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Research Article

Cold-induced ginsenosides accumulation is associated with the alteration in DNA methylation and relative gene expression in perennial American ginseng (*Panax quinquefolius* L.) along with its plant growth and development process

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ABSTRACT

Background: Ginsenosides accumulation responses to temperature are critical to quality formation in cold-dependent American ginseng. However, the studies on cold requirement mechanism relevant to ginsenosides have been limited in this species.
Methods: Two experiments were carried out: one was a multivariate linear regression analysis between the ginsenosides accumulation and the environmental conditions of American ginseng from different sites of China and the other was a synchronous determination of ginsenosides accumulation, overall DNA methylation, and relative gene expression in different tissues during different developmental stages of American ginseng after experiencing different cold exposure duration treatments.
Results: Results showed that the variation of the contents as well as the yields of total and individual ginsenosides Rg1, Re, and Rb1 in the roots were closely associated with environmental temperature conditions which implied that the cold environment plays a decisive role in the ginsenoside accumulation of American ginseng. Further results showed that there is a cyclically reversible dynamism between methylation and demethylation of DNA in the perennial American ginseng in response to temperature seasonality. And sufficient cold exposure duration in winter caused sufficient DNA demethylation in tender leaves in early spring and then accompanied the high expression of flowering gene PqFT in flowering stages and ginsenosides biosynthesis gene PqDDS in green berry stages successively, and finally, maximum ginsenosides accumulation occurred in the roots of American ginseng.
Conclusion: We, therefore, hypothesized that cold-induced DNA methylation changes might regulate relative gene expression involving both plant development and plant secondary metabolites in such cold-dependent perennial plant species.

1. Introduction

The quality formation in medicinal plants is an important basic scientific problem involving the interaction between external and internal factors; study in this area can help to improve the cultivation strategy in medicinal plants [1]. Temperature, one of the major external factors that affect all aspects of plant growth and development, can affect plant secondary metabolism and consequently lead to quality variation in medicinal plants [2]. In some cold-dependent plant species, some secondary metabolites genes may be upregulated by cold temperature during plant growth and development, which might be regulated by an epigenetic mechanism through the alteration of DNA methylation [3–5]. This mechanism might play an important role in facilitating ecological

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adaptation in cold-dependent plants and also result in the differential quality in medicinal plants from habitats with different temperature [3–5]. Given the increasing evidence for dynamic changes of both DNA methylation and secondary metabolites in response to cold stress, we hypothesized that the potential relationship of DNA methylation and secondary metabolism might play a critical role in cold-induced quality formation in cold-dependent medicinal plants.

American ginseng (*Panax quinquefolius* L.), a close relative to Asian ginseng (*Panax ginseng* C. A. Meyer) in the genus *Panax* of Araliaceae family, originally grown perennially in south of Canada and north of the USA, has been introduced successfully in northern China, where cold is regional climate characteristics in every producing site, implying that American ginseng might be a typical cold-dependent plant species, quite similar to its close relative Asian ginseng [6]. Both American ginseng and Asian ginseng produce various ginsenosides with various biological activities such as antifatigue, antioxidant, immunomodulation, antitumor, and so on [7]. Studies have reported that ginsenosides contents in these plants vary depending on light, water, soil, site, plant age, season, and so on [8,9]. However, the effects of temperature factors on ginsenosides in these plants had been paid more and more attention recently. In the case of Asian ginseng, low temperature was found to play a decisive role in ginsenoside accumulation when compared with other factors such as soil moisture, soil nutrition, and light [10–12]. In the case of American ginseng, Wu et al. (2002) [13] preliminary realized that temperature and light were the two main climatic factors affecting the total ginsenosides contents. Huang et al. (2013) [14] further presented that the low-temperature factors promoted ginsenosides accumulation than other factors. However, an opposite experimental result by Jochum et al. [15] (2007) indicated that American ginseng plants grown at relatively high temperature had greater root ginsenosides contents than plants grown at relatively low temperature. Therefore, it is necessary further to clarify the details of the mechanism of temperature-relative ginsenosides accumulation; of which, whether the cold-induced ginsenosides accumulation is associated with alterations of overall DNA methylation and relative gene expression in American ginseng is even more important.

2. Materials and methods

2.1. Plant materials

Experiment 1

The objective of this experiment was to study the relationship between ginsenosides accumulation and environmental factors of American ginseng. Root samples of 3-year-old American ginseng were collected at the same time on 21 October 2016 from different cultivation sites of China including Wendeng, Liuba, Fusong, Jian, Raohe, and Huairou. Each root sample was carefully washed and divided into different parts including rhizomes, outer tissues (periderm and phloem) of main roots, inner tissues (xylem and pith) of main roots and hair roots; each part was used for determination of biomass, ginsenosides contents, and ginsenosides yields, separately. The biomass of each part of root sample was determined by measuring the dry weight with an electronic balance after drying. The ginsenoside contents (Rg1, Re, and Rb1) in different parts of root samples were determined by high performance liquid chromatography (HPLC). The yields of total and individual ginsenosides in root samples were calculated with contents multiply by dry weights.

Experiment 2

The objective of this experiment was to study the effects of cold temperature on ginsenoside accumulation and overall DNA methylation in American ginseng by setting different cold exposure duration treatments in dormant season of American ginseng before seedlings emerged. At the end of October 2016, 2-year-old American ginseng plants strictly uniform in shape, size, and weight were collected from Wendeng (main producing areas of American ginseng in Shandong of China) and were transplanted in pots (30 cm in diameter × 40 cm in depth, each pot one plant, with same volume of fertile sandy loam) under sunshade net in Medicinal Plant Garden of Pharmacy School, Qingdao University. Temperature treatments began at 1 November 2016; plants were randomly divided into six groups ($N = 30$ per group at least, by marking on the pots), which were designated six cold exposure duration treatments including no cold exposure (CE-0), one month (CE-1, November), two months (CE-2, November–December), three months (CE-3, November–January), four months (CE-4, November–February), and cold exposure naturally (CE-N). There was a separate constant temperature chamber (18 ± 3.0 °C) under the same sunshade sheds that provided exactly the same sunshine, soil moisture, and natural photoperiod except temperature. The plants marked with CE-0 to CE-4 were, respectively, transferred to the constant temperature chamber on 1 November 2016, 1 December 2016, 1 January 2017, 1 February 2017, and 1 March 2017. And all the plants were transferred back to the natural temperature conditions on 1 April 2017. The CE-N group was exposed under natural temperature conditions (without any temperature control) from 1 November 2016 to 1 April 2017 (monthly temperature fluctuation range: November −2 °C ~ 18 °C, December −5 °C ~ 13 °C, January −6 °C ~ 9 °C, February −4 °C ~ 10 °C, March −1 °C ~ 15 °C). From 1 April 2017 then on, all the plants in different cold exposure duration treatments were cultured under completely identical natural conditions including temperature, light, photoperiod, soil moisture, soil nutrient, and so on. Plants were sampled during every development stage by schedule, for determination of plant dry weights, ginsenosides contents, overall DNA methylation levels, and relative gene expression in different parts of plants.

2.2. Ginsenoside extraction and HPLC analysis

The determination of ginsenosides was according to the method of Chinese Pharmacopoeia with some modification [16]. American ginseng samples were ground into powder with a grinder, filtered through a 355 μm sieve. One gram of the milled powder was twice soaked in 50 ml of 80% (v/v) methanol solution at 80 °C for 90 min. The extract was filtered and then evaporated to remove the solvent. After the liquid evaporated, the residue was dissolved in distilled water and extracted three times with water-saturated n-butanol. The n-butanol layer was then evaporated to produce a ginsenoside fraction. Each sample was dissolved in HPLC-grade methanol (1.0 g/5 mL) and then filtrated through a 0.45-mm filter for HPLC analysis.

The HPLC system Agilent 1260 (Agilent Technologies Co. Ltd., USA) was equipped with a diode array detector and a OpenLAB Service for data processing. The columns used were Zorbax SB C18-column, 5 μm, 250 × 4.6 mm. Authentic standards of ginsenosides Rb1, Re, and Rg1 were obtained from Chengdu DeSiTe Phytochemicals Ltd. (Chengdu, Sichuan, China). The standards and samples were separated using a gradient mobile phase consisting of acetonitrile (A) and 0.1% phosphoric acid in water (B). The gradient elution program is 0–25 min, 20% A; 25–60 min, 20–40% A; 60–70 min, 40–100% A. The injection volume was 10 *u*L, the flow rate was set at 1.0 mL·min^{-1}, and the column temperature was 40 °C. The detection wavelength was set at 203 nm. Standard curves were constructed by plotting the peak areas (mAU·min^{-1}) versus the amount (ug) of each analyte injected. The content in the samples was calculated by the formula below:

Ginsenoside content ($mg \cdot g^{-1}DW$) = Sample concentration ($mg \cdot L^{-1}$) × Sample volume (L)/Sample dry weight (g)

All analyses were repeated three times, and the mean ± S.E. were calculated.

2.3. DNA extraction, hydrolysis, and HPLC analysis for DNA methylation

The determination of DNA methylation followed some studies with modification [17]. Samples of American ginseng were excised, frozen in liquid nitrogen, and stored at −80 °C. Total cellular DNA was isolated following a modification of the cetyltrimethylammonium bromide extraction method by Murray and Thompson [18]. Proteins were eliminated by extraction with chloroform, whereas carbohydrates were discarded after the precipitation of the cetyltrimethylammonium bromide−DNA complexes by lowering the salt concentration. Concentration and purity of DNA were determined spectrophotometrically (A260 for DNA, A230 for polysaccharides, and A280 for proteins). Approximately 40-mg DNA was hydrolyzed to bases in 50 μl of 70% perchloric acid (95 °C for 50 min). The pH was adjusted to between 3 and 5 with KOH (1 mol/L). The homogenate was centrifuged at 12000 rpm for 5 min and then filtrated through a 0.45-mm filter for HPLC.

An aliquot of the filtrate (20 uL) was injected into the HPLC with a column ZORBAX SB-C18 (4.6 mm × 250 mm, 5 um) (Agilent 1260, Palo Alto, USA). The eluent (pH = 4.6) contained 8% methanol and 50-$mmol \cdot L^{-1}$ potassium dihydrogen phosphate, with a flow rate of 0.3 $mL \cdot min^{-1}$, detection wavelength of 270 nm, and column temperature of 30 °C. The chromatogram was further analyzed using OpenLAB Service computer software. The standard was a mixture of the two bases: cytosine and 5-methylcytosine in a concentration of 10 $ug \cdot mL^{-1}$ each and was used for all measurements. By comparing peak areas of same retention times, the contents of cytosine and 5-methylcytosine in samples were calculated and then the percentages of 5-methylcytosine were calculated by (5-methylcytosine × 100%)/(5-methylcytosine + cytosine). All analyses were repeated three times and the mean ± standard error (S.E.) were calculated.

2.4. RNA extraction, cDNA synthesis, and qRT-PCR for gene expression analysis

Total RNA was extracted from frozen samples of American ginseng rhizomes using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) including DNase I digestion. Afterward, total RNA was reverse transcribed with the ReverTra Ace QPCR RT Kit (TOYOBO, Japan) to synthesize cDNA. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with a SYBR Premix Ex Taq Kit (TaKaRa, Kyoto, Japan) as described in the manufacturer's protocol. Gene-specific primers for PqDDS, (forward) 5′-TAA TGA GTT GGT GGG CAG AAG AT-3′ and (reverse) 5′-GGT GGC GAT AAT TGC TTG AGT AG-3′, and PqFT, (forward) 5′-GGT GGA GAA GAC CTC AGG AA-3′ and (reverse) 5′-GGT TGC TAG GAC TTG GAA CAT C-3′, were used to perform qRT-PCR. According to the manufacturer instructions, the thermal cycler conditions were as follows: 95 °C for 10 min, followed 35 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. The fluorescent product was detected during the final step of each cycle. To determine the relative fold differences in template abundance for each sample, the Ct values for each of the gene-specific primers were normalized to the Ct value of β-actin (5′-CGT GAT CTT ACA GAT AGC TTG ATG A-3′ and 5′-AGA GAA GCT AAG ATT GAT CCT CC-3′).

2.5. Statistical analysis

All the statistical analysis was conducted based on a SPSS 20.0 system (SPSS Inc., Chicago, IL, USA). Hierarchical cluster analysis was used to group the cultivation sites with measured parameters. analysis of variance (ANOVA) followed by Fisher's least significant difference test was used to evaluate the significant differences at $p \leq 0.05$ or $p \leq 0.01$. Multiple linear regression analysis was used to obtain the optimal regression equation for selecting the main temperature factors that contribute to the ginsenoside accumulation of American ginseng. Correlation analysis was used to show the relationship between the measured parameters with the Pearson's correlation coefficient.

3. Results

3.1. Comparison of the contents and yields of ginsenosides in American ginseng from different sites

Hierarchical cluster analysis according to the measured ginsenosides parameters divided the cultivation sites into two clusters: cluster A including Wendeng (north latitude 37.47°, annual mean temperature 11.20 °C), Huairou (north latitude 40.32°, annual mean temperature 10.80 °C), and Liuba (North Latitude 33.07°, Annual mean temperature 14.00 °C) vs Cluster B including Raohe (North Latitude 45.27°, Annual mean temperature 3.40 °C), Jian (North Latitude 41.10°, Annual mean temperature 6.30 °C) and Fusong (North Latitude 42.35°, Annual mean temperature 4.00 °C). As the ginsenosides contents shown in Table 1, results presented similarity in the ginsenosides profiles of American ginseng from different sites. The total contents of three ginsenosides in all the samples follow this order: hair roots > rhizomes > outer tissue of main roots > inner tissue of main roots, and the contents of individual ginsenosides in all the samples follow this order: Rb1 > Re > Rg1 in hair roots; outer tissue of main roots and inner tissue of main roots except that they follow this order: Re > Rb1 > Rg1 in rhizomes. Results presented significant difference ($p \leq 0.05$) in the ginsenoside contents between the two group of cultivation sites, and all presented cluster A < cluster B. As the ginsenosides yields shown in Table 1, results presented that the total yields of three ginsenosides in all the samples follow this order: outer tissue of main roots > hair roots > inner tissue of main roots > rhizomes which are related to the biomass in different root parts of American ginseng, and the yields of individual ginsenosides in all the samples follow this order: Rb1 > Re > Rg1 in hair roots; outer tissue of main roots and inner tissue of main roots except that they follow this order: Re > Rb1 > Rg1 in rhizomes which are consistent with the contents in the samples from different sites. However, results presented extremely significant difference ($p \leq 0.01$) in the ginsenosides yields between the two group of cultivation sites, but all presented Cluster A > Cluster B. So we first realized that the inconsistency between contents and yields of ginsenosides in American ginseng from different sites, which might involve a complex mechanism of ginsenoside accumulation related to different temperature zone.

3.2. Correlation between ginsenosides and temperature factors

As shown in Table 2, multivariate linear regression equations between ginsenosides and temperature factors were derived. In the case of contents, the biggest influences on ginsenosides were January minimum temperature (x_1) and annual mean temperature (x_6), both presented negative correlation with contents, while the minimum influences on ginsenosides were active cumulative temperature (x_5), which presented weak positive correlation with

Table 1
The contents and yields of ginsenosides in the different root parts of 3-year-old American ginseng from different cultivation sites of China

Sites and root parts		Contents (mg/g)				Yields (mg)			
		Rg1	Re	Rb1	Total	Rg1	Re	Rb1	Total
Wendeng	Rhizome	4.96 ± 0.19	24.15 ± 1.16	18.07 ± 0.72	47.19 ± 2.40	2.76 ± 0.29	13.45 ± 0.80	10.06 ± 1.27	26.28 ± 1.11
	OM	2.60 ± 0.11	15.36 ± 0.62	16.76 ± 0.50	34.74 ± 1.20	11.62 ± 0.31	68.46 ± 2.71	74.66 ± 2.23	154.75 ± 5.12
	IM	0.73 ± 0.10	6.77 ± 0.21	8.48 ± 0.38	15.99 ± 0.91	2.03 ± 0.12	18.85 ± 0.90	23.62 ± 1.25	44.50 ± 2.13
	Hair root	3.01 ± 0.15	21.33 ± 1.09	26.92 ± 0.87	51.27 ± 2.22	3.07 ± 0.11	24.12 ± 0.92	30.44 ± 0.97	57.97 ± 2.30
	Total	Total contents per plant			31.76 ± 1.53	Total yields per plant			283.49 ± 10.72
Huairou	Rhizome	8.18 ± 0.22	28.08 ± 1.17	20.38 ± 0.99	56.64 ± 1.89	5.21 ± 0.22	17.87 ± 0.58	12.98 ± 0.58	36.06 ± 1.32
	OM	2.11 ± 0.13	12.31 ± 0.61	19.37 ± 0.71	33.78 ± 1.25	10.73 ± 0.37	62.71 ± 2.12	98.68 ± 4.55	172.12 ± 7.45
	IM	1.28 ± 0.07	5.61 ± 0.27	7.32 ± 0.25	14.21 ± 0.50	4.07 ± 0.27	17.87 ± 0.61	23.30 ± 1.07	45.23 ± 2.17
	Hair root	4.74 ± 0.30	28.48 ± 1.16	38.01 ± 1.20	71.21 ± 2.93	5.53 ± 0.23	33.24 ± 1.67	44.35 ± 2.19	83.12 ± 4.11
	Total	Total contents per plant			32.11 ± 1.18	Total yields per plant			336.53 ± 11.38
Jian	Rhizome	7.39 ± 0.29	31.32 ± 1.02	25.66 ± 1.21	64.39 ± 3.24	3.21 ± 0.14	13.61 ± 0.55	11.15 ± 0.43	27.97 ± 0.91
	OM	2.66 ± 0.21	18.35 ± 0.71	19.77 ± 0.73	40.78 ± 1.60	9.25 ± 0.25	63.77 ± 2.95	68.70 ± 2.64	141.73 ± 6.78
	IM	1.08 ± 0.28	5.41 ± 0.30	8.42 ± 0.38	14.92 ± 0.80	2.34 ± 0.12	11.76 ± 0.41	18.30 ± 0.57	32.40 ± 1.38
	Hair root	4.31 ± 0.14	33.75 ± 1.61	37.99 ± 1.23	70.04 ± 2.71	3.43 ± 0.12	26.88 ± 1.18	30.25 ± 1.26	60.56 ± 2.26
	Total	Total contents per plant			36.73 ± 1.19	Total yields per plant			262.65 ± 10.32
Fusong	Rhizome	6.71 ± 0.20	36.33 ± 1.77	27.09 ± 0.89	70.13 ± 2.31	2.44 ± 0.08	13.23 ± 0.37	9.87 ± 0.38	25.54 ± 1.13
	OM	3.80 ± 0.14	19.77 ± 0.81	25.83 ± 0.91	49.41 ± 2.18	11.08 ± 0.31	57.60 ± 2.15	75.27 ± 3.20	143.95 ± 5.29
	IM	2.09 ± 0.11	8.11 ± 0.30	9.08 ± 0.41	19.29 ± 0.76	3.82 ± 0.30	14.76 ± 0.57	16.55 ± 0.71	35.13 ± 1.78
	Hair root	3.92 ± 0.17	35.71 ± 1.24	38.37 ± 1.28	77.99 ± 2.49	2.62 ± 0.08	23.84 ± 1.21	25.62 ± 1.37	52.08 ± 2.15
	Total	Total contents per plant			42.78 ± 2.05	Total yields per plant			256.70 ± 11.23
Raohe	Rhizome	5.01 ± 0.12	31.70 ± 0.96	25.23 ± 0.73	61.94 ± 2.27	2.13 ± 0.11	13.49 ± 0.48	10.73 ± 0.36	26.35 ± 0.93
	OM	3.96 ± 0.27	17.13 ± 0.79	23.79 ± 0.86	44.88 ± 2.15	13.47 ± 0.67	58.30 ± 2.96	80.96 ± 3.88	152.73 ± 7.41
	IM	0.73 ± 0.04	8.80 ± 0.31	9.69 ± 0.41	19.22 ± 1.05	1.56 ± 0.07	18.73 ± 0.71	20.60 ± 1.25	40.89 ± 1.84
	Hair root	3.79 ± 0.15	33.73 ± 1.21	39.15 ± 1.67	76.68 ± 3.81	2.96 ± 0.08	20.31 ± 1.12	30.54 ± 1.30	59.80 ± 2.57
	Total	Total contents per plant			39.89 ± 2.08	Total yields per plant			279.77 ± 11.35
Liuba	Rhizome	6.97 ± 0.25	26.07 ± 1.26	21.49 ± 0.88	54.53 ± 2.63	4.80 ± 0.22	17.96 ± 0.71	14.80 ± 0.61	37.56 ± 1.81
	OM	2.88 ± 0.11	14.87 ± 0.71	15.91 ± 0.72	33.66 ± 1.38	15.86 ± 0.74	81.94 ± 3.71	87.67 ± 3.36	185.47 ± 7.32
	IM	0.66 ± 0.03	4.54 ± 0.21	7.59 ± 0.21	12.76 ± 0.64	2.16 ± 0.11	15.64 ± 0.72	26.14 ± 1.27	43.94 ± 1.79
	Hair root	4.76 ± 0.13	23.56 ± 1.20	30.13 ± 1.10	58.45 ± 2.12	6.01 ± 0.30	29.75 ± 1.32	38.04 ± 1.31	73.81 ± 2.25
	Total	Total contents per plant			30.07 ± 1.55	Total yields per plant			340.77 ± 14.60

Outer tissue of main roots (OM) including periderm and phloem; inner tissue of main roots (IM) including xylem and pith; total contents/yields per plant is the sum of three individual ginsenosides contents/yields (Rg1 + Re + Rb1) according to their weight coefficient of biomass in the four parts (rhizomes + outer tissue of main roots + inner tissue of main roots + hair roots) of the roots.

contents. In addition, both July maximum temperature (x_3) and July mean temperature (x_4) positively correlated with contents, and January mean temperature (x_2) is negatively correlated with contents. In the case of yields, the biggest influences on ginsenosides were July maximum temperature (x_3) and July mean temperature (x_4); both presented positive correlation with yields, while the minimum influences on ginsenosides were active cumulative temperature (x_5) but presented negative correlation with yields. In addition, January minimum temperature (x_1) positively, whereas January mean temperature (x_2) negatively correlated with yields. Interestingly, the annual mean temperature (x_6) positively correlated with the total ginsenosides, whereas Re and Rb1 yields negatively correlated with Rg1 yields.

3.3. Effect of temperature treatments on ginsenoside accumulation

The contents of three ginsenosides in leaves, stems, and roots at different growing stages of American ginseng were shown in Table 3. Results presented similarities not only in ginsenoside organ-specific distribution but also in fluctuation

Table 2
Multivariate linear regression equation between the temperature factors and the ginsenosides in roots of 3-year-old American ginseng collected from different cultivation sites of China

Parameters		Multivariate linear regression equation
Contents	Rg1	$Y = 0.180 - 0.068\,x_1 + 0.013\,x_3 + 0.001\,x_5$ ($F = 67.958 > F0.05$, $R^2 = 0.936$)
		$Y = 2.178 - 0.039\,x_2 + 0.010\,x_4 - 0.070\,x_6$ ($F = 43.391 > F0.05$, $R^2 = 0.903$)
	Re	$Y = -3.833 - 0.425\,x_1 + 0.038\,x_3 + 0.001\,x_5$ ($F = 105.257 > F0.05$, $R^2 = 0.958$)
		$Y = 15.993 - 0.231\,x_2 + 0.018\,x_4 - 0.452\,x_6$ ($F = 67.899 > F0.05$, $R^2 = 0.936$)
	Rb1	$Y = 12.467 - 0.278\,x_1 + 0.118\,x_3 + 0.001\,x_5$ ($F = 66.231 > F0.05$, $R^2 = 0.934$)
		$Y = 18.373 - 0.129\,x_2 + 0.245\,x_4 - 0.481\,x_6$ ($F = 50.067 > F0.05$, $R^2 = 0.915$)
	Total	$Y = 29.600 - 0.328\,x_1 + 0.076\,x_3 + 0.001\,x_5$ ($F = 77.5 > F0.05$, $R^2 = 0.943$)
		$Y = 30.728 - 0.069\,x_2 + 0.606\,x_4 - 1.185\,x_6$ ($F = 81.007 > F0.05$, $R^2 = 0.946$)
Yields	Rg1	$Y = -2.393 + 0.046\,x_1 + 0.664\,x_3 - 0.001\,x_5$ ($F = 50.983 > F0.05$, $R^2 = 0.916$)
		$Y = -6.63 - 0.169\,x_2 + 0.969\,x_4 - 0.097\,x_6$ ($F = 22.261 > F0.05$, $R^2 = 0.827$)
	Re	$Y = -5.014 + 0.486\,x_1 + 4.220\,x_3 - 0.004\,x_5$ ($F = 11.475 > F0.05$, $R^2 = 0.711$)
		$Y = -34.048 - 1.198\,x_2 + 5.863\,x_4 + 0.104\,x_6$ ($F = 8.461 > F0.05$, $R^2 = 0.645$)
	Rb1	$Y = -12.438 + 3.031\,x_1 + 7.801\,x_3 - 0.009\,x_5$ ($F = 66.320 > F0.05$, $R^2 = 0.934$)
		$Y = -113.998 - 1.250\,x_2 + 10.505\,x_4 + 3.479\,x_6$ ($F = 34.824 > F0.05$, $R^2 = 0.882$)
	Total	$Y = 49.776 + 5.980\,x_1 + 11.949\,x_3 - 0.022\,x_5$ ($F = 70.684 > F0.05$, $R^2 = 0.938$)
		$Y = -185.492 + 0.259\,x_2 + 19.119\,x_4 + 2.362\,x_6$ ($F = 34.349 > F0.05$, $R^2 = 0.880$)

January minimum temperature (x_1); January mean temperature (x_2); July maximum temperature (x_3); July mean temperature (x_4); Active cumulative temperature (x_5); Annual mean temperature (x_6). Data of temperature factors in different cultivation sites were collected from National Meteorological Information Center of China (data did not all show).

Table 3
Comparison of the contents of ginsenosides (mg/g) in the leaves, stems, and roots at different growing stages of 3-year-old American ginseng between CE-N and CE-3

Plant tissues and growing stages		CE-N				CE-3			
		Rg1	Re	Rb1	Total	Rg1	Re	Rb1	Total
Leaves	Early leaf stage	5.78 ± 0.25	12.21 ± 0.41	10.89 ± 0.31	28.88 ± 0.94	3.83 ± 0.17	10.80 ± 0.41	5.78 ± 0.27	20.42 ± 0.89
	Opened leaf stage	5.98 ± 0.23	9.90 ± 0.32	8.97 ± 0.38	24.85 ± 1.08	4.19 ± 0.21	8.92 ± 0.34	6.03 ± 0.22	19.14 ± 0.57
	Flowering stage	6.60 ± 0.28	15.87 ± 0.76	9.32 ± 0.43	31.79 ± 1.12	4.70 ± 0.21	11.09 ± 0.46	7.87 ± 0.31	23.67 ± 1.17
	Green berry stage	3.82 ± 0.13	18.06 ± 0.77	13.34 ± 0.39	35.23 ± 1.61	2.19 ± 0.09	15.07 ± 0.55	8.79 ± 0.23	26.06 ± 1.28
	Red berry stage	2.09 ± 0.07	22.02 ± 0.98	6.91 ± 0.33	31.02 ± 1.15	1.96 ± 0.24	15.01 ± 0.61	5.70 ± 0.27	22.68 ± 0.94
	Yellow leaf stage	1.04 ± 0.04	10.09 ± 0.23	6.89 ± 0.27	18.02 ± 0.61	0.85 ± 0.05	9.32 ± 0.32	2.68 ± 0.19	12.85 ± 0.59
Stems	Early leaf stage	0.33 ± 0.02	4.08 ± 0.15	4.31 ± 0.22	8.73 ± 0.42	0.33 ± 0.07	3.06 ± 0.17	3.10 ± 0.16	6.50 ± 0.41
	Opened leaf stage	0.46 ± 0.03	4.79 ± 0.19	3.15 ± 0.17	8.41 ± 0.23	0.31 ± 0.09	3.24 ± 0.13	2.07 ± 0.11	5.63 ± 0.28
	Flowering stage	0.43 ± 0.14	3.66 ± 0.13	3.34 ± 0.14	7.43 ± 0.31	0.20 ± 0.02	2.92 ± 0.16	2.21 ± 0.11	5.34 ± 0.31
	Green berry stage	0.32 ± 0.09	5.19 ± 0.41	4.32 ± 0.21	9.74 ± 0.21	0.25 ± 0.03	3.36 ± 0.18	2.29 ± 0.15	5.92 ± 0.31
	Red berry stage	0.35 ± 0.05	5.57 ± 0.27	5.01 ± 0.13	10.94 ± 0.41	0.17 ± 0.01	3.95 ± 0.22	2.38 ± 0.17	6.52 ± 0.34
	Yellow leaf stage	0.27 ± 0.08	4.78 ± 0.32	1.65 ± 0.07	6.71 ± 0.31	0.14 ± 0.01	2.46 ± 0.21	1.39 ± 0.11	4.00 ± 0.25
Roots	Early leaf stage	0.71 ± 0.01	8.74 ± 0.28	14.37 ± 0.55	23.83 ± 1.52	0.71 ± 0.02	7.70 ± 0.34	10.21 ± 0.46	18.63 ± 0.82
	Opened leaf stage	0.99 ± 0.11	8.13 ± 0.31	12.09 ± 0.51	21.22 ± 0.95	0.67 ± 0.01	6.37 ± 0.32	9.71 ± 0.51	16.76 ± 0.53
	Flowering stage	0.92 ± 0.05	8.20 ± 0.41	12.22 ± 0.51	22.34 ± 1.29	0.67 ± 0.05	6.90 ± 0.21	9.38 ± 0.42	16.96 ± 0.61
	Green berry stage	1.38 ± 0.05	8.97 ± 0.22	16.81 ± 0.65	27.04 ± 1.25	0.98 ± 0.09	5.99 ± 0.25	9.97 ± 0.34	16.94 ± 0.58
	Red berry stage	1.40 ± 0.08	11.07 ± 0.38	17.73 ± 0.42	30.21 ± 1.41	1.23 ± 0.07	6.33 ± 0.26	10.03 ± 0.44	17.60 ± 0.64
	Yellow leaf stage	1.60 ± 0.11	11.43 ± 0.24	19.63 ± 0.96	32.68 ± 1.29	0.96 ± 0.04	7.83 ± 0.25	12.53 ± 0.38	21.33 ± 0.96

CE-N, cold exposure naturally; CE-3, cold exposure 3 months.

patterns at different growing stages. Rb1 is the main ginsenoside in roots, and it increased to the highest level during the yellow leaf stage. Re is the main ginsenoside in leaves, and it increased to the highest level during flowering stage to the red berry stage and then reduced. Rg1 is relatively fewer both in roots and leaves, whereas it increased to a relatively higher level during flowering stage and then decreased significantly in leaves while it increased gradually to a relatively higher level in roots during the yellow leaf stage. In addition, the three ginsenosides in stems present dramatically lower contents when compared with other two parts, and they fluctuated slightly with the growing stages which were similar to the trend in leaves. However, results also presented significant difference in fluctuation range of ginsenosides contents between CE-N and CE-3, which indicated that insufficient cold treatments resulted in the decline of ginsenoside contents. In addition, always there was a dropping process of ginsenoside contents in leaves (usually Rg1 from flowering stages to yellow leaf stages and Re and Rb1 from red berry stages to yellow leaf stages) while a rising process of ginsenosides contents in roots (usually all the three ginsenosides from flowering stages to yellow leaf stages), which indicated that the leaves were the main biosynthetic organs while the roots were the main storage organ of ginsenosides, and ginsenosides were transported between them.

The ginsenosides yields in leaves, stems, and roots at different developing stages of American ginseng between CE-N and CE-3 were shown in Table 4. Data analysis indicated a more significant difference in the ginsenosides yields than that in the ginsenosides contents between CE-N and CE-3, especially in the roots. These results indicated that cold temperature not only increased the ginsenosides contents but also increased the ginsenosides yields more significantly. In addition, we noticed that the ginsenosides yields increased continuously, whereas the ginsenosides contents decreased in leaves, stems, and roots during the opened leaf stage, which might be related to the quickly growing of plant during this stage.

3.4. Effect of temperature treatments on DNA methylation

Results showed that the DNA methylation gradually increased with the plant developmental stages both in leaves and in hair roots of American ginseng (Table 5). However, there was a significant difference between CE-N and CE-3. Under CE-N, the variation of DNA methylation ranged from 12.63% to 24.23% in leaves and 13.93% to 22.70% in hair roots, whereas under CE-3, they are from 15.78% to 22.12% in leaves and 14.65% to 20.57% in hair roots. Obviously, different cold temperature treatments resulted in different DNA methylation, which indicated insufficient cold temperature treatments might cause abnormal DNA methylation and then affect the plant growth and development as well as the biosynthesis of plant secondary metabolites including ginsenosides.

3.5. Correlation between ginsenosides and DNA methylation

As shown in Table 6, DNA methylation in the rhizomes during dormant season presented an increasing, unchanging, decreasing patterns, which might be related to the temperature changing patterns: autumn cooling, winter cold, spring warming. When rhizomes sprouted into seedlings, DNA methylation in the early leaves presented a further decreasing. Most notably, different cold exposure duration resulted in different DNA methylation levels in the early leaves and presented a significant negative correlation between them. Meanwhile, different cold exposure duration resulted in different ginsenosides yields in the final roots but presented a significant positive correlation between them. These indicated a close relationship among cold temperature, DNA methylation, and ginsenoside yields in American ginseng. In addition, we also noticed the plant ontogeny was seriously affected by cold treatments, and the shorter the cold exposures, the more serious they were affected. Results presented that insufficient cold exposures caused early sprouting, early flowering, early fruiting, and premature senility of above-ground organs, and in too short cold exposures, very few plants sprouted but died rapidly; even no cold exposure during autumn and winter resulted in no sprouted in spring. These indicated a close relationship between the cold temperature and the plant ontogeny in American ginseng.

3.6. Effect of temperature treatments on ginsenoside-related genes expression levels

PqDDS (the dammarenediol synthase gene in *P. quinquefolius*) encodes the first committed enzyme in the ginsenoside biosynthetic pathway for dammarane-type ginsenosides, and PqFT [the

Table 4
Comparison of the yields of ginsenosides (mg) in the leaves, stems, and roots at different growing stages of 3-year-old American ginseng between CE-N and CE-3

Plant tissues and growing stages		CE-N				CE-3			
		Rg1	Re	Rb1	Total	Rg1	Re	Rb1	Total
Leaves	Early leaf stage	7.63 ± 0.21	16.12 ± 0.68	14.38 ± 0.42	38.14 ± 1.23	3.39 ± 0.11	9.56 ± 0.41	5.12 ± 0.25	18.08 ± 1.02
	Opened leaf stage	10.13 ± 0.24	16.77 ± 0.31	15.19 ± 0.64	42.10 ± 2.19	6.39 ± 0.38	13.61 ± 0.66	9.21 ± 0.23	29.21 ± 1.54
	Flowering stage	18.55 ± 0.77	44.59 ± 1.67	26.11 ± 1.02	89.34 ± 3.34	8.25 ± 0.22	19.47 ± 0.95	13.81 ± 0.60	41.55 ± 2.11
	Green berry stage	10.86 ± 0.32	51.38 ± 1.95	37.95 ± 1.24	100.20 ± 3.76	4.63 ± 0.31	31.79 ± 1.31	18.53 ± 0.79	54.96 ± 2.68
	Red berry stage	6.16 ± 0.40	64.93 ± 2.20	20.39 ± 0.75	91.49 ± 3.68	5.20 ± 0.22	39.82 ± 1.51	15.14 ± 0.31	60.16 ± 2.66
	Yellow leaf stage	3.00 ± 0.17	28.94 ± 1.37	19.76 ± 0.83	51.71 ± 2.55	2.05 ± 0.30	22.44 ± 0.98	6.45 ± 0.28	30.94 ± 1.11
Stems	Early leaf stage	0.35 ± 0.09	4.32 ± 0.21	4.56 ± 0.31	9.24 ± 0.32	0.29 ± 0.02	2.69 ± 0.04	2.72 ± 0.03	5.71 ± 0.22
	Opened leaf stage	0.55 ± 0.02	5.66 ± 0.13	3.71 ± 0.08	9.92 ± 0.91	0.33 ± 0.04	3.45 ± 0.19	2.20 ± 0.17	5.99 ± 0.39
	Flowering stage	0.62 ± 0.06	5.30 ± 0.15	4.83 ± 0.21	10.76 ± 0.41	0.23 ± 0.01	3.32 ± 0.03	2.52 ± 0.11	6.08 ± 0.24
	Green berry stage	0.47 ± 0.01	7.61 ± 0.33	6.35 ± 0.21	14.30 ± 0.49	0.32 ± 0.01	4.22 ± 0.17	2.88 ± 0.09	7.43 ± 0.27
	Red berry stage	0.54 ± 0.07	8.50 ± 0.36	7.65 ± 0.14	16.70 ± 0.69	0.23 ± 0.05	5.13 ± 0.08	3.08 ± 0.13	8.46 ± 0.34
	Yellow leaf stage	0.39 ± 0.08	5.73 ± 0.21	3.15 ± 0.13	9.28 ± 0.58	0.18 ± 0.03	3.09 ± 0.17	1.71 ± 0.14	4.99 ± 0.28
Roots	Early leaf stage	5.30 ± 0.13	58.98 ± 2.15	91.91 ± 4.27	156.20 ± 5.28	4.70 ± 0.21	39.70 ± 1.79	66.80 ± 3.77	111.21 ± 4.23
	Opened leaf stage	7.92 ± 0.24	64.92 ± 2.38	96.58 ± 3.79	169.47 ± 8.11	4.82 ± 0.25	43.28 ± 2.01	66.08 ± 2.71	114.19 ± 4.76
	Flowering stage	7.56 ± 0.27	64.94 ± 2.76	106.84 ± 3.91	179.35 ± 6.78	4.95 ± 0.27	45.40 ± 1.81	68.83 ± 3.89	119.19 ± 5.75
	Green berry stage	11.60 ± 0.37	75.10 ± 2.23	140.75 ± 3.99	226.42 ± 8.01	6.49 ± 0.25	47.48 ± 2.01	69.24 ± 2.81	123.22 ± 4.69
	Red berry stage	12.28 ± 0.22	96.83 ± 3.69	154.98 ± 6.51	264.10 ± 9.02	9.28 ± 0.31	50.42 ± 1.91	75.22 ± 3.03	134.93 ± 4.63
	Yellow leaf stage	15.33 ± 0.32	108.92 ± 2.98	187.13 ± 4.71	311.38 ± 12.33	7.62 ± 0.27	62.05 ± 3.11	99.28 ± 3.77	168.95 ± 9.79

CE-N, cold exposure naturally; CE-3, cold exposure 3 months.

Flowering Locus T (FT) homologous gene in *P. quinquefolius*] encodes a small mobile protein in leaves and transmits to the shoot apex for mediating the onset of flowering. As shown in Fig. 1, the plant developmental stages significantly affected expression levels of these two genes in all samples. The PqDDS expression level changed with the plant developmental stages and dramatically increased from flowering stage to the red berry stage and then gradually decreased at the yellow leaf stage; it reached the highest level at the green berry stage. The PqFT expression level also changed with the plant developmental stages, but only detected from the opened leaf stage to the green berry stage, it dramatically increased to the highest level at the flowering stage. However, compared between CE-3 and CE-N, these two genes' expression level were significantly different, especially the expression levels of PqDDS at the green berry stage and PqFT at the flowering stage under CE-N were enhanced 1.17-fold and 1.01-fold, respectively, compared with those under CE-3. In addition, correlation analysis exhibited a highly significant correlation between the two genes, and the two genes correlated significantly with the ginsenosides in leaves and roots ($P \leq 0.01$). The results indicate that cold-induced ginsenoside changes might be involved in not only plant secondary metabolism but also plant development process.

Table 5
Comparison of overall DNA methylation levels in the leaves and hair roots at different growing stages of 3-year-old American ginseng between CE-N and CE-3

Plant tissues and growing stages		CE-N	CE-3
		5-methylcytosine (%)	5-methylcytosine (%)
Leaves	Early leaf stage	12.63 ± 0.45	15.78 ± 0.42
	Opened leaf stage	15.21 ± 0.55	17.32 ± 0.43
	Flowering stage	17.02 ± 0.37	19.23 ± 0.31
	Green berry stage	18.98 ± 0.68	19.41 ± 0.59
	Red berry stage	21.75 ± 0.61	20.75 ± 0.52
	Yellow leaf stage	24.23 ± 0.64	22.12 ± 0.57
Hair roots	Early leaf stage	13.93 ± 0.38	14.65 ± 0.45
	Opened leaf stage	16.45 ± 0.43	15.77 ± 0.37
	Flowering stage	18.25 ± 0.42	17.39 ± 0.28
	Green berry stage	18.97 ± 0.61	18.09 ± 0.55
	Red berry stage	20.67 ± 0.66	19.98 ± 0.49
	Yellow leaf stage	22.70 ± 0.39	20.57 ± 0.47

CE-N, cold exposure naturally; CE-3, cold exposure 3 months.

4. Discussion

The quality of a medicinal plant is inescapably affected by abiotic and biotic factors in the wild habitat or the cultivation site. Plants belonging to the same species but growing in different geographical zones may differ significantly in their secondary metabolites, both qualitatively and quantitatively. This phenomenon is observed in many saponin-containing plants including American ginseng and Asian ginseng [2]. These two species in the same genus *Panax* of Araliaceae family have been originally found in the habitats of Northeast America and Northeast Asia, respectively, where cold temperature prevails in winter [6,7]. Therefore, one naturally speculates that the ginsenosides with diverse structures in these ginseng species might have a close relationship with cold environment. Indeed, different cultivation sites with different temperature conditions have different ginsenoside accumulation in Asian ginseng [10–12] and American ginseng [13–15]. In the present study, results indicated that environmental temperatures have significant impact on ginsenoside biosynthesis because the variation in contents and yields of ginsenosides of American ginseng is closely related to the temperature difference in different sites (Tables 1 and 2). In some sites (cluster A), American ginseng plants might adapt themselves better than other site (cluster B); probably the temperature conditions in such site favor ginsenoside accumulating massively.

Interestingly, as for the effect of temperature on the ginsenosides accumulation in Asian and American ginseng, completely opposite results were reported. Some deduced that the cold temperature were the major factor promoting the ginsenosides biosynthesis [10–12,14], while others concluded that the warm temperature played important roles in it [13,15]. In the present study, multivariate linear regression analysis between temperature factors and the ginsenosides accumulation were determined deliberately (Table 2). These results were partially consistent with that of Wu et al. [13] and Huang et al. [14], who both reported that temperature factors played important roles in the ginsenosides accumulation of American ginseng. These results further indicated that the contents of ginsenosides were significantly increased by cold temperature factors while the yields of ginsenosides were significantly increased by warm temperature factors, implying the presentations between contents and yields of ginsenosides were not completely synchronized (Tables 1 and 2). So we hypothesized

Table 6
Change of DNA methylation, ginsenosides yields and plant ontogeny characteristics after different cold exposure durations

Cold exposure duration	Rhizomes	Early leaves	Final roots	Plant ontogeny characteristics
	5-methylcytosine (%)	5-methylcytosine (%)	Ginsenosides (mg)	
No cold exposure (CE-0)	17.16 ± 0.39	–	–	No plants sprouted
Nov (CE-1)	19.77 ± 0.44	17.88 ± 0.36	–	A very few plants sprouted but died rapidly
Nov to Dec (CE-2)	23.25 ± 0.71	17.09 ± 0.47	90.79 ± 4.09	Partial plants sprouted but prematured early
Nov to Jan (CE-3)	23.38 ± 0.68	15.78 ± 0.22	168.95 ± 9.79	Most plant sprouted but prematured early
Nov to Feb (CE-4)	23.15 ± 0.77	14.27 ± 0.42	188.75 ± 11.41	Most plant sprouted but prematured early
Natural temperature (CE-N)	17.34 ± 0.56	12.63 ± 0.31	311.38 ± 12.33	Normal sprouted, flowered and fruited

Rhizomes are sampled at the end of Oct (CE-0), Nov (CE-1), Dec (CE-2), Jan (CE-3), Feb (CE-4), Mar (CE-N); early leaves are sampled at the fifth day after seedling sprouted; final roots are sampled at the final plant harvest day on 8 October 2017.

that these results can be explained that there might be a switching on/off mechanism of ginsenosides accumulation of American ginseng in response to cold stimuli, because the experience of cold temperature is a necessary process, whereas warm temperature is beneficial to accumulate ginsenosides in American ginseng. As for the increasing of ginsenosides yields caused by relatively higher temperature, it must be with a cold temperature experience as the prerequisite. These enlightenments might explain the result reported by Jochum et al. [15], as the plant material of American ginseng in that experiment had experienced cold temperature in the last winter.

To further verify the temperature effect on the ginsenosides accumulation, a series of cold exposure duration treatments on American ginseng plants during their dormant season were carried out, and then the contents and yields of three ginsenosides in different organs during different development stages were determined. Results further proved that cold temperature treatments did affect the ginsenosides accumulation significantly (Tables 3 and 4). Most noteworthy, compared between CE-N and CE-3, the measured parameters of individual and total ginsenosides presented significant difference in roots, stems, and leaves, which implied that it is the different cold exposure duration that led to the different ginsenosides biosynthesis in leaves and the different ginsenosides storage in roots, and sufficient cold exposure duration could increase the total ginsenosides yields of American ginseng (Tables 3 and 4).

To investigate the mechanism of ginsenosides accumulation, we first determined the overall DNA methylation changes in American ginseng (Tables 5 and 6). Dynamic changes of DNA methylation in response to low temperature represent a potentially mechanism able to regulate gene expression in both annual and perennial plants. DNA methylation changes induced by vernalization promoting flowering have been extensively studied in some overwintering annual plants [19–21]. However, the role of DNA methylation in overwintering perennial plants with different lifestyles is still obscure because of the absence of study, so alternative regulatory mechanisms are expected to occur in perennial plants. In the present investigation, our results presented a cyclically reversible dynamism between methylation and demethylation of DNA in American ginseng in response to temperature seasonality. Especially, we found that DNA methylation levels in the rhizomes of American ginseng during dormant season presented an increasing, unchanging, decreasing patterns. This indicated that cooling in autumn could cause DNA hypermethylation, whereas warming in spring could cause DNA hypomethylation; these might be totally different with that in many annual plant species as many results showed that cold temperature resulted in hypomethylation extensively in many annual plant species [3–5,19–21]. We also noticed the DNA methylation dynamics during major developmental transitions of American ginseng that is a decreasing from rhizome to seedling but an increasing from juvenile to adult. Compared with naturally sufficient cold exposure duration (CE-N), artificially shortening cold exposure duration (CE-1, 2, 3, 4) might cause insufficient DNA demethylation during early leaf stages from rhizome to seedling and then cause insufficient DNA methylation from juvenile to adult (such as CE-3). Corresponding to the different DNA methylation dynamics between different cold exposure duration treatments, the final total ginsenosides yields in roots presented significant difference, and data analysis indicated that DNA hypomethylation in early leaf stage after sufficient cold exposure durations (CE-N) were correlated best with the highest final total ginsenosides yields in the roots. In this study, we observed plant developmental abnormalities including not sprouted, advanced sprouted, advanced flowering, advanced senescence, advanced died, and immature seeds in American ginseng after artificially shortened the cold exposure duration. Therefore, we

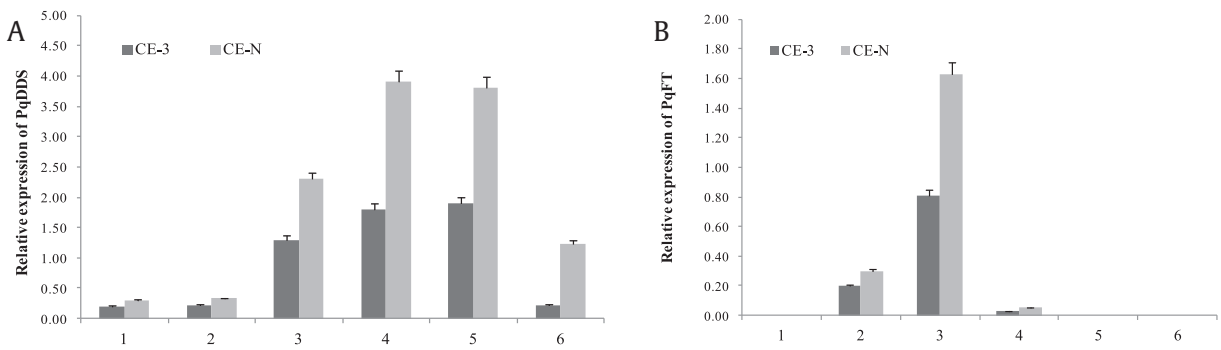

Fig. 1. Comparison of relative expression levels. (A) PqDDS genes and (B) PqFT genes in leaves during different developmental stages of 3-year-old American ginseng between CE-3 and CE-N. 1, early leaf stage; 2, opened leaf stage; 3, flowering stage; 4, green berry stage; 5, red berry stage; 6, yellow leaf stage. CE-N, cold exposure naturally; CE-3, cold exposure 3 months. Vertical bars indicate the mean ± standard error from three independent experiments.

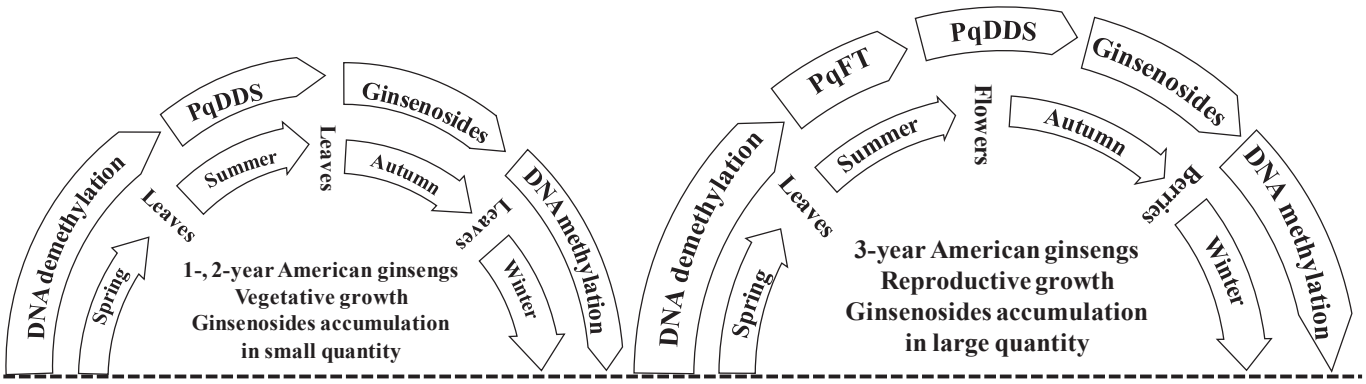

Fig. 2. Schematic representation of proposed mechanism of cold-induced ginsenoside accumulation in perennial American ginseng. Along with the seasonal changes in temperature, the 1-year-old to 2-year-old American ginseng, which are at the stage of plant vegetative growth, experience DNA demethylation, a lower level expression of PqDDS, a small quantity of ginsenosides accumulation and then plant aerial parts wilted and remaining roots entered a winter dormant state with DNA methylation increasing. However, the 3-year-old American ginseng, which is at the stage of plant reproductive growth, experience DNA demethylation, a higher levels expression of PqFT, a higher levels expression of PqDDS, a large quantity of ginsenosides accumulation and then plant aerial parts wilted, and remaining roots entered a winter dormant state with DNA methylation increasing. The cold temperature in winter plays a decisive role in both plant development and ginsenoside accumulation of American ginseng.

speculated that the cold-dependent American ginseng might regulate both plant development and plant secondary metabolism simultaneously by altering DNA methylation state of relative genes.

To further study the molecular mechanism of ginsenoside biosynthesis, we analyzed the expression levels of two ginsenoside-relative genes (PqFT and PqDDS) in American ginseng after different cold exposure duration treatments (Fig. 1). Abiotic stress resistance is mostly quantitative trait with its continuous variation influenced by genotype-environment interaction and has been considered as a polygenic character. Usually, DNA methylation level has overall effect on plant genome. Therefore, change in DNA methylation level is not targeting a certain gene but a set of polygenes; probably many genes may be affected simultaneously and hence increasing the variation in quantitative traits. American ginseng is a polycarpic perennial which can preserve some meristems in the vegetative state overwinter to sustain alive in subsequent years and produce flowers and fruits in its third year and later. This feature probably requires more complex mechanisms to regulate different time-dependent and tissue-specific genes and keep acclimatization to respond to cyclical cold stimuli, which is likely by cold-induced DNA methylation modified dynamically. Plant development is regulated by specific plant hormones, and DNA methylation modulation is one of the molecular mechanisms of phytohormone action in plants. Many studies have proved that vernalization in many cold-dependent plant species represses Flowering Locus C and thus allows the expression of FT. FT is a major component of the florigenic mobile signal produced in the leaves in response to inductive cold temperature and photoperiods and then translocated to the shoot apical meristem, where it promotes the conversion from vegetative to reproductive meristem. The ginsenosides are mainly biosynthesized utilizing the precursor isopentenyl diphosphate and dimethylallyl diphosphate through the mevalonic acid pathway and further including genes encoding farnesyl diphosphate synthase, squalene synthase, squalene epoxidase, dammaranediol-II synthase, and so on. The dammaranediol-II synthase (DDS) transforms 2,3-oxidosqualene to dammarenediol-II as an essential substrate for the biosynthesis of PPT- and PPD-type ginsenosides, which is the key rate-limiting enzymes in the ginsenoside biosynthetic pathway. Comprehensively, we infer that the ability of cold induced DNA methylation to regulate the floral transition and the ginsenosides accumulation in American ginseng is age dependent and stage dependent (Fig. 2). Plants less than 2 years old neither flowered nor accumulated ginsenosides in larger quantities until they develop axillary vegetative shoots and sufficient biomass in its third year and later. The expression of the flowering gene such as PqFT and the ginsenoside biosynthesis gene such as PqDDS might be repressed by DNA methylation in 1-year-old and 2-year-old plants (data did not show). However, as to 3-year-old plants, sufficient cold exposure might induce sufficient DNA demethylation with tissue-specific and stage-specific way and result in an increase of relative gene expression including PqFT and PqDDS. We found that the highest expression of the two relative genes was in sequence: PqFT in the flowering stage and then PqDDS in the green berry stage, which was concomitant with a significant increase in ginsenosides. This finding is partially consistent with the results in Asian ginseng previously reported by Liu et al. (2016) [10] and Kim et al. (2012) [22]. We proposed a hypothesis that the ginsenosides accumulation and the development process were regulated by cold-induced DNA methylation and might be closely related to each other in American ginseng. But up to now, few studies have focused on the relationship between plant secondary metabolism and plant development process in such plants. Further studies are necessary to disclose the detail mechanism of quality formation in this important medicinal plant.

Conflicts of interest

All authors have no conflicts of interest to declare.

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