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Cloning of artemisinin biosynthetic cDNAs and novel ESTs and quantification of low temperature-induced gene overexpression

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To isolate and verify novel genes from qinghao (*Artemisia annua*) based on the development-specific and environment-induced transcriptomics, leaves have been harvested from the flowering *A. annua* plants and exposed to low temperature for isolation of total RNAs and cloning of full-length cDNAs and cDNA fragments, or expressed sequence tags (ESTs). After being sequenced and browsed for homology, these sequences have been submitted to GenBank. Among the accessed 75 sequences, 4 full-length cDNAs are highly homologous to the known *A. annua* genes, but 71 ESTs are absent in the sequence records of *A. annua* genes, in which 34 sequences are homologous to other plant genes, including 24 identified protein-coding sequences and 10 unidentified protein-coding sequences, while other 37 sequences are not present in the sequence records of any plant genes, representing the first cloned plant genes. In order to investigate the responsive patterns of *A. annua* genes to extreme environmental stresses, especially low temperature, the expression levels of 3 critical qinhaosu (artemisinin) biosynthetic genes, *ADS*, *CYP71AV1* and *CPR*, have been measured in pre- and post-chilling *A. annua* seedlings cultured in vitro by semi-quantitative PCR (SQ-PCR). Consequently, *ADS* and *CYP71AV1* genes are strongly induced by chilling, but *CPR* gene is not significantly affected by such treatment. Furthermore, induction of these genes by chilling can be potently suppressed by Ca^{2+} channel inhibitor $LaCl_3$ or Ca^{2+} chelator EGTA, suggesting a putative involvement of Ca^{2+}-CaM signal transduction pathway in chilling-induced overexpression of *ADS* and *CYP71AV1* genes. The real-time fluorescent quantitative PCR (RFQ-PCR) assay of *A. annua* seedlings exposed to chilling has shown that the expression level of *CaM* gene is up-regulated for more than 2.5 folds, thereby confirming our above inference on the relevance of Ca^{2+}-CaM-mediated signal transduction to chilling-induced gene overexpression. Finally, 7 newly isolated *A. annua* ESTs have been functionally annotated by RFQ-PCR, which indicates that the chilling stress-induced overexpression levels of *D/LTSRP*, *UBE*, *AR/DAP* and *POD1* genes are augmented approximately for 8, 5, 2.3, and 1.5 folds, respectively, but those of *CHI* and *RGP* genes are not predominantly up- or down-regulated. The present study has preliminarily explored the chilling-induced overexpression patterns of 3 artemisinin biosynthetic genes and 7 novel *A. annua* ESTs at the transcriptional level, which should further facilitate our understanding of the intrinsic rule and mechanism underlying the coordinative regulation manner of artemisinin biosynthesis and accumulation, and yield substantial insight into improvement of *A. annua* by the metabolic engineering-guided breeding strategy.

Artemisia annua, expressed sequence tag (EST), abiotic stress, signal transduction, fluorescent quantification

Whole genome sequencing (WGS) provides essential and massive sequencing data for probing the structure, function and evolution of genomes, and represents an entire suite of protocols in functional genomics. However, WGS is absolutely a highly technical and extremely cost project in biological system explorations, and cannot be affordable by the most academic institutions or research groups. Despite extensive application in numerous plant species, WGS has been exclusively completed in one model plant with a smaller genome size, *Arabidopsis thaliana*[1], and another food crop plant with great economic implications, *Oryza sativa*[2], while almost all rare medicinal plants, including *Artemisia annua*, *Taxus brevifolia*, and *Catharanthus roseus*, have not been currently chosen as the candidates for WGS.

In order to improve the agronomic traits of *A. annua* and enhance artemisinin production by the genetic engineering-directed breeding technique, it is prerequisite to own copious storages of interested genes. Although researchers worldwide have made their efforts to construct *A. annua* cDNA library toward isolation of target genes[3,4], a vast majority of sequencing data are still unavailable for the public, and few *A. annua* genes have been accessed in GenBank. In the latest decade, some key genes encoding tailoring enzymes specifically responsible for artemisinin biosynthesis have been successfully cloned and identified, which include amorpha-4, 11-diene synthase gene (*ADS*), cytochrome P450 monooxygenase/amorpha-4, 11-diene oxygenase gene (*CYP71AV1*/*AMO*) and cytochrome P450 reductase gene (*CPR*)[5−7]. Nevertheless, questions have been raised to the proposed total biosynthetic pathway from amorpha-4, 11-diene to artemisinin via (dihydro)artemisinic alcohol, (dihydro)artemisinic aldehyde, and (dihydro)artemisinic acid and argues are present regarding the role played by several intermediates or precursors in artemisinin biosynthesis[8−10]. Even someone has suggested that perhaps certain artemisinin biosynthetic genes controlling unknown reaction steps have not been teased out[11]. For example, Wang et al.[12,13] have indicated that artemisinic acid is a common precursor of arteannuin B and artemisinin. Sangwan et al.[14] have demonstrated that arteannuin B is an intermediate during conversion from artemisinic acid to artemisinin. Nair et al.[15] have elucidated that artemisinic acid first forms arteannuin B, then converts to dihydroarteannuin B, and finally produces artemisinin. Although a kind of enzyme catalyzing reaction from artemisinic acid to artemisinin via arteannuin B intermediate has been isolated from *A. annua* cell-free extracts[16 − 19], the corresponding structural gene encoding the enzyme protein has not been cloned up to now. Therefore, for pooling much more useful *A. annua* genes prior to launching of the WGS project, an urgent necessity has been anticipated to initiate the large-scale functional genomic researches in *A. annua*, especially those aiming at isolation of novel *A. annua* genes based on the development-specific and environment-induced transcriptomics. For this consideration, we have gathered low temperature-exposed leaves from the flowering *A. annua* plants for isolation of total RNAs, from which full-length cDNAs and ESTs have been cloned by RT-PCR, anchored RT-PCR and cDNA library construction, and functional annotations to these cDNAs and ESTs have also been conferred by the comparative genomics-based bioinformatic analysis.

On the other hand, the exact regulation mechanism of artemisinin biosynthesis has not been fully understood, and such topics as whether the gene 'switch' controlling induction and suppression of artemisinin biosynthesis remain unsolved; how the 'on-and-off' operations of these genes are functioned through the specific developmental elicitation or extreme environmental stimulation, when and where these intracellular and extracellular signals are functionally transduced, and how many and which genes are involved in signal transduction and promoter-transcription factor interaction, etc. have not been elucidated in spite of extensive surge in recent years. By monitoring the time course associated with the seasonal variation of artemisinin, Wallaart et al.[20] have reported that in *A. annua* undergoing night-frost, gradually increased artemisinin accumulation is accompanyied by correspondingly decreased dihydroartemisinic acid storage, implying that low temperature-mediated cell damage may promote conversion of dihydroartemisinic acid to artemisinin. Afterwards, as having confirmed presence of dihydroartemisinic acid hydroperoxide in *A. annua* upon night-frost, they[21,22] have further suggested that night-frost may benefit to generation of the singlet oxygen ($^1O_2$), a kind of reactive oxygen species (ROS), and dihydroartemisinic acid can act as a scavenger of

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ROS to accept and quench $^1O_2$, thus giving rise to dihydroartemisinic acid hydroperoxide, which is then converted immediately to artemisinin as a final product by a spontaneous auto-oxidation process. Irfan et al.[23] have recently revealed that higher concentrations of salts and heavy metals enhance generation of ROS through augmenting the intracellular osmotic pressure, thereby facilitating rapid transformation of artemisinic acid to yield artemisinin. Nevertheless, few reports have indicated that ROS is likely to induce overexpression of the artemisinin biosynthetic genes.

Indirect evidence supporting possible relevance of the artemisinin biosynthetic genes to ROS has been originated from the unexpected outcome observed by Wallaart et al.[24], who have been aware that *ADS* cDNA can be uniquely amplified from the pre-treated *A. annua* shoots by 30% relative humidity (drought stress) and 6000 lx light (irradiation stress), while no amplified product is available from shoots without such treatments. Because abiotic stresses such as low temperature, extensive irradiation, drought, and saline are able to elicit generation of ROS[25], straightforward experimental confirmation for the hypothesis of environment stress-promoted artemisinin biosynthesis can be readily executed under all the stress conditions described above. Therefore, we have applied *A. annua* seedlings preferably to chilling and choose 3 artemisinin biosynthetic genes (*ADS*, *CYP71AV1*, and *CPR*) as well as 7 newly discovered ESTs for investigating their expression and regulation patterns upon chilling by SQ-PCR and RFQ-PCR, and then exploring the possible routes toward overexpression of these candidate genes initiated by the low temperature signal. The present study should shed light on further elucidation of the biosynthesis mechanism and accumulation fashion of artemisinin and its precursors or intermediates, which is obviously beneficial to opening a gate toward economic and convenient breeding for artemisinin overproduction and paving a path to the great challenge dealing with metabolic engineering in *A. annua*.

1 Materials and methods

1.1 Strains, plasmids and reagents

E. coli DH5α strain was stored in our laboratory; pMD18-T and pSIMPLE-19 plasmids were purchased from Takara. Plasmid DNA extraction kit (Concert Rapid Plasmid Miniprep System) and gel melting/recovery kit (Concert Rapid Gel Extraction System) were from Life Technologies; plant RNA extraction kit (E. Z. N. A. Plant RNA kit) was from Omega; plant mRNA purification kit (PolyAtract mRNA isolation System III) was from Promega; cDNA synthesis kit (M-MuLV RTase cDNA Synthesis Kit), fluorescent quantitative PCR kit (SYBR Premix Ex Taq Perfect Real Time Kit), PCR kit (One Shot LA PCR Mix), T_4 DNA ligase and restrictive enzymes were from Takara; cDNA first strand synthesis kit (RevertAid H Minus First Strand cDNA Synthesis Kit) was from Fermentas; other general biochemical and chemical reagents were from the native local manufacturers. All amplification primers are artificially synthesized as commercial services.

1.2 Amplification of *A. annua* total RNA and cDNA cloning

The flowering *Artemisia annua* leave fractions were collected from Fengshun County of Guangdong Province (originated from Youyang County of Shichuan Province) and transiently exposed to low temperature (4℃, 30 min) prior to total RNA extraction and mRNA purification. Followed by DNase digestion, total RNAs were applied directly to amplification by anchored RT-PCR. The reaction mixtures for reverse transcription containing 8 μL total RNAs, 1 μL oligodeoxythymidylate (oligo dT_{18}) anchor primers (0.5 μg/μL), 4 μL 5× buffer, 1 μL RNase inhibitor, 2 μL 10 mmol/L deoxynucleotide triphosphate (dNTP), 1 μL M-MuLV reverse transcriptase (200 U/μL) and 3 μL diethypyrocarbonate (DEPC)-treated double distilled water (ddH_2O) were incubated in 42℃ bath for 60 min. Then residual primers were depleted by 10% cetyltrimethyl ammonium bromide (CTAB) precipitation, and cDNAs were entailed by polyguanosine (polyG) as incubated at 37℃ for 30 min in the following reaction mixture: 12 μL primer-free cDNA, 1 μL terminal deoxynucleotide transferase (TDT), 4 μL 5×TDT buffer, 2 μL 0.1% bovine serum albumin (BSA) and 1 μL 10 mmol/L deoxyguanosine triphosphate (dGTP).

After being purified by CTAB and hydrolyzed by alkaline, PCR amplification was conducted in the following reaction mixture: 6 μL 10×buffer (25 mmol/L $MgCl_2$), 9.6 μL 10×dNTP (2.5 mmol/L), 1.2 μL 5′-terminal polyG linker primer EST-C [5′-GAGAGTCG-AC$(dC)_{13}$-3′] (33.3 μmol/L), 1.2 μL 3′-terminal polyadenosine (polyA) primer EST-T [5′-GAGAGAGCTC-$(dT)_{18}$-3′] (33.3 μmol/ L), 18 μL cDNA, 0.6 μL *Taq* (5

U/μL) and 23.4 μL ddH_2O. The thermoprofile was 94℃, 5 min; 94℃, 30 s, 40℃, 2 min, 72℃, 3 min; 94℃, 30 s, 58℃, 2 min, 72℃, 3 min, 25 cycles. Once separated by electrophoresis, the amplicons with small size below 300 bp were discarded by briefly cutting the gel and then reversely run to condense the dispersed amplicons as bands and finally recovered by cleavage and dissolving of gel slices. The purified amplicons were applied to double digestion at 37℃ for 2.5 h in the following reaction mixture: 2.5 μL *Sac*I, 2.5 μL *Sal*I, 7.5 μL 10×T buffer, 5 μL BSA, and 32.5 μL amplicons (260 ng/μL) to 50 μL total volume. Meanwhile, pMD18-T was isolated and purified for double digestion at 37℃ for 2.5 h in the following reaction mixture: 1.5 μL *Sac*I, 1.5μL *Sal*I, 4.5 μL 10×T buffer, 3 μL BSA and 15 μL pMD18-T (6.15 μg) to 30 μL total volume. The ligation reaction was executed in the mixture as follows: 1 μL 10×T buffer, 1 μL 100 μg/μL linear vectors, 3 μL 55 ng/μL digested amplicons, and 1 μL DNA ligase to 10 μL total volume, and completed by incubation at 16℃ overnight.

1.3 Purification of *A. annua* mRNAs and cDNA cloning

A. annua mRNAs were purified from total RNAs by a commercial magnetic bead system and subsequently applied to cDNA first-strand synthesis in the following reaction mixture: 11 μL mRNA, 4 μL 5×buffer for the first-strand synthesis, 1 μL dNTP mixture, 1 μL RNase inhibitor, 1 μL reverse transcriptase and 2 μL oligo dT_{18} in 42℃ bath for 1 h incubation. Then the synthesized cDNA first strands were employed for cDNA second-strand synthesis in the following reaction mixture: 30 μL 5×buffer for the second-strand synthesis, 3 μL dNTP mixture, 2 μL DNA polymerase I, 2 μL *E. coli* RNase H + DNA ligase mixture, and 89 μL RNase-free ddH_2O at 16℃ for 2 h incubation. For smoothing the terminals of staggered cDNAs, 4 μL T4 DNA polymerase was added and placed at 37℃ for 10 min incubation. For cDNA cloning, 4 μL blunted cDNA, 1 μL linearized pSIMPLE-19 and 5 μL Solution 1 were mixed and placed at 16℃ for 5 h incubation.

1.4 Bacteria transformation, recombinant identification and homology browsing

E. coli competent cells were prepared and transformed as general. Transformed colonies were picked up by the blue-white colony screening procedure for extraction of plasmids and subsequent identification by restrictive digestion or amplification. Double digestion of recombinant plasmids were performed in the following reaction mixture: 5 μL plasmid DNA, 1 μL 10×buffer, 0.5 μL *Eco*RI, and 0.5 μL *Hin*dIII to 10 μL total volume at 37℃ for 2 h incubation, and finally detected by 1% agarose gel electrophoresis.

Alternatively, the universal primers RV-M and M13-47PCR were used to amplify the culture derived from a single white colony, and the inserted fragment size was estimated on the gel. The verified recombinant plasmids were sequenced and homology browsed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST). For the sequence with an *E* value less than 1, its functional annotation was just given by the definition of compatible homologous gene, and then submitted to GenBank. For the sequence with an *E* value more than 1 or *E* value less than 1 but only with a short homologous polyA sequence, no sequencing record was simply classified.

1.5 Co-treatment by chilling with Ca^{2+} channel inhibitor or Ca^{2+} chelator

The treatment procedure was as described[26] with minor modifications. For the control group, *A. annua* seedlings were cultured at 25℃ in an illuminated growth chamber with a photoperiod of 16 h light and 8 h dark; for the low temperature treatment group, 25℃ and light cultured *A. annua* seedlings were transferred into a 4℃ refrigerator for 30 min, then transferred back to a 25℃ illuminated growth chamber for 24 h, or 25℃ cultured *A. annua* seedlings were kept in a 4℃ illuminated growth chamber for 48 h.

For the co-treatment group of Ca^{2+} channel inhibitor $LaCl_3$ with low temperature, 50 mL 100 mmol/L $LaCl_3$ in 1/2 MS medium was gently poured onto the surface of solid medium with *A. annua* seedlings and incubated for 48 h, then transferred into a 4℃ refrigerator for 30 min, and finally the flask still with $LaCl_3$ was transferred back under 25℃ light for 24 h or first for one 12 h incubation with $LaCl_3$ and then for another 12 h incubation without $LaCl_3$.

For the co- treatment group of Ca^{2+} chelator EGTA with low temperature, two various formats were adopted as follows: 50 mL 10 mmol/L EGTA in 1/2 MS culture medium was gently poured onto the surface of solid medium with *A. annua* seedlings and incubated for 48 h, then transferred to a 4℃ refrigerator for 30 min, and finally transferred back under 25℃ light for 24 h; or *A.*

annua seedlings were briefly transferred into an empty flask without MS medium, then 10 mmol/L EGTA in ddH_2O was added and transferred into a 4℃ refrigerator to stand for 30 min, and finally transferred back under 25℃ light for 24 h.

1.6 SQ-PCR and RFQ-PCR

Table 1 listed the amplification primers for *ADS*, *CYP71AV1* and *CPR* genes from *A. annua* seedlings by SQ-PCR and RFQ-PCR. For SQ-PCR assay, ubiquitin gene (*Ubi*) was chosen as a reference. The thermoprofile for *ADS*, *CYP71AV1* and *CPR* gene amplification was defined as: 94℃ 5 min, 94℃ 30 s, 57℃ 1 min, 72℃ 3 min for 30 cycles. The thermoprofile for *Ubi* gene amplification was as: 94℃ 5 min, 94℃ 20 s, 56℃ 20 s, 72℃ 20 s for 28 cycles.

For RFQ-PCR assay, 18S rRNA gene was used as a reference. For preparation of the reverse transcription reaction system, 5 μg total RNAs were mixed with 0.2 μg random primers, and supplemented with ddH_2O to a total volume of 12 μL, incubated in 70℃ bath for 5 min, and then placed into an ice bath for 2 min. To each sample, 4 μL 5×buffer, 2μL 10 mmol/L dNTP mixture and 20 U RNase inhibitor were added and incubated in 25℃ bath for 5 min. After 1 μL 200 U transcriptase was added, the mixture was incubated respectively at 25℃ for 10 min, then at 42℃ for 60 min, and finally at 70℃ for 10 min to stop the reaction. The RFQ-PCR reaction mixture was adjusted as: 10 μL 2×SYBR Premix Ex Taq mixture, 0.2 μmol/L primers each, 0.4 μL 50×reference staining, and 2 μL 10-fold diluted reverse transcription reaction system in a 20-μL total volume. The amplification was performed by an ABI 7300 fluorescent quantitative PCR instrument based on the following thermoprofile: 95℃ 10 s; 95℃ 5 s, 60℃ 31 s for 40 cycles. Once reaction was completed, a melting curve was plotted as following thermocondition: 95℃ 15 s, 60℃ 30 s, 95℃ 15 s.

1.7 Data analysis

Obeying the relationship principle that the larger Ct value, the less original copy numbers, we have represented difference of the targeted genes as folds of the expression level in 4℃ treatment group samples versus that in control group samples ($2^{-\Delta\Delta Ct}$) after normalization to the inner reference gene (18S rRNA), in which $\Delta\Delta Ct = \Delta Ct_{4℃} - \Delta Ct_{control}$, $\Delta Ct_{4℃} = (Ct_{target\ gene} - Ct_{18S\ rRNA})_{4℃}$, $\Delta Ct_{control} = (Ct_{target\ gene} - Ct_{18S\ rRNA})_{control}$[27]. The paired *t* tests were carried out to measure if difference of the gene copy number is significant or not, in which *P* value less than 0.05 means significantly different, and *P* value less than 0.01 means very significantly different. All the data were dealt with the SPSS software.

2 Results

2.1 Cloning, identification and homology comparison for *A. annua* cDNAs

After being amplified from total RNAs of *A. annua* by anchored RT-PCR, the available cDNA fragments with the size ranging from 300 to 1000 bp were recovered from the gel. Upon ligation and transformation, white colonies were picked up for isolation of recombinant plasmids and applied to double digestion with *Eco*RI and *Hin*d III. By gel electrophoresis, each recombinant plasmid generates one large fragment (i.e. vector DNA) and another small fragment with 200－1000 bp (i.e. insert DNA). Alternatively, when the culture derived from a single white colony was directly amplified, the targeted band with a certain size (200－1000 bp) can be also seen on the gel. These above recombinant plasmids that are verified by restrictive digestion and amplification were sequenced, and the sequencing data were collected for further bioinformatic analysis.

The newly isolated sequences have been browsed for homology with the accessed genes in GenBank by BLAST, thereby conferring homology-based functional annotations to these sequences. Among 75 *A. annua* sequences accessed in a format of either CoreNucleotide or EST in GenBank, four full-length cDNA (AY445506, DQ241826, DQ-872632, DQ984181) are highly homologous to the known *A. annua* genes, other 71 ESTs have no sequencing records in *A. annua*, but in which 34 ESTs are homologous to other plant genes, including 24 known protein-coding sequences and 10 unknown protein-coding sequences, other 27 ESTs have not sequencing records in any plants. Table 2 listed 28 *A. annua* sequences with homology-based functional annotations. Besides, 10 sequences (ES494773, ES582126, ES582130, ES582133, ES582135, ES582140, ES582142, ES582144, ES582151, and ES582155) have been classified as genes encoding unknown proteins and 37 sequences (DQ838799, EF050425, EF050426, EF050428, EF379388, EF494771－EF494773,

Table 1 Primer sequence for amplification and expected length of amplicons

A. annua gene	GenBank accession number	Primer sequence (5′ →3′)	Length of amplicon (bp)
SQ-PCR			
Amorpha-4, 11-diene synthase gene (*ADS*)	DQ241826	ATGTCACTTACAGAAAAACCTATTCGCC TCATATACTCATAGGATAAACGAGTAGAGAC	1641
Cytochrome P450 mono-oxygenase gene (*CYP71AV1*)	DQ872632	ATGAAGAGTATACTAAAAGCAATG CTAGAAACTTGGAACGAGTAAC	1488
Cytochrome P450 reductase gene (*CPR*)	DQ984181	ATGCAATCAACAACTTCCGTTAAG TTACCATACATCACGGAGATATC	2115
Ubiquitin gene (*Ubi*)	EU258763	CTTGGGGGAAGACGGGC GCCAAGATTCAGGACAAGGAAGG	250
RFQ-PCR			
18S rRNA	To be accessed	GCAACAAACCCCGACTTCTG TGCGATCCGTCGAGTTATCA	102
Peroxidase 1 gene (*POD1*)	AY208699	GAAATCTGCCCAAACCAAGC GGAGAATCCGAGGGTGTGTG	116
Chitinase gene (*CHI*)	EF050424	TGTTTCTCACCTCCACCTACTAATG AAATCTCTCCCCTCACAACTCG	110
Calmodulin gene (*CaM*)	EF549582	GCCCGCAAGATGAAAGACAC CATGACGTGACGAAGCTCAG	111
Ubiquitin-conjugating enzyme gene (*UCE*)	EF549585	GATTCCACCAGACCAGCAAC CGTAGCCTCAACACCAAGTGC	117
Drought/low temperature and salt responsive protein gene (*D/LTSRP*)	ES582125	AACTGCATCGACATCATCCTTG CCATCCGAACAAAGTCAACAAC	111
RNA-binding glycine-rich protein gene (*RGP*)	ES582129	TGGTGATTCTGAGGGAAAGTGG AGGTCAAAAACCCTTCAACACC	113
Auxin-repressed/dormancy-associated protein gene (*AR/DAP*)	ES582145	ACCATGACCGACAAAGGTGA AGCTTTACGTGCCGATGATG	117

Table 2 The functional annotation of *A. annua* cDNAs and ESTs by homology comparison

No.	GenBank accession	Homology comparison-based functional annotation
1	AY445506	squalene synthase, SS
2	DQ241826	amorpha-4,11-diene synthase, ADS
3	DQ872632	cytochrome P450 monooxygenase, CYP
4	DQ984181	cytochrome P450 reductase, CPR
5	DQ838800	vacular processing enzyme-1b
6	DQ838801	membrane protein
7	EF050423	structural constituent of ribosome
8	EF050424	chitinase, CHI
9	EF050427	ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, RuBPC/O
10	EF050429	cytosolic NADP-malic enzyme
11	EF549580	40S ribosomal protein S9
12	EF549581	15.9 kDa subunit of RNA polymerase II
13	EF549582	calmodulin, CaM
14	EF549583	histone H4-like protein
15	EF549584	light-harvesting chlorophyll a/b-binding protein, LHCBP
16	EF549585	ubiquitin-conjugating enzyme, UCE
17	EF660343	eukaryotic translational factor TIF3B1
18	ES582125	drought/low temperature and salt responsive protein, D/LTSRP
19	ES582128	hydroxyl praline-rich protein
20	ES582129	RNA-binding glycine-rich protein, RGP
21	ES582131	thioredoxin
22	ES582132	acyl-ACP thioesterase FATA1
23	ES582134	nucleic acid binding protein
24	ES582143	dicer-like 2/3 spliceform 2
25	ES582145	auxin-repressed/dormancy-associated protein, AR/DAP
26	ES582152	cytosolic malate dehydrogenase
27	ES582153	40S ribosomal protein S30-like protein
28	ES582154	secretory peroxidase

ES582127, ES582136 − ES582139, ES582141, ES582146 − ES582150, ES880929 − ES880939, EV780877 − EV780883) have been judged as genes without significant homology to any plant genes.

From the known functions served by those genes listed in Table 2, we are able to find out a lot of genes coding for the environment inducible or stress responsive proteins. The fact that so many stress responsive genes have been cloned may contribute to the choice of low temperature–exposed plants as experimental materials in the study. The genes with diverse functions include those for metabolic engineering-directed breeding aimed at artemisinin overproduction (*ADS*, *CYP71AV1*, *CPR*, *SS*, etc.), those for breeding toward highly effective photosynthesis (*RuBPC/O*, *LHCBP*, etc.), and those for breeding on disease and pest insect resistance (*CHI*, *D/LTSRP*, *AR/DAP*, *CaM*, etc.). Moreover, all other novel genes with unknown protein-encoding function or no sequencing records await identification by the so-called 'gain/lost-of-function' procedures such as site-directed gene mutagenesis, homologous recombination-mediated gene knockout, and antisense interference by microRNAs.

2.2 Chilling-induced overexpression of artemisinin biosynthetic genes

As illustrated by Figure 1, upon transient exposure to low temperature, artemisinin biosynthetic genes in cultured *A. annua* seedlings show different expression patterns, in which the expression levels of *CYP71AV1* and *ADS* genes are significantly elevated (lanes 2 and 4 in the left figure), while whose transcripts are undetectable prior to the low-temperature treatment (lanes 3 and 5 in the left figure). By contrast, the expression level of *CPR* gene is basically equivalent to each other for pre- and post-treatment by low temperature (lanes 6 and 7 in the left figure). As a reference, *Ubi* gene has displayed a constitutive expression manner, i.e. a consistent expression level either with chilling or without chilling (lanes 2−7 in the right figure).

These results have shown that *CYP71AV1* and *ADS* genes are potently induced through exposure to chilling. Simultaneously, *CPR* gene seems not affected by chilling, thus demonstrating a similar expression mode with *Ubi* gene.

2.3 Suppression of chilling-induced overexpression of artemisinin biosynthetic genes

To probing the role of Ca^{2+} and Ca^{2+} channel-mediated signal transduction pathway in chilling-induced overexpression of artemisinin biosynthetic genes, concomitant treatments of cultured *A. annua* seedlings by chilling with Ca^{2+} channel inhibitor $LaCl_3$ or Ca^{2+} chelator EGTA have been performed. As exhibited by Figure 2, the chilling-induced overexpression levels of *CYP71AV1* and *ADS* genes are suppressed although the extent is distinct, i.e. the former is less than the latter (lanes 3 and 7 in the left figure). After depletion of $LaCl_3$, transcription of *CYP71AV1* gene has partially recovered (lane 4 in the left figure), but that of *ADS* gene is not significantly recruited (lane 8 in the left figure). In addition, expression of either *CYP71AV1* gene or *ADS* gene is not fully inhibited by EGTA (lanes 2 and 6 in the left figure). As to *CPR* gene, its expression level seems also attenuated by $LaCl_3$ (lane 3 in the right figure). After depletion of $LaCl_3$, transcription of *CPR* gene is nearly recovered to the level prior to $LaCl_3$ treatment (lanes 4 and 5 in the right figure). Similarly, *CPR* gene is not inhibited by EGTA (lane 2 in the right figure).

The preliminary results exhibited here have demonstrated direct relevance of chilling-induced overexpression of artemisinin biosynthetic genes to Ca^{2+} channel-mediated signal transduction. After blocking the Ca^{2+} channel by $LaCl_3$, expressions of *CYP71AV1* and *ADS* genes are suppressed, suggesting that the chilling stress signal may activate the corresponding transcription factor (s) by Ca^{2+}-involved transduction, thus induce transcription of *CYP71AV1* and *ADS* genes. After depletion of $LaCl_3$, however, expression of *CYP71AV1* gene is partially recovered while that of *ADS* gene is still suppressed. The reason why their transcription mechanics are controlled tightly or loosely requires to be answered.

On the other hand, when being treated by EGTA in 1/2 MS medium, suppression of the above genes in cultured *A. annua* seedlings has not been observed, the possible reason for this phenomenon may be due to presence of Ca^{2+} in the medium, which leads to incomplete chelate of the extracellular Ca^{2+} by EGTA. Therefore, the Ca^{2+}–containing medium has been replaced in our optimized experiment by the medium-free EGTA solution, with which an expected result has been given as Figure 3. Expression of *CYP71AV1* gene is slightly down-regulated (lane 2 in the right figure), while that of *ADS* gene is completely blocked (lane 4 in the right figure). As usual, transcription of *CPR* gene is still intact (lane 6 in the right figure) with either pres-

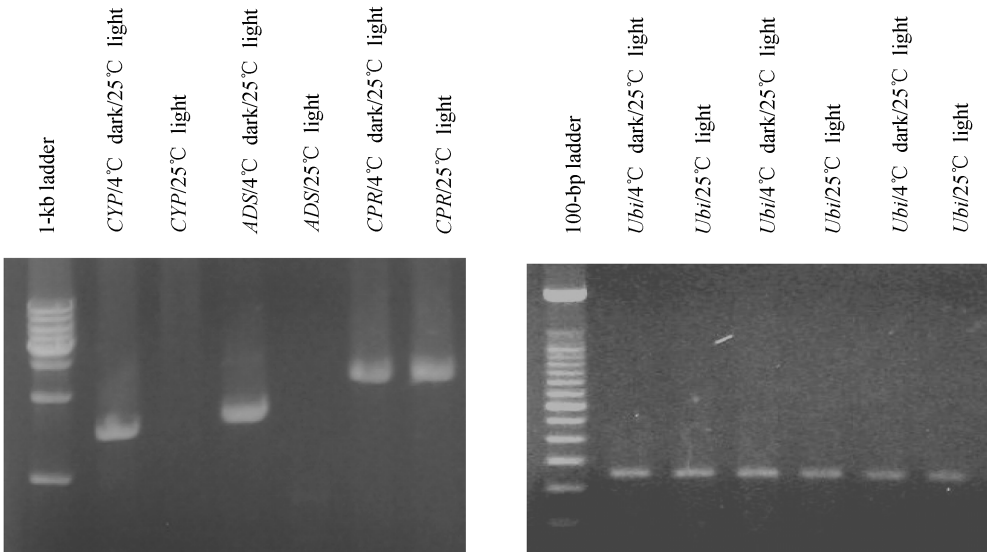

Figure 1 Chilling-induced overexpression of artemisinin biosynthetic genes in *A. annua* seedlings. For the control group, *A. annua* seedlings were cultured at 25℃ in an illuminated growth chamber (16 h light and 8 h dark); For the low temperature treatment group, 25℃ and light cultured *A. annua* seedlings were transferred into a 4℃ refrigerator to stand for 30 min, then transferred back to a 25℃ illuminated growth chamber for 24 h. Total RNAs were isolated and reverse transcription was conducted for further SQ-PCR assay.

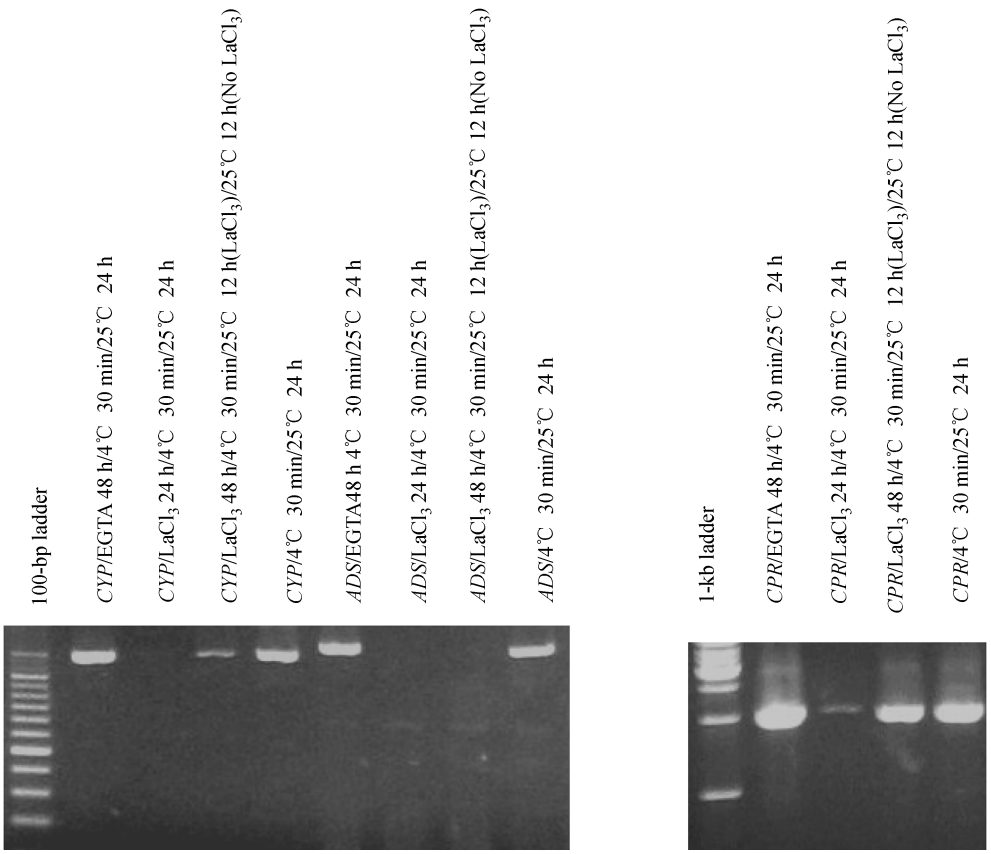

Figure 2 Suppression of chilling stress-induced overexpression of artemisinin biosynthetic genes in *A. annua* seedlings. For the $LaCl_3$ or EGTA and low temperature co-treatment group, 50 mL 100 mmol/L $LaCl_3$ or 10 mmol/L EGTA in 1/2 MS medium was gently poured onto the surface of the solid medium with *A. annua* seedlings and incubated for 48 h, then the flask still with $LaCl_3$ was transferred into a 4℃ refrigerator to stand for 30 min, and finally transferred back under 25℃ light for 24 h, or first for one 12-h incubation with $LaCl_3$ and then for another 12-h incubation without $LaCl_3$. Total RNAs were isolated and reverse transcription was conducted for further SQ-PCR assay.

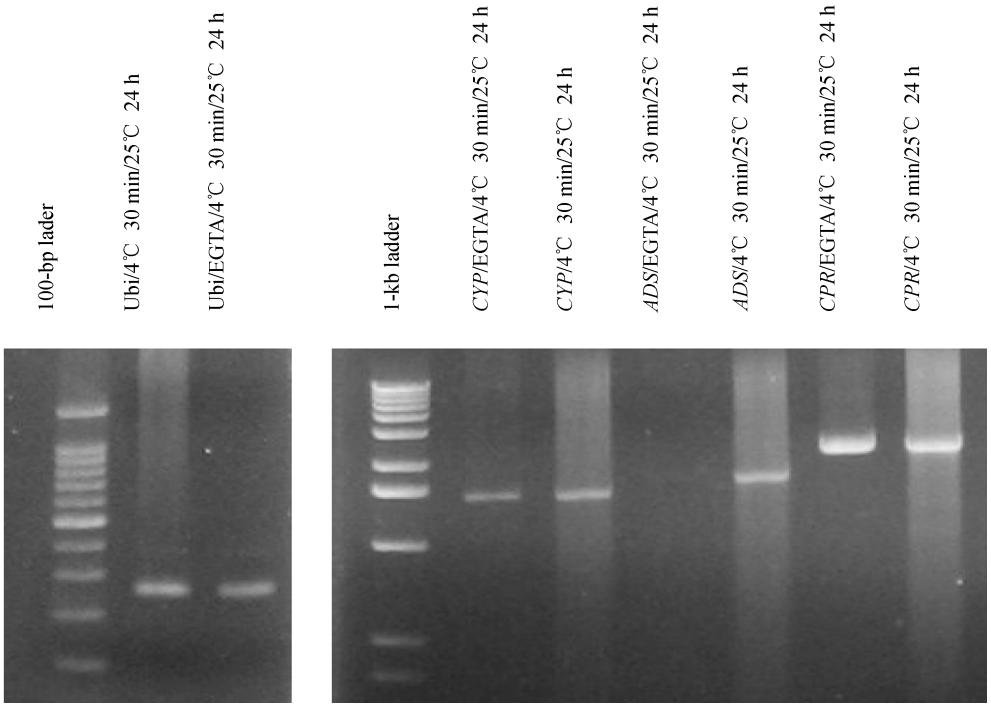

Figure 3 Suppression of chilling stress-induced overexpression by EGTA in *A. annua* seedlings. The cultured *A. annua* seedlings were briefly transferred into an empty flask without MS medium, then 10 mmol/L EGTA in ddH_2O was added and transferred into a 4℃ refrigerator to stand for 30 min, and finally transferred back under 25℃ light for 24 h. Total RNAs were isolated and reverse transcription was conducted for further SQ-PCR assay.

ence or absence of EGTA, which is the case as *Ubi* gene (lanes 2 and 3 in the left figure). Therefore, Ca^{2+} presence in the medium may be thought as the major reason for incomplete chelate of the extracellular Ca^{2+} by EGTA.

Nevertheless, the extent to which the candidate genes are suppressed by Ca^{2+} channel inhibitor $LaCl_3$ or Ca^{2+} chelator EGTA can be feasibly distinguished from comparison of the *CYP71AV1* amplicons in Figures 2 and 3. When the Ca^{2+} channel is blocked by $LaCl_3$, chilling-induced overexpression of *CYP71AV1* gene is almost fully suppressed (lane 3 in the left panel of Figure 2). In contrast, when Ca^{2+} is chelated by EGTA, chilling-induced overexpression of *CYP71AV1* gene is only partially suppressed (lane 2 in the right panel of Figure 3).

2.4 Regulation of novel *A. annua* gene expression by low-temperature treatment

To experimentally verify the function of newly isolated *A. annua* ESTs, the responsive patterns of *A. annua* seedlings to low temperature have been chosen for quantitative evaluation of chilling-induced overexpression of 7 novel ESTs by RFQ-PCR. The results have shown that upon standing at 4℃ for 48 h, the expression levels of 5 tested genes (*D/LTSRP*, *UCE*, *CaM*, *AR/DAP*, and *POD1*) are significantly up-regulated, while those of other 2 tested genes (*CHI* and *RGP*) are not predominantly fluctuated (Figure 4).

The plotted melting curve for RFQ-PCR has shown

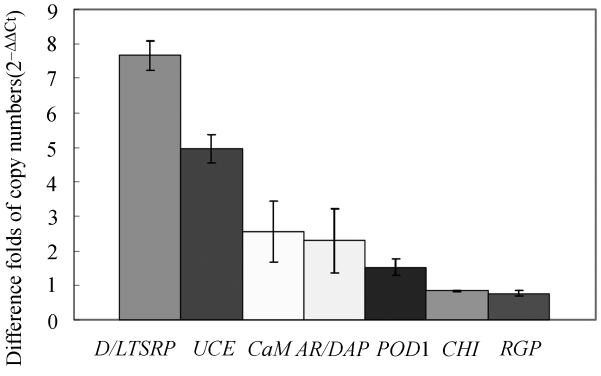

Figure 4 Regulation of chilling stress-induced expression of tested novel genes in *A. annua* seedlings. The 25℃ light cultured *A. annua* seedlings were always kept in a 4℃ illuminated growth chamber for 48 h. Total RNAs were isolated and reverse transcription was conducted for further RFQ-PCR assay.

that the peak of strand disassociation for the amplicon of each gene is a singlet one, demonstrating high specificity for measurement. In general, it can be classified as up-regulation of the expression level as the ratio of mRNAs from the target gene in the treatment group versus that in the control group is higher than 1.5; otherwise, as the ratio is lower than 0.67, it should be thought as down-regulation[27]. Based on this criterion, the expression levels of *D/LTSRP*, *UCE*, *CaM*, *AR/DAP*, *POD1* genes can be judged as up-regulation because their ratios are up to 8, 5, 2.5, 2.3, and 1.5, respectively, at a significantly statistical level ($P<0.05$), while the expression levels of *CHI* and *RGP* genes are unlikely judged as up-regulation or down-regulation because their ratios are 0.85 and 0.77 albeit higher than 0.67 but without statistically significant difference ($P>0.05$).

3 Discussion

Since the first *A. annua* gene, farnesyl perophosphate synthase gene (*FPS*), was cloned[29], only approximately 20 *A. annua* genes have been accessed in GenBank[30], implying that a plenty of sequencing data of cloned *A. annua* genes have not been opened to the public. By cloning, identification and sequencing of cDNAs in leaves collected from the low temperature-pretreated flowering *A. annua* plants, we have submitted and accessed 4 CoreNucleotide full-length sequences, 18 CoreNucleotide sequence fragments and 53 ESTs in GenBank, which constitute 3 sequence types: 4 ESTs with sequencing records in *A. annua* (5.3%); 34 ESTs without sequencing records in *A. annua* but in other plants (45.3%); and 37 sequences without any sequencing records (49.3%). In the newly discovered *A. annua* genes, there are genes encoding structural proteins (histone, ribosomal protein, and hydroproline-rich protein, etc.), genes encoding regulatory proteins (RNA polymerase, nucleotide binding protein, eukaryotic translation factor, and Dicer-like 2/3 spliceform 2, etc.), and genes encoding metabolic enzymes (acyl-ACP thioesterase, cytosolic malate dehydrogenase, and cytosolic NADP-malic enzyme, etc.). Furthermore, the present study has also identified several interested *A. annua* genes with potential implications in plant genetic improvement, for instance, highly effective photosynthetic genes (*RuBPC/O* and *LHCBP*), the fungal disease resistant gene (*CHI*), and stress tolerance genes (*D/LTSRP*, *AR/DAP*, hydroproline-rich protein gene, thioredoxin thioredoxin gene, and secretory peroxide gene, etc.), which should become in the near future potential gene resources for the genetic engineering-guided plant breeding strategy.

There are dozen of reports regarding the environmental responsive genes in *A. annua*. Souret et al.[31] have examined regulation of *A. annua FPS* gene expression by oxygen, light and culture duration. Subsequently, some others have observed the effects of flowering[32,33] and phytohormones including gibberellins[34] and cytokinins[35,36] on artemisinin biosynthesis, but direct evidence supporting involvement of the extreme environmental conditions (abiotic stress such as chilling, drought, hypoxia, and saline, etc.) in expression of the artemisinin biosynthetic genes has not been documented. Our study has disclosed for the first time that *A. annua ADS* and *CYP71AV1* genes are induced by chilling, while *CPR* gene keeps a stable expression level in pre- and post-chilling (see Figure 1). It is worth notice that the amplicons derived from *ADS* mRNA might be undetectable on the gel for *A. annua* seedlings without exposure to chilling, whereas a faint band equivalent to the amplified *ADS* transcript can be sporadically observed on the electrophoregram, which is likely essential consequence of the relatively low resolving power of gel electrophoresis or limitation of the SQ-PCR technique itself. For this sake, we have quantified *ADS* and *CYP71AV1* transcripts after exposure of *A. annua* to chilling. As expected, chilling exposure surely induces the elevated *ADS* and *CYP71AV1* mRNA levels up to tenfold and sevenfold as the control, respectively, coupled with an enhanced artemisinin yield (Yin L L, Zhao C, Huang Y, et al. Abiotic stress-induced expression of artemisinin biosynthesis genes in *Artemisia annua* L. Chin J Appl Environ Biol, 2008, 14(2): in press). Moreover, we have also found that *ADS* and *CYP71AV1* genes are induced not only by chilling, but also by heat shock and ultraviolet irradiation (to be issued elsewhere).

Minorsky[37] first proposed that Ca^{2+} can act as a primary transduction signal for low temperature in plants, which has been later confirmed by others[38−41]. We have revealed that Ca^{2+} chelator EGTA and Ca^{2+} channel inhibitor $LaCl_3$ can suppress chilling-induced overexpression of *A. annua ADS* and *CYP71AV1* genes, implying that the concentration of Ca^{2+} and the function of Ca^{2+} channel may be directly relevant to low temperature elicited signal transduction and chilling-induced gene expression. A striking resemble conclusion has been

drawn for induction of the antioxidant enzyme genes by co-treatment of chilling with $CaCl_2$, and suppression of the antioxidant enzyme genes by EGTA and $LaCl_3$. During cold acclimation, $CaCl_2$-treated *Populus tomentosa* shoots exhibit a dramatically increased CaM content and a substantially enhanced antioxidant enzyme activity, but such promotion can be abolished by EGTA and $LaCl_3$, demonstrating that the Ca^{2+} signal system may be closely related to induction of the cold resistance trait in plants [26]. Evidence of Ca^{2+}-induced overexpression of cold-acclimation specific genes, *cas15* and *cas18*, in *Alfalfa*[39] has also provided support to this conclusion. In our prelimilary tests, however, we have not observed suppression of chilling-induced overexpression of *ADS* and *CYP71AV1* genes by EGTA in the medium containing Ca^{2+}, suggesting incomplete chelate of the extracellular Ca^{2+} pool under such treatment. So we have subsequently adopted the medium-free EGTA solution to treat *A. annua* seedlings, and found that expressions of *ADS* and *CYP71AV1* genes are suppressed at some extent, thus thoroughly confirming our inference described above. Moreover, we have also observed that the chilling-stimulated expression level of *CPR* gene is somewhat declined by the action of $LaCl_3$, seeming to mean that expression of *CPR* gene may be more or less controlled by Ca^{2+}, whether it implys concomitant presence of the baseline and inducible transcription mechanism remains controversial. However, promoters controlling simultaneous baseline and inducible expression activities have been actually identified in *Catharanthus roseus*[42].

RFQ-PCR is a highly reproductive method for quantification of the gene expression level, which includes both absolute and relative approach for quantification of DNAs. The former approach is to compare the difference of copy numbers by a standard curve in relation to the fluorescent signal with original copy numbers of the gene. This approach, however, needs a strict manual procedure for preparing standard samples during which even minor mistakes would lead to fail for standard curve plotting and eventually affect the measurement result. In the contrary, the latter approach focuses on the difference of expression between the compared samples and is unnecessary to know exact copy numbers of the gene. The $2^{-\Delta\Delta Ct}$ method is such a simple one for analyzing the relative difference of gene expression[27,28]. Our work has employed this method for comparing the expression level differences among 7 new ESTs with pre- and post-chilling. As a result, the expression levels of *D/LTSRP*, *UCE*, *CaM*, *AR/DAP*, and *POD1* genes are up-regulated, while those of *CHI* and *RGP* genes are basically kept invariable.

At present, inducible expression and regulation of the above genes have not been reported. However, a higher content of CaM has been assessed in *P. tomentosa* after cold acclimation[26], indirectly demonstrating a chilling-induced expression fashion of *CaM* gene. We have also found a 2.5-fold increase of the *CaM* mRNA level in chilling-treated plants when compared with the control plant, indicating that Ca^{2+}-CaM signal transduction is obviously involved in chilling-induced overexpression of *ADS* and *CYP71AV1* genes. In *Arabidopsis thaliana*, *HOS1* gene encoding E3 ubiquitin conjugating enzyme (E3-UCE) has been identified, which is sequestered in the cytosol as standing at normal temperature, but accumulated in the nuclei upon chilling[43]. Our research results have also demonstrated up-regulation of *UCE* gene up to approximately fivefold upon induction by chilling, thereby confirming the conclusion drawn in other plants. Furthermore, it has been found that light in *Arabidopsis thaliana* prompts phytochrome-mediated tightly binding of corresponding transcription factors (CBF1, 2, 3 and cor15-a) to specific promoters for activating low temperature- and drought-induced gene expressions[44]. In our previous work accomplished by RFQ-PCR, we have placed *A. annua* seedlings in dark at 4℃ for 30 min and then applied to amplify the corresponding mRNAs. In consequence, the expression levels of the tested 7 new genes are not markedly altered, suggesting that the present condition only leads to a slightly inducible expression or does not induce a threshold expression level sufficient to be assessed (unpublished results). Instead, here we have optimized the protocol by extending the duration of chilling exposure from 0.5 to 48 h and also providing continuous light. Consequently, the above novel *A. annua* genes exhibit the cold responsive expression behavior. In the future, there will be a need for elucidating the coordinative effects of light on chilling-induced gene expression.

The present work has explored the transcriptomics-based chilling induction on gene expression and regulation in *A. annua*, and disclosed for the first time the low temperature-responsive expression characteristics of artemisinin biosynthetic genes and other new *A. annua*

genes at the transcriptional level, which should be beneficial to our further exploration on the relationship between low temperature signal transduction and inducible artemisinin biosynthetic gene expression, thereby identifying the specific transcription factors and corresponding regulatory genes controlling these gene expression and shed light on transcription factor-targeted plant as well as microbial genetic engineering (Zeng Q P, Qiu F, Yuan L. Production of artemisinin by genetically modified microbes. Biotechnol Lett, DOI 10.1007/s10529-007-9596-y. See the electronic edition at: http:// www.springerlink.com/content/100138). On the other hand, the attractive goal to reveal the innate regulatory and homeostatic mechanisms of artemisinin biosynthetic gene expression needs to be further emphasized. Once the intrinsic principles of carbon flux rechanneling during artemisinin biosynthesis and accumulation are elucidated, we would eventually find out a highly desirable way toward tremendous enhancement for artemisinin production.

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