

The Study of Variation of Phloridzin Content in Six Wild *Malus* Species

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Abstract Phloridzin is relatively abundant in species of genus *Malus* Miller, of which China is the most important origin and diversification center. In this study, phloridzin contents in fruits and leaves of six wild *Malus* species were analyzed using a RP-HPLC system. The phloridzin content varied significantly among species. The highest phloridzin content in fruits was found in *M. xiaojinensis* (0.63 mg/100mg), whereas the lowest in *M. maerkangensis* (0.04 mg/100mg); meanwhile, the highest phloridzin content in leaves was found in *M. transitoria* (14.36mg/100mg), whereas the lowest in *M. kansuensis* (4.08 mg/100mg). Interestingly, phloridzin content showed no significant distinction among different populations of *M. toringoides*, however, there is a wide range of variation among individuals of the same wild *Malus* species. Different genetic constitution is the most likely reason for variation of phloridzin content among species, whereas variation among individual may be caused by heterogeneous environmental factors. Our preliminary study provides important information for potential novel utilization of wild apple germplasm in China.

Keywords: genus *Malus*, phloridzin content, germplasm, utilization

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1. Introduction

Phloretin is a type of dihydrochalcone belonging to flavonoids family. Phloridzin (Figure 1), the glucoside conjugate of phloretin, is mainly found in *Malus* species, in which a relative high content of phloridzin is accumulated in leaves, tree barks and peel of fruits (Gosch et al. 2010). Since the discovery of phloridzin, a large number of studies which focused on this compound have been conducted for different purposes, including resistance against plant pathogens, antioxidant activity, treatment of metabolic syndrome, glucose transport pathology, renal glycosuria and toxicology (Ehrenkranz et al. 2005; Gosch et al. 2010; Xiang et al. 2011). Phloridzin has also been used as a biochemical marker to indicate the phase transition of apple seedlings (Zhang et al. 2007). Previous studies found that phloridzin has the capability to block glucose uptake in human intestinal and liver cells, improving the hyperglycemia of streptozotocin-induced diabetic mice, and affecting the gene expression related to the citric acid cycle, gluconeogenesis and fatty acid metabolism in liver (Zhao et al. 2004; Ehrenkranz et al. 2005; Manzano and Williamson 2010; Kobori et al. 2012).

Malus toringoides Hughes is a wild species in the genus *Malus* Miller of the family Rosaceae (Yu et al. 1974). The hybrid nature of *M. toringoides* was confirmed by

phylogenetic analysis of a single-copy nuclear gene *SbeI*, and *M. transitoria* was identified as the paternal parent of *M. toringoides* (Tang et al. 2014). Three wild *Malus* species are recognized as close relatives of *M. toringoides*. Two of them, *M. maerkangensis* and *M. xiaojinensis*, have originated through hybridization between *M. toringoides* and *M. kansuensis*, whereas the third one (*M. setok* Vassilcz.) is genetically closely related to *M. toringoides* (Tang et al. 2014). Geographically, *M. toringoides* is widely distributed in mountain regions of Shanxi, Gansu, Ningxia, Qinghai and Sichuan provinces (Zhou 1999). Moreover, its distribution areas are overlapped with *M. setok*, *M. xiaojinensis* and *M. maerkangensis* at west part of Sichuan Province (Li 2001; Cheng et al. 2002, 2003). *Malus toringoides* is a good apple rootstock because of its excellent graft compatibility, promotion of early fruiting, resistance to drought and cold abiotic stresses (Shi et al. 2004). *Malus xiaojinensis* is another good rootstock. It is resistant to latent virus and is the first apple genotype selected for resistance to iron deficiency (Cheng et al. 2000). Our previous studies mainly focused on the morphology, physiology, taxonomy and evolutionary origin of the above mentioned species (Zhou 1999; Tang et al. 2014). A systematic evaluation of the bioactive compound phloridzin in wild *Malus* species will facilitate potential novel utilization of natural apple germplasms in China.

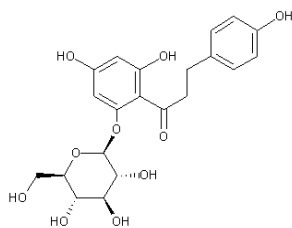


Figure 1. The chemical structure of phloridzin in plants

In the past decades, the content, distribution and biosynthesis of phloridzin in plants have attracted great interests from many research fields. The genetic and environmental factors that influence the content and biosynthesis of phloridzin were investigated (Gosch et al. 2009, 2010; Fromm et al. 2012). In addition, the physiological function of phloridzin and its bioactive nature as a dietary or nutraceutical agent were evaluated (Gosch et al. 2012). To the best of our knowledge, no work has been carried out to evaluate the phloridzin content of wild *Malus* species, especially the variation of phloridzin content among different populations or individuals under natural condition.

The objective of the current study was to determine the phloridzin contents in fruits and leaves of six wild *Malus* species using a RP-HPLC system. We also compared the phloridzin contents between wild and cultivated apple species and explored the variation of phloridzin content among populations and individuals of *Malus* species. The potential factors on such variation were analyzed. Our research should provide valuable information for the better utilization of wild apple germplasms in China.

2. Materials and Methods

2.1. Plant Material

Healthy fruits and leaves, without any damage induced by disease or pests, were collected from wild *Malus* species distributed at the Aba Tibetan Autonomous Region of Sichuan Province, China. Fruits were harvested at natural maturity stage based on external color and size uniformity. Leaves with similar growth status were gathered from roughly the same position on several branches. Detailed sampling information is given in Table 1. Fruits or leaves of the same individual plant were pooled, freeze-dried, then smashed in liquid nitrogen and sieved through a 40-mesh sieve. The powders were stored at -80°C until analysis.

2.2. Reagents and Standards

Phloridzin standard was purchased from Beijing Toby Technology Co., Ltd. (Beijing, China). Acetonitrile (HPLC grade) was purchased from J&K Scientific Ltd. (Beijing, China). All other chemicals used in this study were of analytical grade.

2.3. Quantification Analysis of phloridzin

The method of Lata et al. (2009) was adapted with slight modification for quantification of phloridzin in fruit and leaf samples (Schlag and McIntosh 2006). 0.15 g of smashed sample was mixed with 75% ethanol by the solid/liquid ratio of 1:10 in a 2 ml centrifuge tube. The

extraction of phloridzin was carried out using a KQ-5200DE computer numerical control ultrasonic instrument (Kunshan Ultrasonic Instrument Co., Ltd., Jiangsu, China) at 50 degrees for 45 minutes. After centrifugation (5000 rpm, 15mins), supernatant from extraction of fruit sample was filtered through a microporous membrane (0.22 μm) and then injected into the HPLC system. Supernatant from extraction of leaf sample was first filtered through a microporous membrane (0.22 μm) and then diluted 10-fold by 50% acetone before being injected into the HPLC system.

The phloridzin contents were determined using a Waters e2695 high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) with Venusil ASB C18 HPLC columns (250 mm $\times$ 4.6 mm, 5 μm , Agela, USA) equipped with a C₁₈ Guard column (5 μm , 3.9 mm $\times$ 20 mm, Waters, Milford, MA). The mobile phases were (A) acetonitrile and (B) H₂O with a flow rate of 1.0 ml min⁻¹ according to the following profile: 0-5 min, 0-30% A; 5-20 min, 30% A. The UV diode array detector was set at 280nm. The column was maintained at 25 $^{\circ}\text{C}$ and sample injection volume was 10 μl . Phloridzin was identified by comparing with the retention time of standard. The phloridzin was quantified based on the peak area of the sample and the standard curve (Figure 2). All samples were analyzed three times.

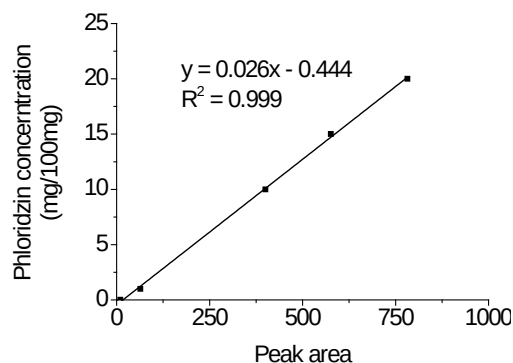


Figure 2. The calibration curve and regression equation of phloridzin standard

2.4. Statistical Analysis

The regression equation of calibration curve was computed with excel 2007. In this study, all data are expressed as means $\pm$ standard deviation of three replicates, and the phloridzin content is expressed on a dry matter basis (mg/100mg dry weight). Following Schlag and McIntosh (2006), the difference of Phloridzin content among wild *Malus* species was test by the Kruskal-wallis test, and the difference of Phloridzin content between *M. torigoides* and other wild *Malus* species was test by the Wilcoxon rank sum test. All statistical tests were performed using SPSS v16.0 (SPSS for Windows, Release 16.0, SPSS Inc.).

3. Results and Discussion

3.1. Phloridzin Contents in Fruits and Leaves of Wild *Malus* Species

In this study, a fast and convenient HPLC system based on the method of Lata et al. (2009) was used to determine

the phloridzin contents in fruits and leaves of *Malus* species. A five-point concentration calibration curve was linear and reproducible for phloridzin standard, and the R-square exceeded 0.999 (Figure 2). The method of Jham (1996) was used to examine the reproducibility (RSD 1.6%) of the HPLC system, and an average recovery rate 98.1% of phloridzin (RSD 2.29%) was obtained. Phloridzin is the main component in ethanol extracts of fruits and leaves of the wild *Malus* species (Figure 3).

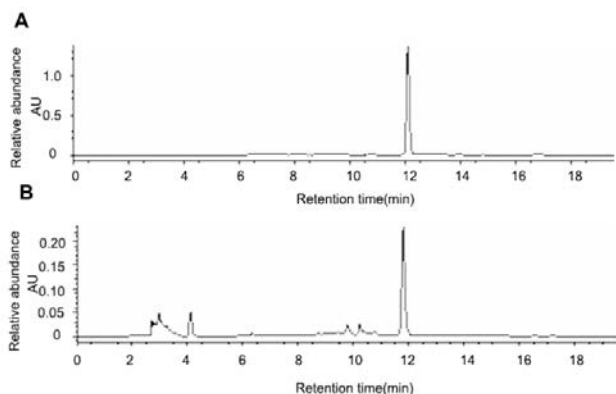


Figure 3. The representative chromatograms of phloridzin samples analyzed in this study

The chromatogram of phloridzin generated from the standard phloridzin sample (A), and the chromatogram of phloridzin generated using the ethanol extract of a fruit sample of *Malus transitoria* (B).

3.2. The Variation of Phloridzin Content among Wild *Malus* Species

To analyze the variation pattern of phloridzin content among wild *Malus* species, the average phloridzin content in leaf and fruit samples were calculated. Phloridzin content showed considerably variation among different *Malus* species (Table 1). In leaf samples, the phloridzin content ranges from 4.95 mg/100mg (*M. maerkangensis*) to 12.90 mg/100mg (*M. transitoria*). The phloridzin content in fruits is much less than that in leaves, and varies from 0.04 mg/100mg (*M. maerkangensis*) to 0.38 mg/100mg (*M. xiaojinensis*).

Phylogenetic analyses of wild *Malus* species demonstrated that *M. transitoria* is the paternal parent of *M. toringoides* (Tang et al. 2014). The phloridzin content in leaves of *M. transitoria* is significant higher than that of *M. toringoides* (Table 1). *Malus xiaojinensis* and *M. maerkangensis* derived from hybridization between *M. toringoides* and *M. kansuensis* (Tang et al. 2014). However, the phloridzin content in leaves of *M.*

xiaojinensis differs remarkably from that of *M. maerkangensis* (Table 1). Three species, *M. xiaojinensis*, *M. setok* and *M. toringoides*, have similar phloridzin content, whereas the phloridzin content in leaves of *M. maerkangensis* is significantly lower than that of *M. toringoides* (Table 1). When measuring the phloridzin content in fruits, we found there was no obvious difference between *M. toringoides* and *M. transitoria*. In addition, *M. xiaojinensis* and *M. setok* have the highest while *M. kansuensis* and *M. maerkangensis* have the lowest phloridzin content, respectively (Table 1). The variation pattern of phloridzin content is distinct between leaves and fruits. In leaves, the highest content is detected in *M. transitoria*, but *M. xiaojinensis* and *M. setok* show the highest content in fruits. However, the phloridzin contents of *M. maerkangensis* and *M. kansuensis* are always low, no matter in fruits or in leaves.

Phloridzin content differed significantly among certain pairs of the six *Malus* species (Table 1). Previous study revealed that apple cultivars had different phloridzin contents (Tsao et al. 2003; Wu et al. 2007; Wojdylo et al. 2008). In addition, Lata et al. (2009) reported that the phloridzin content in fruits of cultivar “Monroe” was almost four times higher than that of cultivar “Gala”. Our study showed the phloridzin contents in fruits of *M. setok* and *M. xiaojinensis* are about nine times higher than that of *M. maerkangensis*. *Malus xiaojinensis* is an excellent wild apple germplasm in the southwest of China. It has been reported that *M. xiaojinensis* could resist latent virus and yellow leaf disease (Cheng et al. 2000). In this study, we found that *M. xiaojinensis* has relative high phloridzin content in both leaves and fruits. When compared the phloridzin content of our study with those of apple cultivars, we found that the phloridzin content in leaves of *M. toringoides* is comparable to that of apple cultivar “Rainha catarina” (10.87 mg/100mg), but roughly four times higher than that of cultivar “Winter banana” (2.49 mg/100mg) (Jham 1996). In addition, the lowest phloridzin content in fruits found in this study is comparable with the highest one reported by Lata et al. (2009), and the phloridzin contents in fruits of wild *Malus* species *M. transitoria*, *M. toringoides*, *M. setok* and *M. xiaojinensis* are all higher than those of common apple cultivars (Tsao et al. 2003). In conclusion, the phloridzin content in wild *Malus* species is generally higher than those have been reported in apple cultivars. Therefore, the wild *Malus* plants could be utilized not only as rootstocks for apple production, but also as potential nutritional supplement for human daily life.

Table 1. Sampling information, median and interquartile ranges of phloridzin contents (mg/100mg dry weight) in leaves and fruits of six wild *Malus* species

Species	Altitude ¹⁾			Number ²⁾	Leaves	Fruits
	Range	Mean	Cv (%)			
<i>Malus toringoides</i> Hughes	2614-3152	2940	7.33	36	10.52 (9.19-11.93)	0.21 (0.11-0.34)
<i>M. transitoria</i> Schneid	2880-3016	2950	1.28	11	12.90 (11.85-14.36)*	0.27 (0.19-0.34)
<i>M. Kansuensis</i> Schneid	3171-3204	3197	0.41	16	5.35 (4.08-6.37)*	0.06 (0.05-0.21)*
<i>M. maerkangensis</i> Cheng et al.	3068-3095	3081	0.49	3	4.95(4.86-9.35)*	0.04 (0.04-0.15)*
<i>M. setok</i> Vassilcz.	2496-2591	2514	1.26	12	9.47 (8.68-12.45)	0.38 (0.29-0.42)*
<i>M. xiaojinensis</i> Cheng & Jiang	3050-3118	3111	0.66	6	9.10 (8.77-9.46)	0.38 (0.34-0.63)*

¹⁾ The range, mean and coefficient of variation of altitudes.

²⁾ The number of samples of each species.

* *Malus* species whose phloridzin content differs significantly from the phloridzin content of *M. toringoides* at $p < 0.05$ according to multiple Wilcoxon rank sum test.

The phloridzin content differs extraordinarily between leaves and fruits in the studied wild *Malus* species, with the ratio of phloridzin content between leaves and fruits varying from 24 to 146 (Table 1). Other studies also reported wide ranges of variation of phloridzin content between leaves and fruits in apple cultivars (Jham 1996; Lata et al. 2009). Moreover, *Malus* species with high phloridzin content in leaves usually have high phloridzin content in fruits (Table 1), suggesting that the phloridzin content in wild *Malus* species are largely controlled by genetic factors.

3.3. The Variation of Phloridzin Content among Different Populations of *M. toringoides*

To analyze variation of phloridzin content among different populations of *M. toringoides*, phloridzin contents of five natural populations with distinct altitude were determined. As showing in Table 2, the highest phloridzin content in leaves is found in Kehe population (11.58 mg/100mg), ranging from 9.83mg/100mg to 11.93 mg/100mg, whereas the highest phloridzin content in

fruits is found in Angqiang population (0.34 mg/100mg), ranging from 0.26 mg/100mg to 0.43mg/100mg. The Nongkesuo population has the lowest phloridzin content both in fruits and leaves. However, neither the phloridzin content in leaves nor in fruits differs significantly between any two of the five sampled *M. toringoides* populations, with the difference of phloridzin content in fruits between Chabei and Angqiang population as the only exception (Table 2). The consistency of phloridzin contents among different populations of the same *Malus* species suggested that genetic constitution may be the principle factor that controls the relative abundance of phloridzin in wild *Malus* species. Additionally, the average phloridzin content of a population is loosely correlated with its altitude. Three populations with altitudes above 3000 meters have roughly higher phloridzin content than other two populations with altitudes below 3000 meters (Table 2), suggesting that altitude may in some extent influence variation of phloridzin content under natural condition. However, such effect is rather small because the content difference among *M. toringoides* populations is not statistically significant.

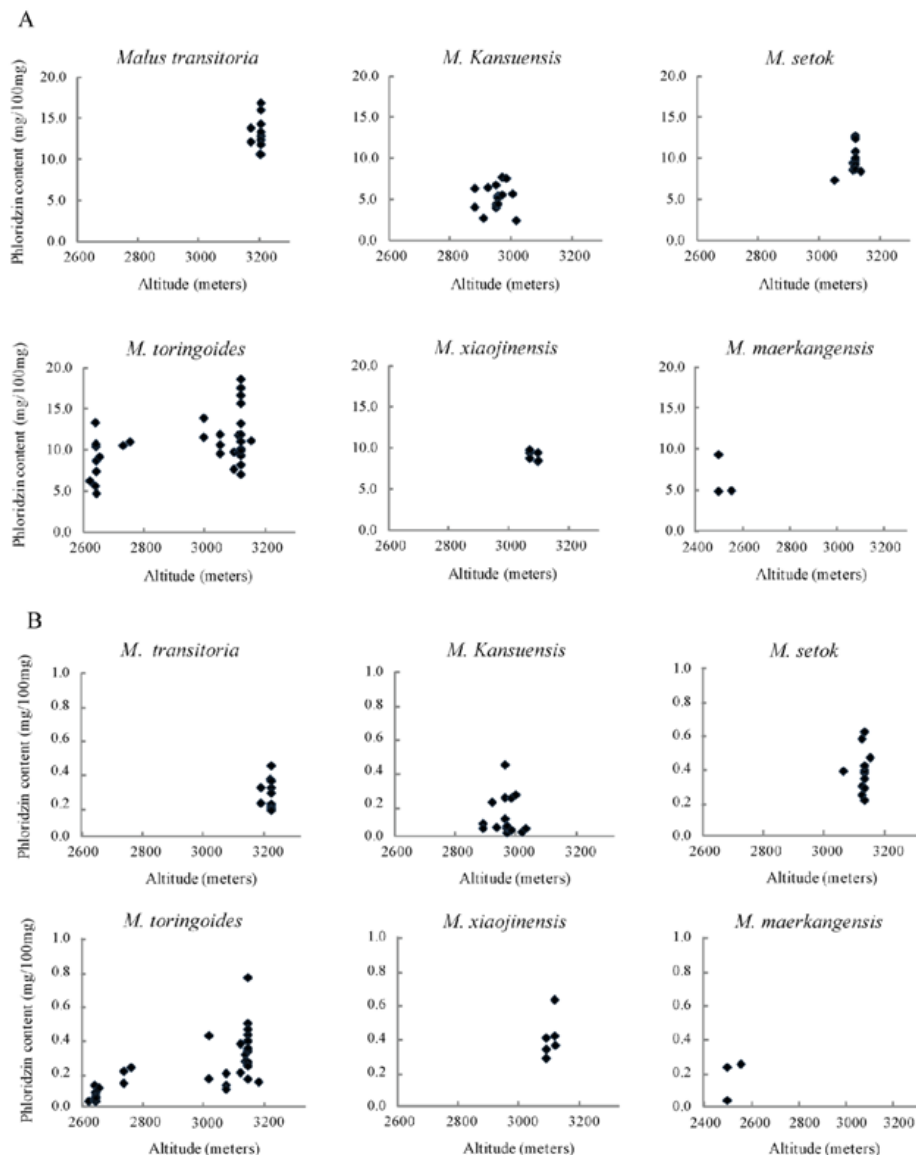


Figure 4. The variation of phloridzin content among individuals of the same *Malus* species. Scatter plot of phloridzin content in leaf samples (A) and fruit samples (B) of different individuals of the six studied wild *Malus* species

3.4. The Variation of Phloridzin Content among Different Individuals of Wild *Malus* Species

To further assess the variation of phloridzin content at individual level, phloridzin content of each individual was analyzed. Phloridzin contents vary considerably among individuals within the same species (Figure 4). Specifically, individuals grown at the same altitude differ remarkably on the phloridzin content, and a few individuals of *M. toringoides* distributed at low altitude even have higher phloridzin content than those grown at high altitude (Figure 4). As we discuss above, the distinction of phloridzin content among species is mainly due to genetic factors (Table 1, Table 2). However, the variation among individuals from the same species is more likely induced by heterogeneous environment factors. First of all, the genetic constitution could not serve as the primary cause of variation of phloridzin content among individuals. Besides *M. transitoria* and *M. Kansuensis*, other wild *Malus* species possess a single species specific genotype due to their apomictic reproduction (Tang et al.

2014). Thus, the fluctuation of content among individuals cannot be resulted from distinct genetic constitution. Moreover, environmental factors have been reported to influence the biosynthesis of phloridzin. For example, sunshine is an indispensable factor for phloridzin synthesis (Gosch et al. 2009), and Awad et al. (2000) found that the phloridzin content in fruit of shaded individuals is similar to ones under a canopy. Ehrenkranz et al. (2005) reported that phloridzin was responsible for both disease resistance and pathogen defense. Moreover, Mikulic-Petkovsek et al. (2011) showed that apple leaves infected with apple scab had accumulated higher content of phloridzin than those were not infected. In our study, individuals distributed under different micro-environment conditions, where the sunshine, soil, water and plant pathogens that affect the phloridzin content would be heterogeneous. Thus, the variation of phloridzin content in individuals may be caused by these heterogeneous environmental factors. Nevertheless, the underlying factors that influence the variation of phloridzin content may be complicated and need further investigation.

Table 2. Median and interquartile ranges of phloridzin contents (mg/100mg dry weight) from different populations of *M. toringoides*

Populations	Altitude ¹⁾	Number ²⁾	Leaves	Fruits
Chabei	2708	4	10.58 (6.28-11.03)	0.17 (0.03-0.21)
Nongkesuo	2642	9	8.78 (7.42-13.37)	0.06 (0.04-0.13)
Lianghe	3114	3	9.78 (7.71-11.15)	0.20 (0.14-0.37)
Kehe	3051	7	11.59 (9.83-11.93)	0.20 (0.13-0.31)
Angqiang	3118	13	11.08 (9.97-15.71)	0.34 (0.26-0.43)*

¹⁾ The mean value of altitudes of each population.

²⁾ The number of samples in each population.

* Population whose phloridzin content differs significantly from the phloridzin content of Chabei population at $p < 0.05$ according to multiple Wilcoxon rank sum test.

4. Conclusion

The phloridzin content in leaves and fruits of six wild *Malus* species are reported. The variation of phloridzin content among species is revealed, and genetic constitution is likely the primary cause of such variation. Significant difference of phloridzin content among individuals of the same species were indicated, which is probably resulted from environmental heterogeneity. Most interestingly, we found that wild *Malus* species have much higher phloridzin contents in leaves and fruits than various apple cultivars. These results shed some light on the factors that influence the variation of phloridzin content in wild *Malus* species, and provided useful information for novel utilization of valuable wild apple germplasm in China in future.

Acknowledgements

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