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, and another food crop plant with great economic implications, Oryza sativa, 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, 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 amorph-4, 11-diene synthase gene (ADS), cytochrome P450 monooxygenase/amorpha-4, 11-diene oxygenase gene (CYP71AV1/AMO) and cytochrome P450 reductase gene (CPR). Nevertheless, questions have been raised to the proposed total biosynthetic pathway from amorph-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. Even someone has suggested that perhaps certain artemisinin biosynthetic genes controlling unknown reaction steps have not been teased out. For example, Wang et al. have indicated that artemisinic acid is a common precursor of arteannuin B and artemisinin. Sangwan et al. have demonstrated that arteannuin B is an intermediate during conversion from artemisinic acid to artemisinin. Nair et al. have elucidated that artemisinic acid first forms arteannuin B, then converts to dihydroartemannuin B, and finally produces artemisinin. Although a kind of enzyme catalyzing reaction from artemisinic acid to arteannuin B intermediate has been isolated from A. annua cell-free extracts, 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. have reported that in A. annua undergoing night-frost, gradually increased artemisinin accumulation is accompanied 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 have further suggested that night-frost may benefit to generation of the singlet oxygen (1O2), a kind of reactive oxygen species (ROS), and dihydroartemisinic acid can act as a scavenger of...
Therefore, we have applied cuted under all the stress conditions described above. 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 Wal- laart et al., 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, 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 RQF-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/recov-

ery 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), T4 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 oligodeoxynucleotidylate (oligo dT18) 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 diethylyrocarbonate (DEPC)-treated double distilled water (ddH2O) were incubated in 42 ℃ bath for 60 min. Then residual primers were depleted by 10% cetyltrimethyl ammonium bromide (CTAB) precipitation, and cDNAs were en- tailed by polyguanosine (polyG) as incubated at 37 ℃ for 30 min in the following reaction mixture: 12 μL primer-free cDNA, 1 μL terminal deoxynucleotide transférase (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 alkali, PCR amplification was conducted in the follow- ing reaction mixture: 6 μL 10×buffer (25 mmol/L MgCl2), 9.6 μL 10×dNTP (2.5 mmol/L), 1.2 μL 5′-termi- nal polyG linker primer EST-C [5′-GAGAGTCG-AC(dC)13-3′] (33.3 μmol/L), 1.2 μL 3′-terminal polyad- enosine (polyA) primer EST-T [5′-GAGAGGCTC- (dT)19-3′] (33.3 μmol/ L), 18 μL cDNA, 0.6 μL Taq (5
overnight.

ng/

reaction was executed in the mixture as follows: 1.5

pMD18-T (6.15

in the following reaction mixture: 1.5

μL SacI, 2.5 μL SalI, 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 iso-
lated and purified for double digestion at 37°C

to 50°C

terminates of staggered cDNAs, 4 μL RNase-free

dNTP mixture, 2 μL DNA polymerase I, 2 μL E. coli

RNase H + DNA ligase mixture, and 89 μL 10×buffer, 0.5

μL 100 mmol/L LaCl3 with low temperature, 50 mL 100 mmol/L LaCl3

time in 1/2 MS medium was gently poured onto the surface

digested amplicons, and 1 μL DNA ligase to 10

buffer, 0.5 μL EcoRI, and 0.5 μL HindIII to 10 μL total volume at

37°C 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 an-

notation 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 se-

quence, no sequencing record was simply classified.

1.5 Co-treatment by chilling with Ca2+ channel in-

hibitor or Ca2+ chelator

The treatment procedure was as described[26] with minor

modifications. For the control group, A. annua

seedlings were cultured at 25°C in an illuminated growth chamber

with a photoperiod of 16 h light and 8 h dark; for the

low temperature treatment group, 25°C and light cul-
tured A. annua seedlings were transferred into a 4°C

refrigerator for 30 min, then transferred back to a 25°C

illuminated growth chamber for 24 h, or 25°C cultured

A. annua seedlings were kept in a 4°C illuminated

growth chamber for 48 h.

For the co-treatment group of Ca2+ channel inhibitor

LaCl3 with low temperature, 50 mL 100 mmol/L LaCl3

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°C refrigerator for 30

min, and finally the flask still with LaCl3 was transferred

back under 25°C light for 24 h or first for one 12 h incu-
bation with LaCl3 and then for another 12 h incuba-
tion without LaCl3.

For the co- treatment group of Ca2+ 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 me-
dium with A. annua seedlings and incubated for 48 h,

then transferred to a 4°C refrigerator for 30 min, and

finally transferred back under 25°C light for 24 h; or A.
annua seedlings were briefly transferred into an empty flask without MS medium, then 10 mmol/L EGTA in ddH2O was added and transferred into a 4°C refrigerator to stand for 30 min, and finally transferred back under 25°C 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°C 5 min, 94°C 30 s, 57°C 1 min, 72°C 3 min for 30 cycles. The thermoprofile for Ubi gene amplification was as: 94°C 5 min, 94°C 20 s, 56°C 20 s, 72°C 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 ddH2O to a total volume of 12 μL, incubated in 70°C 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°C bath for 5 min. After 1 μL 200 U transcriptase was added, the mixture was incubated respectively at 25°C for 10 min, then at 42°C for 60 min, and finally at 70°C 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°C 10 s; 95°C 5 s, 60°C 31 s for 40 cycles. Once reaction was completed, a melting curve was plotted as following thermocondition: 95°C 15 s, 60°C 30 s, 95°C 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°C treatment group samples versus that in control group samples (2−ΔΔCt) after normalization to the inner reference gene (18S rRNA), in which ΔΔCt=ΔCt−ΔCtcontrol, ΔCt= (Ct−Ctcontrol18S rRNA)4

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 EcoRI and Hind 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 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, ΔΔCtcontrol=(Ct−Ct18S rRNA)control. 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.
### Table 1  Primer sequence for amplification and expected length of amplicons

<table>
<thead>
<tr>
<th><strong>A. annua</strong> gene</th>
<th>GenBank accession number</th>
<th>Primer sequence (5′ → 3′)</th>
<th>Length of amplicon (bp)</th>
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<tbody>
<tr>
<td><strong>SQ-PCR</strong></td>
<td></td>
<td></td>
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<tr>
<td>Amorpha-4, 11-diene synthase gene (<strong>ADS</strong>)</td>
<td>DQ241826</td>
<td>ATGTCACTTACAGAAAAACCTATTCGCC TCGATCCTCAGAAAAACCTATTCG</td>
<td>1641</td>
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<tr>
<td>Cytochrome P450 mono-oxygenase gene (<strong>CYP71AV1</strong>)</td>
<td>DQ872632</td>
<td>ATGAGAAGTATACTAAACGCAATG CGAGGTATGAGTAAAC</td>
<td>1488</td>
</tr>
<tr>
<td>Cytochrome P450 reductase gene (<strong>CPR</strong>)</td>
<td>DQ984181</td>
<td>ATGCAATCAACAACTTCCGTTAAG CCAGGATGAGGCACG</td>
<td>2115</td>
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<tr>
<td>Ubiquitin gene (<strong>Ubi</strong>)</td>
<td>EU258763</td>
<td>GCCAAGATTCAGGACAAGGAG</td>
<td>250</td>
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<tr>
<td><strong>RFQ-PCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>18S rRNA</strong></td>
<td>To be accessed</td>
<td>GCAACAAAAACCCGACACTCTG</td>
<td>102</td>
</tr>
<tr>
<td>Peroxidase 1 gene (<strong>POD1</strong>)</td>
<td>AY208699</td>
<td>CACCGGAGATGAAAGACCAC GACAAGGAG</td>
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<tr>
<td>Chitinase gene (<strong>CHI</strong>)</td>
<td>EF050424</td>
<td>TTCTCTACCTCCATCTAAATG AAATCTCCACCTAACTTGG</td>
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<td>Calmodulin gene (<strong>CaM</strong>)</td>
<td>EF549582</td>
<td>GCCGCAAGATGAAAGACAC CATGACGTCGCAAG</td>
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<tr>
<td>Ubiquitin-conjugating enzyme gene (<strong>UCE</strong>)</td>
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<td>GATTTCCACCAAGACACAAAGCAC CGTAGCTCAAAACAACTTGG</td>
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<td><strong>11S rRNA</strong></td>
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<tr>
<td>Drought/low temperature and salt responsive protein gene (<strong>D/LTSRP</strong>)</td>
<td>ES582125</td>
<td>AACTGACGTCGTCGATTCGGAACAC CCATCAGGAGAAGGCCG</td>
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<td>RNA-binding glycine-rich protein gene (<strong>RGP</strong>)</td>
<td>ES582129</td>
<td>TGGTGTTCGTAGGGAAAGTGG AGGTCAAAAACCCTTCAACCC</td>
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<tr>
<td>Auxin-repressed/dormancy-associated protein gene (<strong>AR/DAP</strong>)</td>
<td>ES582145</td>
<td>ACCATGACCGAACAAGGTGA AGCCACGCGGGAGGGCC</td>
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</table>

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

<table>
<thead>
<tr>
<th>No.</th>
<th>GenBank accession</th>
<th>Homology comparison-based functional annotation</th>
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<tr>
<td>1</td>
<td>AY445506</td>
<td>squalene synthase, SS</td>
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<td>DQ241826</td>
<td>amorpha-4.11-diene synthase, ADS</td>
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<td>cytochrome P450 mono-oxygenase, CYP</td>
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<td>4</td>
<td>DQ984181</td>
<td>cytochrome P450 reductase, CPR</td>
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<td>5</td>
<td>DQ5383000</td>
<td>vacuolar processing enzyme-1b</td>
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<tr>
<td>6</td>
<td>DQ538301</td>
<td>membrane protein</td>
</tr>
<tr>
<td>7</td>
<td>EF050423</td>
<td>structural constituent of ribosome</td>
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<tr>
<td>8</td>
<td>EF050424</td>
<td>chitinase, CHI</td>
</tr>
<tr>
<td>9</td>
<td>EF050427</td>
<td>ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, RuBPC/O</td>
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<td>10</td>
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<td>cytosolic NADP-malic enzyme</td>
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<td>13</td>
<td>EF549582</td>
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<td>EF549583</td>
<td>histone H4-like protein</td>
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<td>15</td>
<td>EF549584</td>
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<td>16</td>
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<td>ES582125</td>
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<td>auxin-repressed/dormancy-associated protein, AR/DAP</td>
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<td>ES582152</td>
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<td>ES582153</td>
<td>40S ribosomal protein S30-like protein</td>
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<td>28</td>
<td>ES582154</td>
<td>secretory peroxidase</td>
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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 mechanisms 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°C in an illuminated growth chamber (16 h light and 8 h dark). For the low temperature treatment group, 25°C and light cultured *A. annua* seedlings were transferred into a 4°C refrigerator to stand for 30 min, then transferred back to a 25°C 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 LaCl₃ or EGTA and low temperature co-treatment group, 50 mL 100 mmol/L LaCl₃ 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 LaCl₃ was transferred into a 4°C refrigerator to stand for 30 min, and finally transferred back under 25°C light for 24 h, or first for one 12-h incubation with LaCl₃ and then for another 12-h incubation without LaCl₃. Total RNAs were isolated and reverse transcription was conducted for further SQ-PCR assay.
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 ddH2O was added and transferred into a 4°C refrigerator to stand for 30 min, and finally transferred back under 25°C light for 24 h. Total RNAs were isolated and reverse transcription was conducted for further SQ-PCR assay.

Figure 4 Regulation of chilling stress-induced expression of tested novel genes in *A. annua* seedlings. The 25°C light cultured *A. annua* seedlings were always kept in a 4°C 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[22]. 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 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.[22] 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[24] 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[27] 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$^{2+}$ exchanger 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 CaCl₂, and suppression of the antioxidant enzyme genes by EGTA and LaCl₃. During cold acclimation, CaCl₂-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 LaCl₃, demonstrating that the Ca²⁺ signal system may be closely related to induction of the cold resistance trait in plants. Evidence of Ca²⁺-induced overexpression of cold-acclimation specific genes, cas15 and cas18, in *Alfalfa* has also provided support to this conclusion. In our preliminary tests, however, we have not observed suppression of chilling-induced overexpression of ADS and *CYP71AV1* genes by EGTA in the medium containing CaCl₂, suggesting incomplete chelate of the extracellular Ca²⁺ 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 LaCl₃, seeming to mean that expression of CPR gene may be more or less controlled by CaM, whether it implies 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*.

RFQ-PCR is a highly representative 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⁻ΔΔCt method is such a simple one for analyzing the relative difference of gene expression. 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, 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 Ca²⁺-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. 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. In our previous work accomplished by RFQ-PCR, we have placed *A. annua* seedlings in dark at 4°C 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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