Expression response of chalcone synthase gene to inducing conditions and its effect on flavonoids accumulation in two medicinal species of *Anoectochilus*

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*Anoectochilus roxburghii* and *A. formasanus* are the major species of genus *Anoectochilus* used in traditional Chinese medicine for their abundant content of flavonoids and some other medicinal constituents. In recent years, their wild resources are gradually exhausted due to over-collection and ecological deterioration. Artificial cultivation and tissue culture are employed to increase production. In this study, the open reading frame, promoter and genomic sequences of the chalcone synthase (CHS) gene were cloned from these two species according to their transcriptome information, and used for expression analysis in response to the induction of phenylalanine, ultraviolet light and NaCl, and its effect investigation on accumulation of flavonoids. The results showed that the expression of the CHS genes was upregulated in response to these inductions and resulted in increasing accumulation of total flavonoids. However, the increased flavonoids induced by phenylalanine and ultraviolet light were mainly allocated into the anthocyanidin branch of flavonoids biosynthesis. Not only did it improved the medicinal value, but might have inhibitory effect on plant growth because of the increased malondialdehyde accumulation. Under the induction of appropriate concentration of NaCl, the medicinal constituents of flavonoids were increased without inhibition to plant growth.

*Anoectochilus roxburghii* and *A. formasanus*, native to Fujian and Taiwan provinces of China respectively, are the major species of genus *Anoectochilus* of family Orchidaceae, used as herbal drug and health food in traditional Chinese medicine1. They are used to treat tumor, cardiovascular disease, fatty liver, hepatitis and other diseases because of their abundant medicinal constituents such as flavonoids and polysaccharides2-7. Due to over-harvesting and ecological deterioration, their wild populations have been gradually exhausted, and a lot of effort has been made for artificial cultivation and tissue culture for rapid propagation8-10. However, the accumulation of the medicinal constituents in the cultivated plants may be different from the wild plants. It becomes important to investigate the effect of cultivation or tissue culture conditions to the accumulation of the medicinal constituents, as well as its molecular mechanism10-14.

Flavonoids are a large category of aromatic secondary metabolites ubiquitous in plant and fungus. These compounds include six major subgroups that are found in higher plants: the chalcones, flavones, flavonols, flavan-ol, anthocyanins, and condensed tannins (or proanthocyanidins); a seventh group, the aurones, is widespread, but not ubiquitous15,16. Most of these constituents have antioxidant properties17. The flavonol subgroup, including rutin (rutinoside), quercetin and kaempferol, is the major medicinal constituents of *A. roxburghii* and *A. formasanus*18,19. In plants, flavonoids are biosynthesized through phenylpropanoid pathway from initial substrate phenylalanine (Phe). Chalcone synthase (CHS) catalyzes the conversion of 4-coumaryl-CoA and 3 units of

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malonyl-CoA to chalcone, 4 units of coenzyme A (CoA-SH) and carbon dioxide (CO₂). This is the committed step branching to biosynthesis of flavonol, isoflavone and anthocyanidin (Fig. S1)²⁰. The CHS gene has been cloned and characterized from Phalaenopsis hybrid²¹, a relative species of another genus of family Anoectochilus, as well as many other plants²²-²⁷. Its expression is responsive to induction of Phe²⁸-²⁹, ultraviolet light (UV)³¹,³², NaCl³³-³⁵ and some other biotic and abiotic conditions.

However, genomic information has not been available for any species of genus Anoectochilus, and even there are few reports about their chromosome ploidy³⁶. It is difficult for homologous cloning of genes from any species of this genus. In the present study, transcripts were assembled from RNA sequencing (RNA-seq) data, and used to clone the open reading frame (ORF) and the genomic sequences of the CHS gene from A. roxburghii and A. formosanus. After function evaluation by bioinformatics analysis, subcellular localization, enzyme activity assay and heterologous expression, their expression response to induction of Phe, UV, and NaCl was measured by real-time quantitative PCR (RT-qPCR). Their promoter sequences were amplified by thermal asymmetrical interlaced PCR (TAIL-PCR) and used for prediction of cis-acting elements. The contents of total flavonoids, free radical scavenging activity (FRSA), content of anthocyanidin and flavonols (rutin, quercetin, and kaempferol), as well as malondialdehyde (MDA), were measured by spectrophotometry and high performance liquid chromatography (HPLC), respectively. The possibility was explored to upregulate the expression of the CHS gene and to increase the accumulation of total flavonoids or flavonols in A. roxburghii and A. formosanus by induction in artificial cultivation or tissue culture.

Results
The CHS genes and their putative proteins. 8.76 and 10.42 Gb raw bases, and 29213781 and 34742855 raw reads were obtained by RNA-seq of the A. roxburghii and A. formosanus samples. Through quality inspection and filtration, 8.28 and 9.85 Gb clean bases, and 27597990 and 32836885 clean reads were retained, respectively, with Q20 (sequence error rate below 1%) greater than 96% and Q30 (sequence error rate below 0.1%) greater than 90% (Fig. S2). From these clean reads, 130024 and 116423 transcripts were assembled, 30265 and 24425 of them were annotated by SwissProt, and 35176 and 28691 of them were annotated by KGO, respectively. One of them was annotated as the CHS gene for A. roxburghii and A. formosanus, respectively. These two transcripts shared 94.80% similarity (Fig. S3).

With the primers homologous to the assembled transcripts, specific fragments of more than 1000 bp were amplified from the cDNA and the genomic DNA samples of A. roxburghii and A. formosanus, respectively (Fig. S4). The sequence analysis showed that their genomic fragments were both 1260 bp long and shared similarity of 94.92%. Their ORFs (1173 bp) were interrupted by an intron of 87 bp from the 180th to the 267th bp (Fig. S5). Their putative proteins (390 aa) were highly homologous (90.75% and 90.00%) with that of the CHS gene (AAV70116.1) in P. hybrid of family Anoectochilus (Fig. 1A)²¹. The predicted molecular weight (42.9 kDa), isoelectric points (pI 6.04 and 6.24), grand averages of hydropathy (−0.116 and −0.057), secondary structure (31.54% and 32.82% α-helices, 21.54% and 20.51% extended strands, 46.92% and 46.67% random coils, respectively), and three-dimensional structure models were all similar to that (42.5 kDa, pl 6.04 and −0.057, 29.74% α-helices, 21.03% extended strands, and 49.23% random coils) of the putative CHS protein in P. hybrid (Fig. S6)²¹. In the phylogenetic tree, the putative proteins of these two ORFs were also clustered into the same group with the putative CHS protein in Phalaenopsis hybrid (Fig. 1B). All the thirteen activation sites (G²⁹, G³³, G³⁵, L²¹, L²⁴, D²⁷, G²³, P³⁰, G³⁶, G³⁷, G³⁸, and G³⁵, P³⁰, G³⁶, G³⁸), the four catalytic residues (Cys²¹, Phe²⁵, His³⁴, and Asp³⁷), and the conserved domain of six residues (G²⁷⁹-V³⁸⁰-L³⁸¹-E³³²-G³³³-L³³⁴) were the same, except the three amino acids different in the malonyl-CoA binding motif (V³¹⁴-E³¹⁵-E³¹⁶-R³¹⁷-V³¹⁸-G³¹⁹-L³²⁰-K³²¹-P³²²-E³²³-K³²⁴-L³²⁵-T³²⁶-T³²⁷-G³²⁸-G³³⁰)²¹.

Function evaluation of the CHS genes. The green fluorescence signal was observed in the nucleus of the inner epidermis cell of onion transiently expressing vectors pCAMBIA2300-35S-CHS-eGFP, whereas it distributed in the whole cell infiltrated by the negative control vector pCAMBIA2300-35S-eGFP (Fig. 2). This result indicated the subcellular localization of the CHS protein in nucleus of A. roxburghii and A. formosanus.

By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), an obvious additional band about 44.3 kDa was separated from the Escherichia coli lines transformed by the CHS genes of these two medicinal species, and induced by isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. S7). The in vivo CHS enzyme activities of the transformed E. coli lines of the A. roxburghii CHS gene was significantly higher than that transformed by the A. Formosanus CHS gene, while the in vitro activities showed no significant difference (Figs. 3A,B and S8).

From the rice calli transformed by the CHS genes, three T₁ lines (two of A. roxburghii and one of A. formosanus) were identified as positive by PCR amplification of the CHS gene fragment (Fig. S9). Their contents of total flavonoids were 1.40, 1.68 and 1.26 times higher than that of the untransformed acceptor line, respectively. All these results confirmed that the two amplified ORF sequences were the CHS genes of A. roxburghii and A. formosanus, respectively, and registered at GenBank with accession numbers MK370742 and MK370743.

Differential expression in response to inducing conditions. Under the induction of Phe and UV, the expression of the CHS genes was upregulated significantly in A. roxburghii and A. formosanus, and reached its peak values (81.53 and 7.49 times of the 0 h control, respectively) at 4 and 8 h of the Phe induction, and (21994.7 and 3654.7 times of the 0 h control, respectively) at 8 and 12 h of the UV induction (Fig. 4A,B). In response to the NaCl induction, the expression of the CHS genes was continually upregulated and reached about three times of the 0 h control in A. roxburghii, while the upregulated expression reached 36.2 and 19.5 times of the 0 h control at 2 and 12 h of the NaCl induction in A. formosanus (Fig. 4C). This result indicated the strong expression response of these two CHS genes.
From the products of the second and the third rounds of thermal asymmetric interlaced PCR (TAIL-PCR) amplification from the genomic DNA samples, specific fragments were separated and sequenced for prediction of promoters and \textit{cis}-acting elements of the \textit{CHS} genes by Plant CARE software (Fig. S10). The promoter sequence of the \textit{CHS} gene of \textit{A. roxburghii} was 342 bp long containing enhance elements TATA-box (4), 5'-UTR Py-rich stretch (1) and CAAT-motif (6), light responsive elements G-box (2), \textit{sp1} (18) and I-box (1), and anaerobic inducing element ARE (1). The promoter sequence of the \textit{CHS} gene of \textit{A. formosanus} was 314 bp long containing enhance elements TATA-box (1), AT-rich sequence (1) and CAAT-box (5), light responsive elements G-box (2), CATT-motif (1), CGT-motif (5), GA-motif (1) and \textit{sp1} (2), MeJA-responsive elements CGTCA-motif (1) and TGACG-motif (1), and anaerobic inducing element ARE (1) (Fig. S11). This result might partially explain the expression response of the \textit{CHS} genes to the UV and NaCl induction.

\textbf{Flavonoids accumulation under inducing conditions.} Under non-inducing conditions (the 0 h control), the accumulation of total flavonoids, FRSA, and the accumulation of three flavonols (rutin, quercetin, and kaempferol) of \textit{A. roxburghii} was 1.73, 1.52, 25.76, 5.48 and 6.02 times of that of \textit{A. formosanus}, respectively (Fig. 5), indicating its higher antioxidant activity and medicinal value.

In response to Phe induction, the accumulation of total flavonoids and FRSA in \textit{A. roxburghii} and \textit{A. formosanus} was both increased and reached their peak values (1.38, 1.51, 1.30, and 2.06 times of the 0 d control) on the 5th, 3rd, and 4th day, respectively (Fig. 6A,B). The difference of total flavonoids content was not significant between these two species, but the increased range of FRSA in \textit{A. roxburghii} was significantly lower than that in \textit{A. formosanus}. The accumulation of rutin, quercetin and kaempferol was decreased during the first three days of the induction but rebounded after that with different ranges between the two species (Fig. 6C–E). The accumulation of anthocyanidin and MDA was increased continually with different ranges between the two species (Fig. 6F,G).
Under UV induction, the accumulation of total flavonoids and FRSA in the two species was increased and reached their peak values (2.09, 2.01, 1.47, and 1.82 times of the 0 h control) on the 5th, 4th, 3rd, and 2nd day, respectively (Fig. 7A,B). The accumulation of rutin, quercetin and kaempferol was decreased and rapidly descend to their valley values on the 1st to 5th day (Fig. 7C–E). The accumulation of anthocyanidin and MAD was increased continually (Fig. 7F,G).

In response to NaCl induction, the accumulation of total flavonoids and FRSA was increased continually with different ranges between A. roxburghii and A. formasanus (Fig. 8A,B). The rutin accumulation in A. roxburghii was increased sharply on the 5th and 6th day and reached as high as 21.28 times of the 0 h control, whereas that in A. formasanus did not changed obviously (Fig. 8C). The accumulation of quercetin, kaempferol and anthocyanidin was increased with different ranges between the two species (Fig. 8D–F). The MAD content was only increased slightly (1.23 and 1.26 times of the 0 h control, Fig. 8G).

**Discussion**

Numerous studies showed that the biosynthesis of flavonoids, as well as flavonols, took place exclusively in endoplasmic reticulum, cytosol, and other cytoplasmic organelles. In this study, the subcellular localization of the CHS proteins of A. roxburghii and A. formasanus was targeted to the nucleus (Fig. 2). This result was also evidenced by the bioinformatics prediction of a nuclear localization signal at the N-terminal of their primary structure (Fig. 1A). Some recent reports from a number of different plant species...
not only documented the presence of flavonoids in nuclei\(^39,40\), but also localized at least two of their biosynthetic enzymes to nuclei in several cell types in *Arabidopsis*\(^41\). It was speculated that the *Anoectochilus* CHS proteins might catalyze flavonoids synthesis of the phenylpropane metabolism pathway in nucleus, and their products might be involved not only in the basal metabolism, stress response, reproductive development and many other growth and development processes\(^30–35\), but also in the transcriptional regulation of related genes.

Under Phe, UV and NaCl induction, the expression of the CHS genes of *A. roxburghii* and *A. formosanus* were upregulated with different ranges (Fig. 4). The *cis*-acting elements related to light and anaerobic response were predicted in the promoter sequences of the two CHS genes (Fig. S11)\(^42–44\). These results not only explain the increased accumulation of total flavonoids in the two species under these inducing conditions, but also confirm the rate-limiting role of the CHS enzyme for flavonoids biosynthesis of phenylpropanoid pathway (Fig. S1)\(^16,20–27\).
Under Phe and UV induction, the decreased flavonols (Figs. 6C–E and 7C–E) and the increased accumulation of anthocyanidin (Figs. 6F and 7F) demonstrated that the increased accumulation of total flavonoids (Figs. 6A and 7A) was mainly allocated into the anthocyanidin branch of the phenylpropanoid pathway (Fig. S1). This was not conducive to improving medicinal value of these two species. Even more, the increased MDA accumulation revealed the inhibitory effect of Phe and UV on plant growth (Figs. 6G and 7G). Similar effect was also found in cell suspension culture of strawberry and calli of *Hydrocotyle bonariensis* \(^{28,29}\). In response to NaCl induction, the increased flavonols (Fig. 8C–E), especially the sharp peak content of rutin on the 5th and 6th day of induction in *A. roxburghii*, indicated that the increased accumulation of total flavonoids (Fig. 8A) was mostly allocated into the flavonol branch (Fig. S1), while the MDA content was only increased slightly (Fig. 8G). Along with the higher accumulation of total flavonoids, FRSA, and the accumulation of flavonols in *A. roxburghii* under non-inducing conditions (Fig. 5), it was suggested that NaCl induction of appreciate concentration (100 mmol/L) in artificial cultivation or tissue culture of *A. roxburghii* could efficiently increase the accumulation of total flavonoids and flavonols, and improved the medicinal value.
conclusion
The Phe, UV and NaCl induction upregulates the expression of the chalcone synthase genes and affects the accumulation of total flavonoids, FRSA, flavonols, anthocyanidin and MAD in A. roxburghii and A. formosanus. NaCl induction of appreciate concentration (100 mmol/L) is suggested to apply in artificial cultivation or tissue culture of A. roxburghii to increase accumulation of total flavonoids and flavonols and improve the medicinal value.

Materials and Methods
Sample preparation. The seedlings of A. roxburghii and A. formosanus were cultured on MS medium for 18 weeks. The uniform seedlings were divided into three groups. Two groups were transplanted into a plastic mesh grid for aquaculture. On the fifth day, Phe and NaCl were added into the nutrient solution with final concentration of 4 mg/L and 100 mmol/L, respectively. The other group was transplanted into plastic pots (five seedlings per pot) with nutritional soil and vermiculite (3:1), and submitted to UV induction of 253.7 nm after recovering. At 0 (control), 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h (1 d), 2 d, 3 d, 4 d, 5 d and 6 d of the Phe, UV and NaCl induction, two leaves were sampled from each of the five induced plants.

One leaf of each sample of 0 to 24 h of the induction was pulverized to fine powder in liquid nitrogen, and used for RNA extraction with RNeasy Plant Mini Kit (Qiagen, China). After release of probable DNA contamination by RNase-free DNase I (Qiagen, China), detection for concentration, purity and integrity on spectrophotometer (NanoDrop One, Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA) respectively, part of each RNA samples was reverse transcribed to cDNA by using PrimeScript RT Reagent Kit (TaKaRa Japan). Part of pulverized leaf samples of the control (0 h) were mixed and used for extraction of genomic DNA with the method of cetyl trimethylammonium bromide (CTAB).

Transcriptome sequencing and CHS gene cloning. Part of the RNA samples of the control (0 h) were mixed and sequenced by Illumina HiseqXten platform at MajorbioBioTech Co., Ltd. The clean reads were assembled by Trinity v2.4.0 (https://github.com/trinityrnaseq/trinityrnaseq/wiki), and used to search for transcript sequences of the CHS genes by SWISSPROT (https://swissmodel.expasy.org/) and KOG (https://genome.jgi.doe.gov/Tutorial/tutorial/kog.html). According to the transcript sequences, a pair of specific primers (Table S1) was synthesized and used to amplify the ORF and genomic sequences of the CHS genes from the cDNA and the genomic DNA samples, respectively, by using Prime STAR HS DNA Polymerase (TaKaRa, Japan) with proof reading activity. The amplified products were purified by using Universal DNA Purification Kit (Tiangen, China), added dATP at the 3’ ends by using Taq™ (TaKaRa, China), cloned into pMD19-T vector (TaKaRa, Japan), and sequenced at Sangon Biotech Co., Ltd (Shanghai, China). The sequencing results were aligned for gene structure on NCBI website (http://www.ncbi.nlm.nih.gov), and predicted for putative proteins by using online tool SWISS-MODEL (https://swissmodel.expasy.org/). Phylogenetic analysis was conducted among the putative proteins by using MEGA7.0 software (https://www.megasoftware.net/).

Function evaluation of the CHS genes. Three pairs of specific primers with appropriate recognition sites (Table S1) were used to amplify the ORF sequences without or with the termination codons from the harbored pMD19-T vectors by using Prime STAR HS DNA Polymerase (TaKaRa, Japan). The amplified products were purified as above, and inserted into transient expression vector pCAMBIA2300, prokaryotic expression vector pET-28a (+) and monocotyledonous expression vector pZZ00026, respectively, by using CloneExpress One Step Cloning Kit (Vazyme, China, Fig. S13).
The transient expression vectors pCAMBIA2300-35S-CHS-eGFP harboring the CHS genes of the two species, as well as the empty plasmid pCAMBIA2300-35S-eGFP (negative control), was infiltrated into the inner epidermis of onion. After incubation at 28 °C under dark for 24 h, the green fluorescence signal for subcellular localization was observed and photographed under fluorescence microscope (Olympus BX63, Japan).

The prokaryotic expression vectors pET-28a (+)–CHS were transformed into E. coli train BL21 with freeze-thaw method. Until OD600 ≈ 0.6, the transformant cultures were added with IPTG to a final concentration of 0.5 mmol L⁻¹, and incubated at 37 °C for 2 h. The heterologous expression of the CHS genes was detected by SDS-PAGE. The proteins were purified by using Ni-NTA Sefinose™ Resin Kit (Sangon China), and determined for concentration by using NanoDrop™ One/OneC B-50Q (Thermo, USA). According to the manual (Table S2) of CHS enzyme activity kit (Gemmed Sciences Inc. USA), the purified protein and the IPTG-induced E. coli cells were reacted with 4-coumaric-CoA and 3 untis malonyl-CoA in the presence or absence of luteolin (a sensitive inhibitor of CHS), respectively. Along with the synthesis of chalcone, the released 4 units of CoA-SH with Ellman reagent 5,5-dithiobis (2-nitrobenzoic acid, DTNB) to produced yellow 5-thio-2-nitrobenzoic acid (TNB). The activity of chalcone synthase was quantitatively analyzed by the change of its absorption peak (412 nm wavelength). The in vitro and in vivo CHS enzyme activities were analyzed by the change of the absorption peak at 412 nm.

The monocotyledonous expression vector pZZ00026-Ubi-CHS-T-nos was mobilized into Agrobacterium tumefaciens strain EHA105, and used to transform rice calli of variety ZH11. The transformed lines were identified by PCR amplification of a 1205 bp fragment of the CHS genes, and detected for content of total flavonoids by spectrophotography (described later).

Expression analysis under inducing conditions. Two pairs of RT-qPCR primers (Table S1) was synthesized to amplify a 136 bp and a 221 bp fragment of the CHS ORFand the Actin2 gene (used as internal reference), respectively, from the cDNA samples of the three replicates of each time point of the inducing treatments. The amplification reaction was conducted by using SsoFastEvaGreenSupermix (Bio-Rad, USA) in Bio-Rad iCycler iQ5 RT-qPCR System. The 2⁻ΔΔCT method of the CFX Manager™ software version 2.0 (Bio-Rad, USA) was used to normalize the expression differentiation between the internal reference and the CHS gene. The relation expression levels under the inducing conditions were calculated by their expression level to those under the non-inducing control.

As described by Liu et al., six arbitrary degenerate primers (AD) and three nested primers (Table S1) complementary to the coding sequences of the CHS genes were synthesized, and used to amplify the promoter sequences of the CHS genes from the genomic DNA samples by TAIL-PCR. The products of the second and third rounds of amplification were separated by 1.2% agarose gel electrophoresis, purified and sequenced as above. The sequenced results were used for prediction of cis-affecting elements by Plant CARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Quantification of total flavonoids and free radical scavenging activity. The other leaf of each sample of 0 to 6 d of the induction was dried at 55°C, ground to fine powder (extraction quality), extracted in 95% alcohol in an ultrasonic instrument at 25 °C for 30 min. The extracts were filtered through Whatman No. 1 paper filter under reduced pressure (extraction volume). Referring to China national standardization for determination of total flavonoids in propolis, 1 mL of each of the filtrates was added with 0.4 ml of 5% NaNO₂, kept for 5 min, added with 0.4 ml of 10% Al(NO₃)₃, kept for 5 min, added with 4 ml of 4% NaOH for coloration, incubated at room temperature for 20 min, and determined for absorbency at 420 nm in a UV-1800 spectrophotometer (Shimadzu, Japan). The content of total flavonoids was calculated as:

\[
\text{Content of flavonoids} = \frac{A_{420} \times V}{m \times d}
\]

where, \(A_{420}\) were the absorbance at 420 nm, \(V\) were total volume of the extract, \(m\) were the extraction quality from the leaf of each sample (1 g), \(d\) were the dilution multiple.

As described by Sharma and Bhat, 2 mL of each of the filtrates, as well as two milliliters of ethanol (blank control), were added with 2 ml of 0.04 mmol/L ethanol solution of 1-diphenyl-2-picrylhydrazyl (DPPH), incubated in dark for 20 min, and detected the absorbance at 517 nm in an UV-1800 spectrophotometer. The free radical scavenging activities were calculated as:

\[
\text{Free radical scavenging activity} = \frac{1 - (A_i - A_b)}{A_n} \times 100
\]

where, \(A_i\), \(A_b\), and \(A_n\) were the absorbance of the induced samples, the non-inducing samples and the blank control.

Quantification of flavonal constituents and anthocyanidin. Ten microliters of each of the filtrates were loaded into HPLC (Agilent 1260) equipped with Brownlee SPP C18 column (E-Merck, 2.7 μm, 4.6 × 150 nm). Gradient elution was conducted with 80% mobile phase A (98% H₂O and 2% H₃PO₄) and 20% mobile phase B (CH₃Cl) for 5 min, 55% mobile phase A and 45% mobile phase B for 43 min, and 80% mobile phase A and 20% mobile phase B for 2 min at a flow rate of 1 mL/min and column temperature of 30 °C. The flavonal constituents (rutin, quercetin and kaempferol) were monitored at 360 nm and identified by comparison of retention time with their authentic samples. Their relative contents were quantified and expressed as percentage peak area.
As described by Tanaka et al.52, one leaf of each sample of 0 (control), 1, 2, 3, 4, 5 and 6 d of the induction, was pulverized to fine powder in liquid nitrogen, extracted with acidified (1% HCl) methanol in dark with shaking for 48 h, and centrifuged at 4000 g for 10 min. The supernatant was used to determine for absorbance at 535 nm in an UV-1800 spectrophotometer. The anthocyanidin content was indicated by absorbance value.

Determination of MDA content. As described by Yu et al.39, the other leaf of the each sample of 0 (control), 1, 2, 3, 4, 5 and 6 d of the induction was pulverized with 10% trichloroacetic acid (TCA) and centrifuged at 4000 g for 10 min. Two milliliters of the supernatant was added with 2 mL of 0.06% thiobarbiuric acid (TBA), and bathed in boiling water for 10 min. After cooled, the samples were centrifuged at 7000 g for 5 min. The supernatant was determined for absorbance at 450, 532, and 600 nm, respectively, in an UV-1800 spectrophotometer. The MDA content was calculated as:

\[
\text{MDA content} = \left[ 6.452 \times (A_{532} - A_{600}) - 0.559 \times A_{450} \times \left( V - (V' + W) \right) \right]
\]

where, A450, A532, and A600 were absorbance at 450, 532, and 600 nm, respectively. V, V', and W were total volume of the extract, the determined volume (2 mL), and the fresh weight of the sample.

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Author contributions
L.Y. carried out the experiments, interpreted the results and wrote the manuscript. J.C.Z., J.T.Q. and G.H. took part in some of the experiments. H.Q.Y. provided technical support. W.C.L. and F.L.F. conceived and designed the research. All the authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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