig. 2a), response to stimulus (972; 48.1% of 2022), ‘response to stress (652; 32.3% of 2022),’ ‘defense response (329; 16.3% of 2022),’ and ‘hormone-mediated signaling (164; 8.11% of 2022)’ were the most representative terms in the biological process category. During the 24-h (Fig. 2b) and 48-h (Fig. 2c) treatment, ‘response to stimulus’ and ‘response to stress’ remained relatively stable in the biological process category, while ‘response to hormone’ and ‘response to endogenous stimulus’ skyrocketed in 24-h treatment samples. In general, ‘response to stimulus,’ ‘response to stress,’ ‘membrane,’ ‘intrinsic component of membrane,’ and ‘oxidoreductase activity’ remained predominating during the whole cold stress, while some biological process categories related to endogenous hormone response skyrocketed in the early stress stage, such as ‘hormone-mediated signaling,’ ‘response to hormone,’ and ‘response to endogenous stimulus’ in 12 h and 24 h. ‘Catalytic activity’ dramatically predominated in the late stress stage.

**KEGG Pathways Enrichment Analysis of DEGs**

A total of 12,936 unigenes were assigned to 272 KEGG pathways. Pathways displaying significant changes (Q value ≤ 0.05) in response to the cold treatment were identified in each comparison group. The numbers of up-regulated genes and down-regulated genes in enriched pathways for each comparison group are listed in Fig. 3. In 12-h (Fig. 3a), 24-h (Fig. 3b), and 48-h (Fig. 3c) treatment groups, 12, 6, and 11 KEGG pathways were significantly enriched, respectively.

In general, ‘metabolic pathways’ and ‘biosynthesis of secondary metabolites’ were commonly regulated during the cold stress. The cold responses were characterized by protective response through stimulating secondary metabolism and inhibiting primary metabolism to modulate cellular metabolic homeostasis. In the early stress stage, primary metabolism still remained vigorous, such as ‘starch and glucose metabolism’ and ‘phenylalanine metabolism,’ while the related secondary metabolism enzyme activities were gradually activated. It would lead to the stimulation of secondary metabolisms such as ‘steroid biosynthesis,’ ‘diterpenoid biosynthesis,’ and so on. With the increase of cold stress time, many degradation pathways were strengthened, such as ‘bisphenol degradation’ and ‘polycyclic aromatic hydrocarbon degradation.’

**Responses of Hormone-Related Genes**

Based on the above overview of GO and KEGG analyses, we speculated that hormone-mediated signaling process would play an important role in early-time responses of *T. hemsleyanum* under cold exposure. So here we mainly focused on groups of DEGs involved in plant hormones biosynthesis pathways, such as abscisic acid (ABA), jasmonic acid (JA), gibberellic acid (GA), brassinosteroid (BR), cytokinin (CK), and ethylene (ET) pathways. The representative hormone-related genes differentially expressed during the cold treatment are shown in supplementary file 1–7. The heat maps of hormones-related transcripts of *T. hemsleyanum* during cold stress are shown in Fig. 4.

Among the mapped enzymes, we identified four 9-cis-epoxycarotenoid dioxygenase (*NCED*) homologs of the ABA biosynthesis pathway, and all of them were highly expressed with RPKM values > 100. Two of them were significantly up-regulated by 2.0–2.5 folds at 24 h. Three highly expressed Zeaxanthin epoxidase (*ZEP*) genes in ABA biosynthesis were identified, with c95330_g1 and c81504_g1 being the most abundantly and the differentially expressed. c35330_g2 was the most particularly noteworthy *ZEP* gene, which was almost undetectable in 0 h and was significantly up-regulated by 9.8 folds and 21 folds under 24 h and 48 h of cold treatment, respectively. Four indole-3-acetaldehyde oxidase (*AAO1*) genes, two cellulose synthase A catalytic (*CESA*) genes, and three abscisic-aldehyde oxidase (*AAO3*) genes were all down-regulated significantly under cold stress. So in general, in the ABA biosynthesis pathway, most of *ZEP* genes were strongly induced significantly during the early cold stress stage, most of *NCED* genes were up-regulated significantly during the medium stress stage, while other genes were down-regulated significantly. But with the increase of cold stress time, all the above genes were down-regulated significantly.

Four Gibberellin 20-oxidase (*GA20ox*) genes were identified, and the facts of c98675_g1 and c93517_g2 were the most highly expressed suggested that they may be crucial for GA biosynthesis, with a decreased RPKM value from 214 to 21 and from 430 to 20 under cold stress, respectively. Six Gibberellin 2-beta-dioxygenase (*GA2ox*) genes were identified. c92581_g1 was the most highly expressed *GA2ox* gene, with an increased RPKM value from 1051 to 3426 under cold stress, c93844_g1 was the most remarkably differentially expressed *GA2ox* gene, with a 7.6-folds significant up-regulation under cold stress. Four *GA3ox* were identified, among which c78816_g1 was the most highly expressed, with a 6.2-folds significant up-regulation under cold stress.
Fig. 2  GO classifications of differentially expressed unigenes of T. hemsleyanum in response to 12 h (a), 24 h (b), and 48 h (c) of cold exposure
All differentially expressed genes in the auxin biosynthesis pathway were down-regulated, including three indole-3-acetaldehyde oxidase (AAO1) genes, four indole-3-pyruvate monooxygenase (YUCCA) genes, and three abscisic-aldehyde oxidase (AAO3) genes. Five CK riboside 5’-monophosphate phosphoribohydrolase (CRMP_LOG) genes were differentially expressed. Among them, LOG1, 5, 7 were up-regulated, while LOG3, 8 were down-regulated significantly, and the fact of c72906_g1 was the most abundantly and the most remarkable differentially expressed suggested that it might be crucial for CK biosynthesis, with an increased RPKM value from 624 to 2292 after 24 h of cold treatment.

Eight 1-aminocyclopropane-1-carboxylate oxidase (ACO) genes of ET biosynthesis pathway were differentially expressed under cold treatment. Most of them were up-regulated in early stress stage and then maintained a high level, among which c90679_g3 was the most highly and the most remarkably up-regulated ACO gene, with an increased RPKM value from 12,152 to 68,974 after 24 h of cold treatment.

Three differentially expressed 1-aminocyclopropane-1-carboxylate synthase (ACS) genes of ET biosynthesis pathway were identified. KCT and 4CL genes were highly expressed with RPKM values > 100 and were significantly down-regulated after 24 h of cold treatment. AOC gene was significantly up-regulated by 10.3- and 9.2-folds at 24 h and 48 h of cold treatment, respectively.

Validation of RNA-seq Via RT-qPCR

To validate the RNA-seq data, qRT-PCR was performed of 14 selected key genes of six endogenous hormone synthesis pathways in every treatment group. The consistency between qRT-PCR and RNA-seq was measured by scatter plotting log2-fold changes, which showed a high similarity (Fig. 5).
by 88.3% and then decreased to a very low level. IAA content basically maintained constant. The ratio of ABA to IAA increased by 151.8%, 238.5%, and 63.7% at 12 h, 24 h, and 48 h, respectively. On the contrary, ZR levels decreased significantly at 24 h by 42.8% ($P < 0.05$) and then maintained a low level. Accordingly, the elevated ratio of ABA to ZR ranged from 121.5 to 395.8% under cold stress.

In general, ABA content was strongly induced by cold stress in the early stages, GA$_{1+3}$ content was significantly higher in the middle-late stages, while ZR content was significantly lower, especially in the middle-late stages. The changes of ABA/GA, ABA/IAA, and ABA/ZR ratio under cold stress showed an increasing trend in the early-middle stages but then gradually decreased as the stress time prolonged.

**Effect of Exogenous Phytohormones and Biosynthesis Inhibitors on Cold Stress Response**

A systematic pilot experiment was conducted by pot experiments to determine the effects of plant growth regulators on cold stress response of *T. hemsleyanum*. The results showed that GA$_3$ and ABA were the most important phytohormones.

After exogenous hormonal applications, the stress-related indicators, including MDA contents, soluble protein content, and electrolyte leakage, were simultaneously analyzed. As shown in Fig. 7a, ABA-treated seedlings had more soluble protein than control. ABA spraying dramatically decreased MDA content and electrolyte leakage. MDA content plummeted from $7.1 \pm 1.4$ μmol/g (CK) to $5.1 \pm 1.1$ μmol/g (ABA). Electrolyte leakage plummeted...
from 73.5 ± 8.4% (CK) to 50.0 ± 11.2% (ABA). FLU-treatment drastically increased electrolyte leakage and MDA content to 94.1 ± 13.2% and 11.2 ± 1.3 μmol/g, respectively. By contrast, the combined use of ABA and FLU had relatively stable MDA content, electrolyte leakage, and soluble protein content. On the other hand, GA₃-treatment had less soluble protein than control. GA₃ spraying dramatically increased the MDA

**Fig. 4** A heat map indicating expression patterns of hormones-related transcripts in ABA (a), auxin (b), CK (c), GA (d), JA (e), and ET (f) biosynthesis pathways during cold stress obtained from RNA-seq data. A color bar is presented at the bottom right. Data represent the log2 values (RPKM) of the *T. hemsleyanum* of 0-h, 12-h, 24-h, and 48-h treatment (from top to bottom) (Color figure online)
content and electrolyte leakage. MDA content soared from 7.1 ± 1.4 μmol/g (CK) to 11.8 ± 1.3 μmol/g (GA3). Electrolyte leakage soared from 73.5 ± 8.4% (CK) to 92.3 ± 16.2% (GA3). Compared with GA3-treated seedlings, Electrolyte leakage and MDA content were greatly decreased to 59.9 ± 8.1% and 6.6 ± 1.4 μmol/g in the presence of PAC, by 35.5% and 44.1%, respectively. The combined use of GA3 and PAC had relatively stable MDA content and electrolyte leakage. We found that treatment with ABA or GA can suppress or enhance cold damage, respectively. However, we thought therefore that ABA-mediated suppression and GA-mediated enhancement of cold damage could be reversed by the application of exogenous FLU and PAC, respectively.

The interaction effects of GA and ABA on cold stress response were further tested (Fig. 7b, c). Both GA-mediated promotion and ABA-mediated repression of cold damage could be attenuated by the combined application of ABA and GA3, respectively, suggesting that GA3 and ABA act antagonistically. Interestingly, the GA-mediated promotion of cold damage was significantly attenuated only when ABA was sprayed within 6 h of the initial GA3 application. No significant attenuation was observed if ABA was applied at 12 or 24 h (Fig. 7b). Moreover, ABA-mediated repression of cold damage was aggravated by supplementation with GA3 at 2 or 6 h after the initial ABA application. No significant attenuation was observed if GA3 was applied at 12 or 24 h (Fig. 7c).

Discussion

In the present study, the transcriptomes of T. hemsleyanum during 0–48 h of cold treatment duration were compared. GO and KEGG enrichment analysis of the DEGs indicated that phytohormones regulatory network was a significant mechanism in response to cold stress of T. hemsleyanum.

Metabolic Changes of Endogenous Hormones in Response to Cold Stress

The first step in the ABA biosynthesis pathway is catalyzed by a zeaxanthin epoxidase (ZEP) (Park et al. 2008). The 9-cis epoxycarotenoid dioxygenase (NCED) is considered to be a key rate-limiting enzyme in ABA biosynthesis (González et al. 2011), which cleaves the ABA precursor to produce xanthoxin, the direct precursor of ABA (Boursiac et al. 2013). Various studies reported that transcriptional regulation of NCED occurred at the time of abiotic stress (Espasandin et al. 2014). In T. hemsleyanum, three ZEP genes were up-regulated significantly after 12 h of cold treatment, and two NCED genes were up-regulated after 24 h of cold treatment. The results implied that the synthesis of ABA might be elevated under cold. It is notable that most of these ABA biosynthesis-related genes were suddenly down-regulated after 24 h of cold.
treatment. Accordingly, the ABA content was also first strongly increased to a ‘peak value’ at 24 h and then gradually decreased to a comparatively low level. It is suggested that the elevated levels of ABA-responsive genes and an increased content of ABA would be beneficial for plants under environmental stress (Luo et al. 2014). Thus, a series of related genes involved in ABA biosynthesis pathway of *T. hemsleyanum* were expressed at low levels after 48 h of treatment, which might be one factor contributing to cold sensitivity of *T. hemsleyanum*.

Plant growth could be inhibited partly by the accumulation of DELLA proteins, an important component in GA signaling (Zhou et al. 2017), suggesting that the plants could balance their growth and the resistance to abiotic stress by down-regulating GA levels (Zentella et al. 2007).

The final step of bioactive GA synthesis is catalyzed by *GA20ox* and *GA3ox*. GA content in rice would be reduced by the suppression of *GA3ox2*, or the activation of *GA2ox1* and *GA2ox3* (Liu et al. 2011). In our study, two *GA2ox* genes were down-regulated significantly under cold stress, which are consistent with this theory that growth retardation is a typical evolution characteristic of plant adaptation to environmental stress (Achard et al. 2006). But there were still some opposite reports, *GA20ox* genes of citrus were induced by cold treatment (Vidal et al. 2003). GA2ox could transform bioactive GA1 and GA3 to inactive GA8 and GA34. Over-expression of *GA2ox* would inhibit GA synthesis. The up-regulation of *GA2ox* genes was found to be in connection with the high tolerance to drought (Li et al. 2016a, b). Similarly, in our study, four putative members of the *GA2ox*

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Fig. 6 Variation of endogenous phytohormones level and ratio of ABA/GA (a); ABA/IAA (b); ABA/ZR (c) under 24 °C or 0 °C. Values are presented as mean ± SD of three independent experiments. Bars annotated by the different lowercase letters indicate the significant differences between the samples (P<0.05). Curves annotated by * indicate the significant differences between the treated and control groups (P<0.05).
gene family were significantly up-regulated following cold treatment, with a peak at 12 h. But then their expression level declined rapidly with the extension of stress time.

On the other hand, \textit{GA3ox} was a positive regulator of GA synthesis. In our study, a significantly up-regulated \textit{GA3ox} was annotated, with a peak at 24 h. Accordingly, GA content increased to peak at 24 h by 161% and then maintained at the level. Previous studies on \textit{Zea mays} under cold stress indicated that the abundance of bioactive GAs was reduced, suggesting a negative effects of GA in stress tolerance (Lu et al. 2017). This is in disagreement with our results. The up-regulation of \textit{GA3ox} and the down-regulation of \textit{GA20ox} were induced by cold, suggesting an inconsistent role of GA in \textit{T. hemsleyanum}. Thus, we speculated that there was a more complicated genes network of GA biosynthesis involving in cold-induced responses, besides these conventional rate-limiting enzymes.

The application of methyl jasmonate (MeJA) in \textit{Camel- lia japonica} (Li et al. 2016a, b) significantly enhanced cold tolerance by increasing endogenous JA accumulation.
AOCs are known as key enzymes in JA biosynthesis. AOC is required for formation of cis-(+)-enantiomer (9S, 13S) of OPDA, which is thought to be the precursor of JA. Suppressing AOC1 expression in Medicago truncatula could reduce JA levels significantly (Isayenkov et al. 2005). Biotic or abiotic stresses that lead to endogenous increases in JAs are usually accompanied by up-regulation of AOC and AOS (Wang et al. 2015). Our results also showed that AOC were up-regulated significantly, indicating a high level of JA in T. hemsleyanum during cold stress.

ET is synthesized in response to stresses such as freezing (Sun et al. 2016). The precursor S-adenosylmethionine (AdoMet) of ET is converted into ACC by the enzyme ACS (Roje 2006). ACO, termed as ET synthase, can directly catalyze the conversion of ACC to ET. An increase in ACC level was observed to coincide with an increase in ET production during cold stress.

The expression levels of ACS genes were increased dramatically under cold stress, yet simultaneously, a significant decrease of ACS genes occurred in T. hemsleyanum. It was interesting to note that the expression trends of ACO and ACS genes were divergent. Similar to our study, the cold tolerance of grapevine was increased by the treatment of the ET precursor ACC (Sun et al. 2016). But on the contrary (Shi et al. 2012), freezing tolerance of Arabidopsis was decreased by the application of ACC. In general, these results suggested that the role of ET in cold tolerance varied in different species.

The Proportion Change and Interaction of Various Endogenous Hormones in Response to Cold Stress

It is generally accepted that ABA/GA ratio could affect seed germination, ABA/IAA ratio could affect growing potential, and ABA/ZA could affect stomatal oscillation. However, it remains less understood whether such an antagonistic relationship exists in the regulation of plant stress response.

Many genes have been reported to be involved in cold stress in plants (Ritonga and Chen 2020). ABA and GA were thought to function via an intricate network in which transcription factors. It has been reported that restricting GA-promoted processes would promote ABA accumulation in arabidopsis (Achard et al. 2008). In contrast, the negative role of an A20/AN1-type zinc finger protein in ABA and GA biosynthesis was found, which led to the reduction of stress resistance of rice (Zhang et al. 2016). Both ABA levels and the ratio of ABA/GA in cold-tolerant bamboo species were higher than those of cold-sensitive species (Zhang et al. 2012). Similarly Huang et al. 2015 verified that exogenous ABA treatment could promote the ratio of ABA/GA, and thereby enhanced cold stress tolerance of sugarcane seedling. In accordance with the previous reports, our study indicated that ratio of ABA/GA was increased by 1.9-fold at 12 h but then declined sharply to about the level of control group.

Previous reports suggested that a cross talk of the signaling pathway also occurred between the ABA and auxin responses. For example, ABL1, an an ABA signaling component of rice, could adjust auxin responses by regulating the expression of ABRE-containing genes associated with auxin metabolism (Yang et al. 2011). Endogenous IAA levels were decreased in the carotenoid-deficient rice mutants, indicating a relationship between ABA and IAA at the biosynthesis level, and the deficiency in IAA would in turn increase the resistance to cold stress (Du et al. 2013). A balanced JA/ABA status was reported to be correlated with adaptation to osmotic stress in Vitis cells (Ismail et al. 2015). In the current study, we found that endogenous IAA levels decreased slightly in T. hemsleyanum under cold stress, but the change was not significant. Ratio of ABA/IAA was increased gradually from 0 to 24 h and then declined at 48 h, but the value was still higher than control. The increase amplitude of ABA/GA ratio was greater than that of ABA/IAA, and as well the peak time of ABA/GA ratio was earlier than that of ABA/IAA, which demonstrated that ABA/GA had a greater impact on the stress response than ABA/IAA. Ratio of ABA/GA3 was one of the crucial factors that could effectively increase cold tolerance in sugarcane plants, along with the increased ratio of ABA/IAA and the ratio of ABA/ZR (Huang et al. 2015; Guo et al. 2013). The increased ZT/IAA ratio was the basis for the enhanced shoot morphogenesis in cold-treated Sausurea involucrata explants. In our study, ABA/ZR ratio in T. hemsleyanum also showed an obviously increasing trend, which was correlated with the increase of ABA content and the decrease of ZR content under prolonged cold stress. On this basis, it could be inferred that other phytohormones also could influence stress tolerance through crosstalk mechanisms.

The Antagonistic Relationship of ABA and GA3 in Response to Cold Stress

Hormones co-regulate plant growth and stress response either synergistically or antagonistically (Ahammed et al. 2020), among which ABA and GA could be the most effective hormones in cold stress response of T. hemsleyanum. ABA has been shown to be antagonized by the function of GA in many developmental processes (Hu et al. 2016). However, it remains less understood whether such an antagonistic relationship exists in the regulation of stress response.

The electrolyte leakage rate and MDA content are negatively correlated with stress tolerance of plants. The soluble protein is positively correlated with stress tolerance of plants. In this study, the electrolyte leakage rate and MDA content of the GA-treated plantlets were promoted, while the soluble
protein content was reduced than control. And instead, ABA treatment had contrary influences on these stress-related indicators. Moreover, we also found that when the biosynthesis of endogenous ABA and GA were inhibited by FLU and PAC, respectively, the effects of GA and ABA treatment were reversed. The combined effects of GA and ABA on cold response were further tested. Two findings in our current study showed that GA and ABA had antagonistic effects on cold tolerance of *T. hemsleyanum*: (1) cold tolerance was enhanced by ABA but weakened by GA when each phytohormone was applied alone; (2) both the ABA-mediated promotion and GA-mediated repression of cold tolerance could be attenuated by the co-application of the two phytohormones, respectively. However, the antagonistic action of GA and ABA was no longer significant when the treatment was extended to longer than 6 h, indicating that transcriptional regulation of hormone-associated pathways was an early and transient event during stress response.

**Author Contributions** XP conceived the study. ML performed GO and KEGG Pathways enrichment analysis; HW performed physiological indicators determination and endogenous hormone determination; HC performed RNA Extraction and quality determination. XP wrote the manuscript and designed the experiments; ZZ carried out the analysis. All authors have read and approved the manuscript.

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**Compliance with Ethical Standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Appendix**

Supplementary data: The datasets generated during the current study are available in Supplementary files 1–7 were unigenes expression matrix data of ABA, Auxin, GA, Ethylene, JA, Cytokinin, and brassinosteroid synthesis pathways in *T. hemsleyanum* transcriptome, respectively.

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