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Variation of anthocyanin content in fruits of wild and cultivated *Lycium ruthenicum*

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ABSTRACT

Lycium ruthenicum Murray (LR) is an important medicinal and edible fruit tree in Northwest China and contains a large number of anthocyanins in ripe fruits. To search for content of the anthocyanins, petunidin-3-O-rutinoside (trans-p-coumaroyl)-5-O-glucoside (PRG) and anthocyanidin (delphinidin, petunidin and malvidin) in fruits of 423 LR samples were determined and analyzed quantificationally by high-performance liquid chromatography. There was a great variation in fruits total anthocyanins content (PRG and anthocyanidins) among LR germplasm. A total of ten significant correlations between anthocyanins and seven ecogeographic factors were detected by bivariate analysis ($p < 0.05$). The content of PRG was slightly lower in cultivated fruits than that in wild fruits, but the cultivated fruits contained more anthocyanidins than wild fruits. Anthocyanins contents of LR fruits were positively correlated with fruit weight and diameter. Therefore, the difference in anthocyanins concentration between the cultivated and wild fruits may be attributed to the indirectly consequence of human selection for heavier fruits and bigger fruit size during LR domestication. Additionally, PRG content was relatively low in LR fruit at the green fruit stage, but significantly increased during fruit development and maturation. The PRG accumulation could be regulated and controlled by *LrAN2* in LR fruit, and the content of PRG was directly proportional to the expression level of *LrAN2*, which suggested a feedback regulation mechanism in PRG-related gene expression. In a word, the result of this work could provide reliable data on the further study and utilization of LR.

1. Introduction

Anthocyanin and anthocyanidin are flavonoids in vegetables and fruits that render them greyish-green to red or purple-black (He and Giusti, 2010). To date, 635 natural anthocyanins have been discovered, and various anthocyanidins are more commonly glycosylated and acylated (Andersen and Jordheim, 2008). About 95 % of the anthocyanins found in nature are derived from the following 6 types of anthocyanidins (aglycones): malvidin, peonidin, pelargonidin, cyanidin, petunidin and petunidin (Andersen and Jordheim, 2006; Castañeda-Ovando et al., 2009). In recent years, a large number of reports show that anthocyanins have anti-inflammatory effect (Seeram et al., 2001; Rossi et al., 2003), anti-tumor (Jing et al., 2008), neuroprotective effect (Chen et al., 2019), obesity control (Tsuda, 2008), cardiovascular disease prevention (Renaud and Lorgeril, 1992; Liobikas et al., 2016), gouty arthritis protection (Zhang et al., 2019), and diabetes alleviation properties (Ghosh and Konishi, 2007). All of these activities could be attributed to antioxidant property of anthocyanins. Additionally, food products contained abundant anthocyanins are increasingly prevalent owing to their attractive colors and the suggested benefits for human health (Pojer et al., 2013).

Lycium ruthenicum (LR) is a medicinal and edible fruit tree highlighted in the classical “The Four Medical Tantras” “Compendium of Materia Medica”. The whole plant of LR has been used for the treatment of abnormal menstruation, menopause, hypertension, and eye disease for thousands of years in China (Wang et al., 2018a). Interestingly, many studies have found that anthocyanin-rich black fruit of LR is the distinctness characteristically different to other *Lyciums*, and therefore

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Table 1
Sampling details of *L. ruthenicum* collections in the present study in Northwest China[a].

No.	Locality	Code	Lt[b] (N)	Ln[c] (E)	Al[d] (m)	AMT[e] (°C)	AMP[f] (mm)	AMH[g] (%)	AMSH[h] (h)
1	Xiangride, Qinghai	XRD	36.01	97.89	3070	3.8	166.8	35.6	3050
2	Nuomuhong, Qinghai	NMH	36.43	96.25	2775	4.4	150.9	33.0	3094
3	KeluKehu, Qinghai	KLKH	37.29	96.86	2814	3.6	178.3	35.6	3170
4	Geermu, Qinghai	GEM	36.46	94.90	2773	5.3	42.2	31.9	3096
5	Dagele, Qinghai	DGL	36.27	95.45	2780	3.6	38.3	31.5	3090
6	Urt Moron, Qinghai	WTMR	36.88	93.13	2883	3.9	45.8	32.7	3100
7	Hongliugou, Xinjiang	HLG	39.14	89.98	2010	11.4	28.4	39.3	3087
8	Ruoqiang, Xinjiang	RQ	39.06	88.13	840	11.8	28.5	39.0	3103
9	Hetian, Xinjiang	HT	37.12	79.93	1430	12.5	35.4	42.6	2587
10	Kashgar, Xinjiang	KS	39.41	76.09	1262	11.9	64.0	43.0	2822
11	Alaer, Xinjiang	ALE	40.54	81.30	1078	10.7	52.0	51.9	2915
12	Yuli, Xinjiang	YL	41.29	86.25	887	10.1	43.2	44.9	2975
13	Turpan, Xinjiang	TUP	42.93	89.20	10	14.4	16.4	41.1	3040
14	Changji, Xinjiang	CJ	44.10	87.47	493	6.8	190.0	55.4	2700
15	Jinghe, Xinjiang	JH	44.64	82.85	274	7.6	102.0	56.7	2730
16	Dunhuang, Gansu	DH	40.11	94.63	1162	9.9	42.2	37.8	3220
17	Guazhou, Gansu	GZ	40.52	95.85	1178	8.8	45.3	40.2	3200
18	Jiayuguan, Gansu	JYG	39.73	98.18	1770	8.0	80.5	43.3	3031
19	Jinta, Gansu	JT	40.38	99.72	1150	9.6	43.5	43.0	3368
20	Shandan, Gansu	SD	38.76	101.06	1773	5.1	22.8	49.6	2823
21	Minqin, Gansu	MQ	38.64	103.07	1360	8.3	113.0	44.5	3073
22	EjinaQi, Inner Mongolia	EQ	42.02	101.06	920	8.6	37.0	34.3	3260
23	AlxaYouqi, Inner Mongolia	ALYQ	39.21	101.65	1480	8.4	89.0	37.5	3205
24	Alxa Zuoqi, Inner Mongolia	ALZQ	39.03	105.67	1419	7.4	136.3	41.8	3152
25	Bayan Nur, Inner Mongolia	BYN	40.85	107.22	1037	7.0	195.7	42.1	3170
26	Qingtongxia, Ningxia	QTX	38.05	105.79	1215	8.6	186.6	53.0	2906
27	Pingluo, Ningxia	PL	38.90	106.58	1100	8.4	173.2	54.6	3002

[a] The climatic data were collected from China Meteorological Data Sharing Service System, based on 1971 – 2000 year after year.
[b] Lt: latitude.
[c] Ln: longitude.
[d] Al: altitude.
[e] AMT: annual mean temperature.
[f] AMP: annual mean precipitation.
[g] AMH: annual mean humidity.
[h] AMSH: annual mean sunshine hours.

they have more important value and can be used for therapeutic or medical treatment (Zheng et al., 2011; Wang et al., 2018a). Furthermore, owing to its characteristic pleasing color, juiciness, sweetness, in addition to effective antioxidant activity, most ripe LR fruits can be consumed directly, and some are used to make functional nutritious foods or beverages (Hu et al., 2014; Wang et al., 2018b).

Generally, the structural genes for anthocyanin production included chalcone synthase (*CHS*), phenylalanine ammonia-lyase (*PAL*), chalcone isomerase (*CHI*), flavonoid-3′-hydroxylase (*F3'H*), flavonoid-3-hydroxylase (*F3H*), and flavonoid-3′,5′-hydroxylase (*F3'5'H*) (Heinzmann and Seitz, 1974; Forkmann and Kuhn, 1979; Shimada et al., 1999; Seitz et al., 2007; Deng et al., 2014; Shi and Hu, 2016). The regulatory functions of structural genes are mainly achieved through transcription factors, including V-myb avian myeloblastosis virus oncogene homolog (MYB) and basic Helix-Loop-Helix (bHLH) (Zhang et al., 2014). Our previous work had identified and analyzed the genes associated with anthocyanin biosynthesis in the LR fruits. And we found that *LrAN2*, as a MYB transcription factor, was expressed only in the black LR fruits (Zong et al., 2019a). In addition, overexpression of *LrAN2* caused visible anthocyanin accumulation in various parts of the tobacco plant. And the relative anthocyanin contents of roots, stems, leaves, flowers, and seeds were significantly higher in the transgenic lines than that of the wild-type (Zong et al., 2019b).

LR is an important economic fruit crop, mainly distributed in Central Asia, including the Caucasus and northwestern China. They usually grow in the environment of arid climates and saline-alkali soil, and possess anti-drought and salt-resistant characteristics (Liu et al., 2012). In recent years, the wild LR resources were decreasing dramatically, which could be caused by the rising price of wild LR fruits, destructive picking, and the deterioration of the ecological environment. As a result, the artificial cultivation of LR has been greatly developed at Qaidam and Tarim Basin. However, few researches related to the anthocyanin composition and content in cultivated LR fruits have been published, and there is no report for comparative assessment of anthocyanin concentration in wild and cultivated LR berries. In this paper, we report on the determination of anthocyanins content in ripe LR fruits from 27 regions, including cultivated and wild varieties in the northwest of China. And the difference in anthocyanin content of LR fruits was also analyzed and evaluated. The main purpose of our study is to clearly investigate the relationship among ecogeographic factor, fruit size, and genetic variation for fruit anthocyanin content in LR germplasm, which can enrich the database of anthocyanin nutrition in LR fruits. The results of this study will have a positive impact on the edible and medicinal value of LR and contribute to the development of novel LR anthocyanin-rich varieties.

2. Materials and methods

2.1. Chemicals and reagents

The high purity of the anthocyanin compound, petunidin-3-*O*-rutinoside (*trans*-*p*-coumaroyl)-5-*O*-glucoside (PRG), was successfully prepared from the LR fruits through extraction and purification by chromatography of macroporous resin, silica gel and reversed-phase C18 column. The spectral data obtained from the experimental results (Fig. S1-S3) are consistent with those of the previously reported PRG compounds (Tang et al., 2017; Jin et al., 2015a,b). Delphinidin (CAS: 528-53-0), petunidin (CAS: 1429-30-7) and malvidin (CAS: 643-84-5), were mainly purchased from Macklin China. The HPLC grade solvents for acetonitrile and methanol were purchased primarily from Xinlanjing

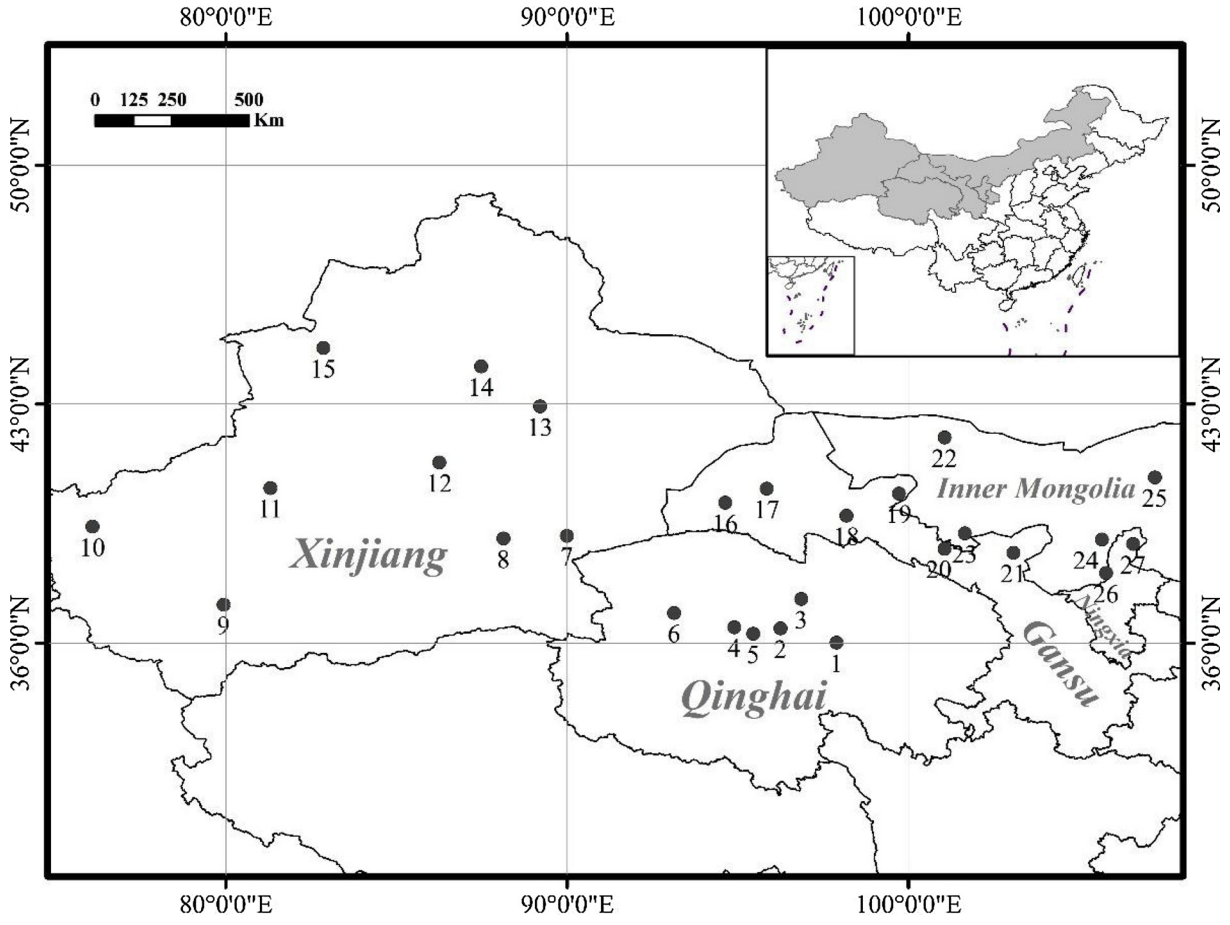

Fig. 1. Geographic distribution of different sampled positions of *L. ruthenicum* in Northwest China. For names of the numbered positions see list in Table 1.

Chemical Co., Ltd. (Yuxi, China). Formic acid, hydrochloric acid, and ethanol (anhydrous) were purchased at the analytical grade, primarily from Sigma-Aldrich. The preparation of HPLC grade water was carried out mainly by the Milli-Q system (Millipore, Billerica, MA, USA). RNAprep pure plant kits and TIANgel midi purification kits were from TianGen Biotech Co. (Beijing, China). Phushion DNA polymerase, plant genomic DNA extraction kits, the PCR products purification kits, and other biochemical reagents purchased from Thermo Fisher Scientifc Inc. (Shanghai, China).

2.2. Plant materials

In our study, a total of 423 LR accessions located at 27 areas were collected, which represent the natural populations of genus (LR) in China. The information of these samples was presented in Table 1 and Fig. 1. Of 423 accessions, 234 and 189 were cultivated and wild samples, respectively. Ripe fruits were randomly collected for evaluation of genotypic variation of anthocyanin content in fruits of LR germplasm in late August and early September 2017. When picking, it is judged whether fruit is mature or not based on the previous record and the color of the fruit. The deeper the color of the fruit, the more mature the fruit is. Fourteen fruit samples (7 cultivated, 7 wild) were collected at each distributed site, and all these ripe black fruits were manually picked and dried at a constant temperature of 45℃ for 72 h. After drying treatment, the fruit samples were stored at a temperature of -18℃ for later analysis in laboratory.

Furthermore, fresh LR cultivated fruit samples located at five provinces, KLKH (Qinghai), CJ (Xinjiang), DH (Gansu), EQ (Inner Mongolia), and PL (Ningxia), were selected randomly for assessment of the dynamic of anthocyanins accumulation during fruit growth, and the fruits materials from three developmental stages (juvenile, expanding and mature stage) were duly collected. The juvenile and expanding stages were corresponding to probably 30 and 75 d after full bloom (DAFB), respectively. The fresh fruit samples were collected and kept in liquid nitrogen immediately, then stored at -80℃ for further analysis, and the anthocyanin in these fruits were determined and analyzed by high-performance liquid chromatography (HPLC).

2.3. Extraction and measurement of anthocyanins

The processes of extraction and preparation of anthocyanins and anthocyanidins were performed according to Zhang et al. (2019) with some modifications. The dried fruit samples were milled into powder, and then sieved for use. And the fresh fruit samples were smashed in liquid nitrogen. Approximately 2.0 g of samples was extracted with 50 mL of 85 % ethanol using ultrasonic extraction method for 30 min at room temperature. After centrifugation at 5000 g for 20 min, the supernatants were poured out, and concentrated by a rotary evaporator at 50℃, and then the concentrated supernatants were subjected to lyophilization. Anthocyanins and anthocyanidins were enriched in AB-8 macroporous resins, and 60 % ethanol (0.5 % HCl) solution was used as eluent. After concentration and lyophilization, the corresponding anthocyanin extracts were obtained. Based on the extracts hydrolyzed in boiling-water bath (1.0 % hydrochloric acid) for 60 min, the anthocyanidin hydrolysate was collected, and then filtered by 0.22 μm membrane for HPLC determination.

The anthocyanins extracts were subjected for HPLC analysis using the SPD-M20A system (Shimadzu, Japan), equipped with a LC-20CE HPLC pump, a SIL-20AC autosampler, and CTO-20AC thermostatic column room. The determination was carried out using a Megres

analytical C18 column with an inner diameter of 5 μm and a cylinder of 250 × 4.6 m. 10 μL of the solution was injected in an aliquot. A chromatogram was obtained at 525 or 530 nm (Fig. S4), and for the photodiode array spectrum, effective recording was possible at 190 nm–800 nm. Analysis of anthocyanins and anthocyanidins can be performed efficiently by applying a gradient program. The eluents were A (2 % of formic acid in water) and B (2 % of formic acid in acetonitrile). The applied elution program for separation of anthocyanins was: 0 − 10 min, linear gradient from 7 % to 13 % B; 10 − 30 min, linear gradient from 13 % to 15 % B; 30 − 50 min, 15 % B isocratic. The flow rate was 1.0 mL/min, and temperature was at 35℃. Additionally, The gradient conditions for separation of anthocyanidins were as follows: 0 − 2 min, 8–12 % B; 2 − 5 min, 12–18 % B; 5 − 10 min, 18–20 % B; 10 − 12 min, 20–25 % B; 12 − 15 min, 25–30 % B; 15 − 18 min, 30–45 % B; 18 − 20 min, 45–80 % B; 20 − 22 min, 80-8 % B; 22 − 30 min, 8 % B. This flow rate was 0.8 mL/min, and temperature 35℃. Based on the standard curve for comparison and calculation, the anthocyanins and anthocyanidins content were obtained, and expressed in mg/g dry weight (DW). However, when we analyzed the dynamic change in PRG content in LR fruit at different growth stages, mg/g fresh weight (FW) could be used to indicate anthocyanin (PRG) content.

2.4. RNA isolation and gene expression analysis

For RNA extraction, fruits were collected from corresponding plants, and the RNAprep pure plant kits were used in strict accordance with the manufacturer's requirements. The determination of RNA content and quality was mainly performed by NanoDrop 2000 (Thermo-Scientific). The cDNA was synthesized by using a reverse transcription kit according to the instructions. The RT-PCR experiments were conducted using previously reported methods (Zong et al., 2019a). The PCR reactions were carried out using high fidelity Phushion DNA polymerase. The primer sequences of the test genes were consistent with previous studies (Zong et al., 2019a). The cycle threshold (Ct) $2^{-\Delta\Delta CT}$ method was used to measure the relative expression of tested gene. All of samples were analyzed in triplicate.

2.5. Statistical analysis

Based on the triplicate analyses, all data were expressed in the form of mean ± standard deviation. The experimental data were statistically analyzed using GraphPad Prism 5.1 software (San Diego, California, USA) and SPSS 16.0 (IBM, New York, USA). One-way analysis of variance (ANOVA) was used for estimation of statistical comparisons of data, and the Pearson's rank correlation coefficients were used to accurately determine the relationship between experimental variables. When $p < 0.05$, the differences estimated using two-tailed tests were significantly highlighted.

3. Results and discussion

3.1. Variation in anthocyanins composition amongst LR germplasm

In the previous work, 16 anthocyanins from LR fruits were identified, and petunidin derivatives in the total anthocyanin content reached approximately 97 % (Jin et al., 2015a, b,c; Wang et al., 2018b).

Moreover, almost 80 % of the total anthocyanins was petunidin-3-*O*-rutinoside (*trans*-*p*-coumaroyl)-5-*O*-glucoside (PRG), which rarely appear in the berries we usually eat or often see (Wu et al., 2006; Zheng et al., 2011). PRG consequently proved to be the predominant anthocyanin in LR fruits (Fig. S4). And through the hydrolyzation of the anthocyanin extracts, petunidin which belonged anthocyanidin, was the main product, then followed by malvidin and delphinidin.

In this work, the content of PRG and anthocyanidins (delphinidin, petunidin and malvidin) in ripe fruits were determined and analyzed systematically in a collection of LR with different regions (Fig.S5). PRG content ranged from 8.29 to 31.51 mg/g DW, with 17.24 mg/g DW as an average value. Most LR accessions (about 95 %) had a PRG concentration above 10 mg/g DW. This is significantly higher than that of other anthocyanin-rich fruits, such as blueberry, blackberry, and blackcurrants (Ogawa et al., 2008; Neveu et al., 2010; Pojer et al., 2013). Total anthocyanidins content ranged from 2.09 to 15.68 mg/g DW, and 6.46 mg/g DW is the average value. The average contents of delphinidin, petunidin and malvidin were 0.29, 5.71, and 0.47 mg/g, respectively. This indicates that petunidin is the major component of LR fruit anthocyanidins, which is consistent with the result of a previous study (Zhang et al., 2019). Notably, there was approximately 4-fold variation in PRG concentration and 7-fold variation in anthocyanidins content among the fruit samples examined. Therefore, this study preliminarily shows a dramatic genetic variation for anthocyanins content in LR plant.

Table S1 exhibited the Pearson correlation coefficients between PRG and anthocyanidins (delphinidin, petunidin and malvidin) traits. It showed that PRG concentration was proportional to the concentration of delphinidin ($r = 0.738$, $p < 0.01$) and petunidin ($r = 0.901$, $p < 0.01$), and insignificantly correlated with malvidin concentration ($r = 0.301$, $p > 0.05$). In addition, delphinidin and malvidin concentrations were significantly proportional to petunidin content, and the correlation coefficients were 0.741 and 0.457, respectively. This result was consistent with the previous report that anthocyanidins content was positively correlated with PRG content, but it showed no significant correlation between malvidin content and PRG content (Wang et al., 2018a, b).

3.2. Correlation between anthocyanins contents and ecogeographic variables

Although the exact reasons have not been clarified yet for the high content of anthocyanins in LR fruits, it has been speculated that unique geographical environments (strong sunshine, drought, and high altitude) and genomic difference could greatly influence the biosynthesis and accumulation of anthocyanins. Therefore, the correlations of variation of LR fruit anthocyanins concentration and ecogeographic variables were also investigated in this study (Table 2). The anthocyanins content of LR fruits increased with altitude and decreased with latitude, which may be due to changes in temperature or humidity conditions. The annual average temperature and precipitation were also studied, which can contribute to clarify this phenomenon. The results showed that there was a close relationship between the anthocyanin content and these two factors. Increased anthocyanins content variation in LR with higher altitude provided secondary metabolites to adapt the high altitude, strong ultraviolet and low temperature environment. The factors of temperature and humidity could greatly influence the germination rate of LR, which further explained the natural distribution of LR plants and the adaptability of these species to alpine cold and arid conditions. At relatively high altitudes, LR seed germination depends on relatively low temperatures which can also make plants more genetically diverse (Liu et al., 2012). In addition, the content of anthocyanins in LR fruit is also positively affected by the annual mean sunshine hours. The more the annual mean sunshine hours, the higher the anthocyanin content in the fruit is. This shows that there is a proportional relationship between the duration of sunshine and the accumulation of anthocyanins. Moreover, the seedling survival and growth of LR plants depend on more sunshine hours which have great impact on genetic diversity and biosynthesis of secondary metabolites such as anthocyanins (Gairola et al., 2010; Wallis et al., 2011; Liu et al., 2012).

3.3. Comparison of anthocyanins concentrations between cultivated and wild LR

As shown in Fig. 2, the difference in the distribution between the anthocyanins content in the cultivated and wild fruits is clearly visible.

Table 2
Pearson correlations (r_s) between anthocyanin components (PRG, delphinidin, petunidin and malvidin) and ecogeographic factors.

		Lt[a]	Ln[b]	Al[c]	AMT[d]	AMP[e]	AMH[f]	AMSH[g]
PRG	r_s	−0.313	−0.044	0.487[m]	−0.509[m]	0.235	−0.377	0.026
	p	0.112	0.829	0.010	0.007	0.238	0.053	0.899
Delphinidin	r_s	−0.392[n]	0.324	0.488[m]	−0.533[m]	0.388[n]	−0.367	0.311
	p	0.043	0.099	0.010	0.004	0.046	0.059	0.114
Petunidin	r_s	−0.390[n]	0.093	0.509[m]	−0.520[m]	0.201	−0.447[n]	0.144
	p	0.044	0.644	0.007	0.005	0.315	0.019	0.472
Malvidin	r_s	0.069	−0.222	−0.092	0.055	−0.202	−0.005	−0.131
	p	0.733	0.266	0.647	0.785	0.312	0.979	0.514

[a] Lt: Latitude.
[b] Ln: Longitude.
[c] Al: Altitude.
[d] AMT: Annual mean temperature.
[e] AMP: Annual mean precipitation.
[f] AMH: Annual mean humidity.
[g] AMSH: Annual mean sunshine hours.
[m] $p < 0.01$.
[n] $p < 0.05$.

In the cultivated fruits, the content of PRG was 8.29–28.54 mg/g DW, and the content of PRG in wild fruits ranged from 9.78 to 31.51 mg/g DW. This indicates the wild fruits have greater variation in PRG content than cultivated fruits. The average PRG contents of the planted and wild fruits were 17.05 and 17.42 mg/g DW, respectively, and their difference was not statistically significant ($p > 0.05$). The average concentration of malvidin was significantly lower in wild fruits than in cultivated fruits ($p = 0.03$). And the average concentrations of the delphinidin and petunidin were slightly lower in wild fruits (0.27 and 5.42 mg/g DW, respectively) than in planted fruits (0.30 and 5.99 mg/g DW, respectively), but these differences were also not statistically significant ($p = 0.53$ and $p = 0.35$, respectively). Moreover, the cultivated fruits had higher variation in both petunidin and malvidin content than the wild fruits. The content of petunidin in wild fruits was 1.74–10.78 mg/g DW, and the petunidin content in cultivated fruits was 2.91–13.81 mg/g DW. Additionally, among the wild fruits, the content of malvidin was 0.28 to 0.77 mg/g DW; and malvidin content could reach 0.31–1.02 mg/g DW in cultivated fruits.

As we know, planted fruits are often bigger than wild fruits in fruits crops (Fang et al., 2017). The correlations between anthocyanins concentration and LR fruit size (fruit weight and diameter) were investigated (Table 3), which could help to be in-depth understanding the effect of fruit quality characteristics on anthocyanins accumulation. It was showed that positive correlations were detected between anthocyanins contents and the weight or diameter of LR fruits. Especially, significant positive correlations were detected between PRG

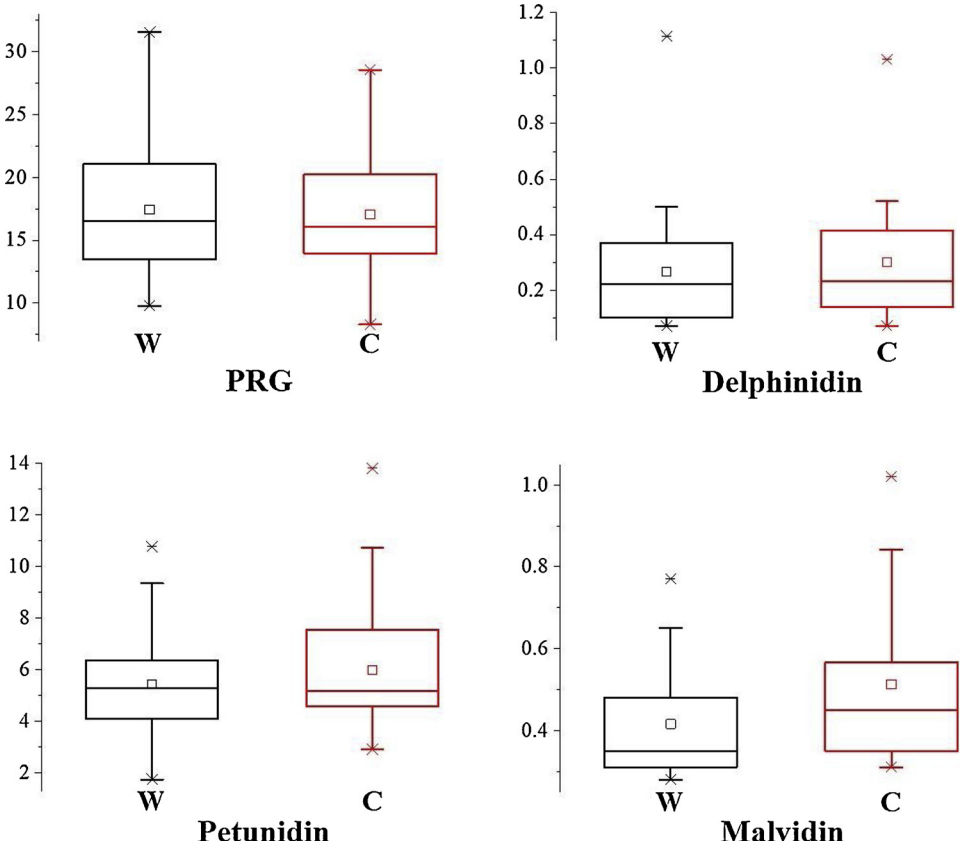

Fig. 2. Range and distribution of anthocyanins (PRG, delphinidin, petunidin and malvidin) contents (mg/g DW) in wild and cultivated fruits of *L. ruthenicum*. The horizontal lines and the small boxes in the interior of the box are the median values and mean values, respectively. Approximately 99 % of the data fall inside the whiskers. The data outside these whiskers are indicated by crosses. W, wild fruits; C, cultivated fruits.

Table 3
Pearson correlation coefficients between anthocyanins (PRG, delphinidin, petunidin and malvidin) and LR fruit size.

		Wild fruits		Cultivated fruits	
		HFW[a]	FD[b]	HFW	FD
PRG	r_s	0.176	0.546[c]	0.358	0.399[d]
	p	0.379	0.003	0.067	0.039
Delphinidin	r_s	−0.095	0.260	0.089	0.104
	p	0.637	0.191	0.660	0.607
Petunidin	r_s	0.088	0.509[c]	0.327	0.279
	p	0.662	0.007	0.096	0.158
Malvidin	r_s	0.271	0.426[d]	0.385[d]	0.395[d]
	p	0.171	0.027	0.047	0.041

[a] HFW: hundred-fruit weight.
[b] FD: fruits diameter.
[c] $p < 0.01$.
[d] $p < 0.05$.

concentration and fruit diameter in both cultivated ($r_s = 0.399$, $p < 0.05$) and wild fruits ($r_s = 0.546$, $p < 0.01$). According to the researches, fruit size is seen as an important trait for selection during LR domestication process. And the artificial selection on LR fruit size may be the most prevalent reason for explaining the difference in anthocyanin content between the cultivated and wild fruits. Therefore, the fruit size and anthocyanins content in fruits are considered to be critical properties in LR breeding and domestication.

3.4. The dynamic change in PRG content at different developmental stages

To investigate the dynamic change in PRG content in LR fruit at different growth stages (juvenile, expanding, and mature), we carried out a random method for the selection of LR varieties. The cultivated fruit samples were selected from five provinces (Fig. 3a and c), KLKH (Qinghai), CJ (Xinjiang), DH (Gansu), EQ (Inner Mongolia), and PL (Ningxia). The results of the study showed that the PRG contents in all fruits of the juvenile stage were the lowest, however, PRG accumulated continuously and the content of PRG increased dramatically with the development of the fruits. PRG content at green fruit stage ranged from 0.22 to 0.37 mg/g FW, with an average of 0.27 mg/g FW. When the fruits were at the expanding stage, PRG content significantly increased, indicating an average of 0.85 mg/g FW; and when fruits entered the last stages of growth, the average content of LR fruits reached 2.97 mg/g FW. While compared with the juvenile stage, the PRG concentration increased nearly 3- and 11-fold at the next two development stages, respectively. Similar result has been published in fruit of commercial apple cultivars, in which the anthocyanin content increased obviously during fruit maturation (Ju et al., 1999). In a word, our work reveals that the biosynthesis and accumulation of anthocyanin (PRG) in LR fruit are very slow in the green fruit stage, but increase significantly during fruit development, especially at the mature stage.

3.5. Expression LrAN2 responsible for anthocyanin content in LR fruit

In our previous study, one allelic gene *LrAN2* was isolated from *L. ruthenicum*, which encoded for a R2R3-MYB transcription factor involved in regulating the biosynthesis and accumulation of anthocyanin in LR fruits. The main reason why LR fruit contained high content of anthocyanins could be caused by high expression level of *LrAN2* (Zong et al., 2019ab). Thus, *LrAN2* was selected to investigate its role in anthocyanin accumulation in LR fruit throughout different growth stages, and the expression level of *LrAN2* was determined in the same fruit samples. In terms of development stages, the expression of the *LrAN2* gene was relatively low in the juvenile stage. However, the PRG contents increased dramatically as the fruit maturation, which significantly related with the high expression level of the target gene *LrAN2*. Finally,

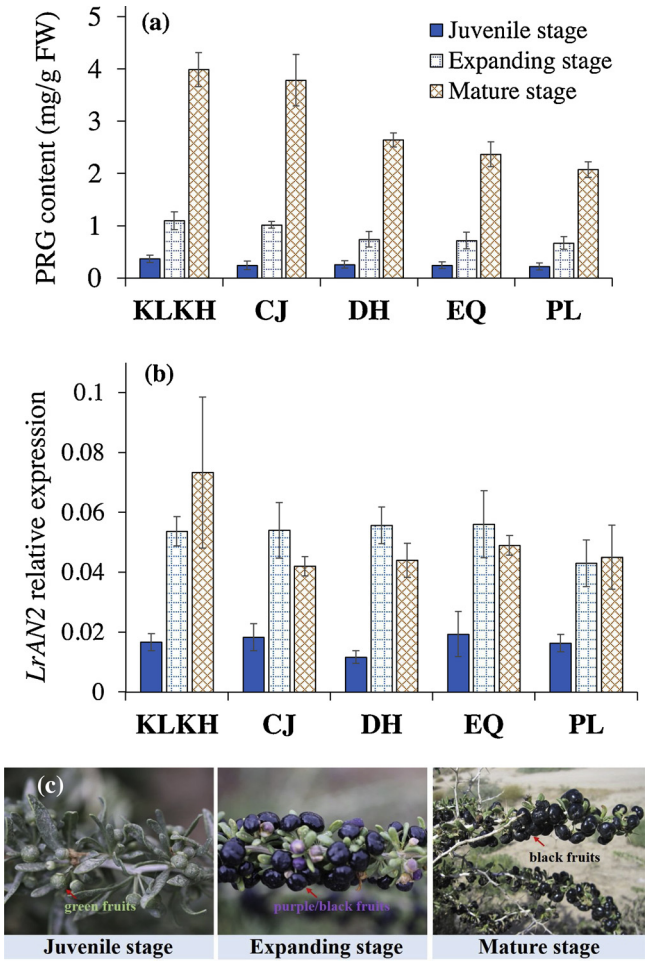

Fig. 3. (a) Changes in the anthocyanin (PRG) content in fruits of different cultivars of *L. ruthenicum* during fruit development. (b) Expression *LrAN2* related to PRG accumulation in different regions of LR fruits at three developmental stages (c).

the expression of the *LrAN2* and the PRG contents in LR fruits could reach the maximum at the mature stage (Fig. 3b).

Further using Pearson's theory for correlation analysis, the relationship between PRG content and *LrAN2* expression in LR fruits was obtained. This was as shown in Table 4. In different stages of fruit development, there was significant correlation between the expression level of *LrAN2* and PRG concentration in LR fruits. And it was notable that this correlation was positive, which also indicated that the *LrAN2* expression may be caused by high PRG concentration in LR fruits during the fruit maturation via feedback mechanism.

In the early green fruits, *LrAN2* expression level was negatively correlated with PRG content. This could be concluded that the structural genes associated with the phenylpropanoid acid pathway in the fruit might be not effectively activated, which led to the down-

Table 4
Pearson correlation coefficients between PRG contents and the *LrAN2* expression level related to PRG accumulation in LR fruits at three developmental stages.

Fruit developmental stage	*LrAN2* expression level
Juvenile	−0.072
Expanding	0.344
Mature	0.544
All	0.613[a]

[a] $p < 0.05$.

accumulation of anthocyanin at juvenile stage (Zong et al., 2019b). However, the expression level of *LrAN2* showed a positive correlation with the content of PRG in fruits at the expanding and mature stages. This speculates that the high PRG concentration in LR fruit at the later stages is probably attributed to synergistic effect of PRG biosynthesis, regeneration, and translocation from other tissues.

4. Conclusion

For the first time, a large-scale study and evaluation of anthocyanins (PRG, delphinidin, petunidin and malvidin) contents in wild and cultivated LR fruits were carried out. LR fruits contain abundant anthocyanins, which demonstrate that the fruits are an excellent source of anthocyanins. Our study also exhibits that different varieties of LR plants have a great variation in fruit anthocyanins content. And the biosynthesis and accumulation of anthocyanins could be influenced greatly by ecogeographic variables. Additionally, wild LR fruits showed greater variation in anthocyanin (PRG) content than planted fruits. This suggests that wild LR germplasms are useful sources of genes that can be fully utilized for genetic improvement of anthocyanins concentrations in LR breeding and domestication in the future.

Moreover, LR fruit size (weight and diameter) and anthocyanin content play important roles during domestication relative to wild relatives. The average content of anthocyanins in the wild and cultivated fruits is quite similar. However, the size of LR fruits was significantly different in wild and cultivated LR. This could be speculated that the difference in LR fruit size was ascribed to the influence of cultivation associated with domestication process.

In LR fruit, PRG contents are relatively low at the juvenile stage, but dramatically increased with fruit growth. The biosynthesis and operation of PRG are relative lack at the juvenile stage, which could lead to extremely low content of PRG in early green fruits. However, the significant increases in PRG concentrations during fruits maturation are probably due to the synergistic effects of PRG biosynthesis, regeneration, and translocation from other tissues.

CRediT authorship contribution statement

Zenggen Liu: Conceptualization, Methodology, Software, Writing - original draft, Data curation. **Banmacailang Dong:** Data curation. **Chuang Liu:** Visualization, Investigation. **Yuan Zong:** Methodology. **Yun Shao:** Supervision. **Baolong Liu:** Software, Validation. **Huilan Yue:** Writing - review & editing.

Declaration of Competing Interest

In the process of research, the authors have no competition and no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2020.112208.

References

Andersen, O.M., Jordheim, M., 2006. The anthocyanins. In: Andersen, O.M., Markham, K.R. (Eds.), Flavonoids Chemistry, Biochemistry and Applications. CRC Press, Taylor and Francis, Boca Raton, pp. 471–552.

Andersen, O.M., Jordheim, M., 2008. Anthocyanins-food applications. In: Proc. 5th Int. Congr. Pigments Foods: For Quality and Health. 14-16 Aug., Helsinki, Finl..

Castañeda-Ovando, A., Pacheco-Hernández, M.L., Páez-Hernández, M.E., Rodríguez, J.A., Galán-Vidal, C.A., 2009. Chemical studies of anthocyanins: a review. Food Chem. 113 (4), 859–871.

Chen, S.S., Zhou, H.N., Zhang, G., Meng, J., Deng, K., Zhou, W., Wang, H.L., Wang, Z.H., Hu, N., Suo, Y.R., 2019. Anthocyanins from *Lycium ruthenicum* Murr. Ameliorated d-galactose-induced memory impairment, oxidative stress, and neuroinflammation in adult rats. J. Agric. Food Chem. 67 (11), 3140–3149.

Deng, X., Bashandy, H., Ainasoja, M., Kontturi, J., Pietiäinen, M., Laitinen, R.A., Albert, V.A., Valkonen, J.P., Elomaa, P., Teeri, T.H., 2014. Functional diversification of duplicated chalcone synthase genes in anthocyanin biosynthesis of *Gerbera hybrida*. New Phytol. 201 (4), 1469–1483.

Fang, T., Zhen, Q.L., Liao, L., Owiti, A., Zhao, L., Korban, S.S., Han, Y.P., 2017. Variation of ascorbic acid concentration in fruits of cultivated and wild apples. Food Chem. 225, 132–137.

Forkmann, G., Kuhn, B., 1979. Genetic control of chalcone isomerase activity in anthers of *Petunia hybrida*. Planta 144 (2), 189–192.

Gairola, S., Shariff, N.M., Bhatt, A., Kala, C.P., 2010. Influence of climate change on production of secondary chemicals in high altitude medicinal plants: issues needs immediate attention. J. Med. Plants Res. 4 (18), 1825–1829.

Ghosh, D., Konishi, T., 2007. Anthocyanins and anthocyanin-rich extracts: role in diabetes and eye function. Asia Pac. J. Clin. Nutr. 16, 200–208.

He, J., Giusti, M.M., 2010. Anthocyanins: natural colorants with health-promoting properties. Annu. Rev. Food Sci. Technol. 1, 163–187.

Heinzmann, U., Seitz, U., 1974. Relationship between anthocyanin synthesis and activity of phenylalanine ammonia-lyase (PAL) in callus cells of *Daucus carota*. Planta 117 (1), 75–81.

Hu, N., Zheng, J., Li, W.C., Suo, Y.R., 2014. Isolation, stability and antioxidant activity of anthocyanins from *Lycium ruthencium* Murray and *Nitraria Tangutorum* Bobr of Qinghai-Tibetan Plateau. Sep. Sci. Technol. 49 (18), 2897–2906.

Jin, H.L., Liu, Y.F., Guo, Z.M., Yang, F., Wang, J.X., Li, X.L., Peng, X.J., Liang, X.M., 2015a. High-performance liquid chromatography separation of cis-trans anthocyanin isomers from wild *Lycium ruthenicum* Murr. Employing a mixed-mode reversed-phase/strong anion-exchange stationary phase. J. Agric. Food Chem. 63 (2), 500–508.

Jin, H.L., Liu, Y.F., Yang, F., Wang, J.X., Fu, D.M., Zhang, X.L., Peng, X.J., Liang, X.M., 2015b. Characterization of anthocyanins in wild *Lycium ruthenicum* Murray by HPLC-DAD/QTOF-MS/MS. Anal. Method. 7, 4947–4956.

Jin, H.L., Zhao, J.Q., Zhou, W.J., Shen, A.J., Yang, F., Liu, Y.F., Guo, Z.M., Zhang, X.L., Tao, Y.D., Peng, X.J., Liang, X.M., 2015c. Preparative separation of a challenging anthocyanin from *Lycium ruthenicum* Murr. By two-dimensional reversed-phase liquid chromatography/hydrophilic interaction chromatography. RSC Adv. 5, 62134–62141.

Jing, P., Bomser, J.A., Schwartz, S.J., He, J., Magnuson, B.A., Giusti, M.M., 2008. Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. J. Agric. Food Chem. 56 (20), 9391–9398.

Ju, Z.G., Liu, C.L., Yuan, Y.B., Wang, Y.Z., Liu, G.S., 1999. Coloration potential, anthocyanin accumulation, and enzyme activity in fruit of commercial apple cultivars and their F1 progeny. Sci. Hortic. 79, 39–50.

Liobikas, J., Skemiene, K., Trumbeckaite, S., Borutaite, V., 2016. Anthocyanins in cardioprotection: a path through mitochondria. Pharmacol. Res. 113, 805–815.

Liu, Z.G., Shu, Q.Y., Wang, L., Yu, M.F., Hu, Y.P., Zhang, H.G., Tao, Y.D., Shao, Y., 2012. Genetic diversity of the endangered and medically important *Lycium ruthenicum* Murr. Revealed by sequence-related amplified polymorphism (SRAP) markers. Biochem. Syst. Ecol. 45, 86–97.

Neveu, V., Perez-Jimenez, J., Vos, F., Crespy, V., Chaffaut, L., Mennen, L., Knox, C., Eisner, R., Cruz, J., Wishart, D., Scalbert, A., 2010. Phenol-Explorer: an Online Comprehensive Database on Polyphenol Contents in Foods. Database. Available from: http://www.phenol-explorer.eu/. Accessed 2012 July 05.

Ogawa, K., Sakakibara, H., Iwata, R., Ishii, T., Sato, T., Goda, T., Shimoi, K., Kumazawa, S., 2008. Anthocyanin composition and antioxidant activity of the crowberry (*Empetrum nigrum*) and other berries. J. Agric. Food Chem. 56 (12), 4457–4462.

Pojer, E., Mattivi, F., Johnson, D., Stockley, C.S., 2013. The case for anthocyanin consumption to promote human health: a Review. Compr. Rev. Food Sci. Food Saf. 12, 483–508.

Renaud, S., Lorgeril, M., 1992. Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339, 1523–1526.

Rossi, A., Serraino, I., Dugo, P., Paola, D.R., Mondello, L., Genovese, T., Morabito, D., Dugo, G., Sautebin, L., Caputi, A.P., Cuzzocrea, S., 2003. Protective effects of anthocyanins from blackberry in a rat model of acute lung inflammation. Free Radic. Res. 37 (8), 891–900.

Seeram, N.P., Momin, R.A., Nair, M.G., Bourquin, L.D., 2001. Cyclooxygenase inhibitory and antioxidant cyanidin glycosides in cherries and berries. Phytomedicine 8, 362–369.

Seitz, C., Ameres, S., Forkmann, G., 2007. Identification of the molecular basis for the functional difference between flavonoid 3′-hydroxylase and flavonoid 3′,5′-hydroxylase. FEBS Lett. 581 (18), 3429–3434.

Shi, S.G., Hu, T.T., 2016. Cloning and bioinformatics analysis of the jujube fruit

flavonoid-3-hydroxylase gene (F3H). North. Hortic. 14, 89–93.
Shimada, Y., Nakano-Shimada, R., Ohbayashi, M., Okinaka, Y., Kiyokawa, S., Kikuchi, Y., 1999. Expression of chimeric P450 genes encoding flavonoid-3′,5′-hydroxylase in transgenic tobacco and petunia plants. FEBS Lett. 461 (3), 241–245.
Tang, J.L., Yan, Y.M., Ran, L.W., Mi, J., Sun, Y., Lu, L., Gao, Y.X., Zeng, X.X., Cao, Y.L., 2017. Isolation, antioxidant property and protective effect on PC12 cell of the main anthocyanin in fruit of *Lycium ruthenicum* Murray. J. Funct. Foods 30, 97–107.
Tsuda, T., 2008. Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome. J. Agric. Food Chem. 56 (3), 642–646.
Wallis, C.M., Huber, D.P., Lewis, K.J., 2011. Ecosystem, location, and climate effects on foliar secondary metabolites of lodgepole pine populations from central British Columbia. J. Chem. Ecol. 37 (6), 607–621.
Wang, H.Q., Li, J.N., Tao, W.W., Zhang, X., Gao, X.J., Yong, J.J., Zhao, J.J., Zhang, L.M., Li, Y.Z., Duan, J.A., 2018a. *Lycium ruthenicum* studies: molecular biology, phytochemistry and pharmacology. Food Chem. 240, 759–766.
Wang, Z.C., Yan, Y.Z., Nisar, T., Zou, L., Yang, X., Niu, P.F., Sun, L.J., Guo, Y.R., 2018b. Comparison and multivariate statistical analysis of anthocyanin composition in *Lycium ruthenicum* Murray from different regions to trace geographical origins: the case of China. Food Chem. 246, 233–241.
Wu, X., Beecher, G.R., Holden, J.M., Haytowitz, D.B., Gebhardt, S.E., Prior, R.L., 2006. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. J. Agric. Food Chem. 54 (11), 4069–4075.
Zhang, Y., Butelli, E., Martin, C., 2014. Engineering anthocyanin biosynthesis in plants. Curr. Opin. Plant Biol. 19, 81–90.
Zhang, G., Chen, S.S., Zhou, W., Meng, J., Deng, K., Zhou, H.N., Hu, N., Suo, Y.R., 2019. Anthocyanin composition of fruit extracts from *Lycium ruthenicum* and their protective effect for gouty arthritis. Ind. Crop. Prod. 129, 414–423.
Zheng, J., Ding, C., Wang, L., Li, G., Shi, J., Li, H., Wang, H., Suo, Y., 2011. Anthocyanins composition and antioxidant activity of wild *Lycium ruthenicum* Murr. From Qinghai-Tibet Plateau. Food Chem. 126, 859–865.
Zong, Y., Zhu, X.B., Liu, Z.G., Xi, X.Y., Li, G.M., Cao, D., Wei, L., Li, J.M., Liu, B.L., 2019a. Functional MYB transcription factor encoding gene *AN2* is associated with anthocyanin biosynthesis in *Lycium ruthenicum* Murray. BMC Plant Biol. 19, 169–177.
Zong, Y., Li, S.M., Xi, X.Y., Cao, D., Wang, Z., Wang, R., Liu, B.L., 2019b. Comprehensive influences of overexpression of a MYB transcriptor regulating anthocyanin biosynthesis on transcriptome and metabolome of tobacco leaves. Int. J. Mol. Sci. 20, 5123–5135.