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Varietal Differences in the Flavonol Content of Mulberry (*Morus* spp.) Leaves and Genetic Analysis of Quercetin 3-(6-Malonylglucoside) for Component Breeding

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Supporting Information

ABSTRACT: The varietal differences in the flavonol glycosides rutin, isoquercitrin, kaempferol 3-(6-rhamnosylglucoside), quercetin 3-(6-malonylglucoside), astragalin, quercetin 3-(6-acetylglucoside), and kaempferol 3-(6-malonylglucoside) contained in mulberry leaves were elucidated. This information was used for breeding mulberry cultivars with a high concentration of functional components. The flavonol content, composition, and proportion in leaves varied widely. 'Kobuchizawa 1' had the highest level of total flavonols (1819 mg/100 g of dry weight), 5 times higher than that of 'Mikurasima 15' (393 mg/100 g of dry weight). Quercetin 3-(6-malonylglucoside) was the most abundant flavonol, although it was not found in all cultivars. Quercetin 3-(6-acetylglucoside) was only found in 'Keguwa'. From the quercetin 3-(6-malonylglucoside) content in crossbred offspring, malonyltransferase, an enzyme involved in quercetin 3-(6-malonylglucoside) synthesis, was acquired according to Mendelian inheritance. An offspring with a higher quercetin 3-(6-malonylglucoside) level than both parents was obtained from the crossing. This suggested that crossbreeding was effective for acquiring cultivars with a higher content of quercetin 3-(6-malonylglucoside).

KEYWORDS: *Flavonol, mulberry leaves, varietal difference, crossbreeding, quercetin 3-(6-malonylglucoside)*

INTRODUCTION

Mulberry is a crop plant traditionally used for silk-worm feed in Japan and has also been used as a food, herbal medicine, and tea, although no supporting scientific evidence of its beneficial effects had been shown. Recently, however, the functional component in mulberry leaves has attracted attention because of its preventive and treatment effects on lifestyle-related diseases, Alzheimer's disease, and cancer based on medical evidence from studies of its nutritional and active constituents.[1−6] It has been well-known that 1-deoxynojirimycin (DNJ), the functional component of mulberry, inhibits elevation of blood glucose levels. Several researchers studied the antioxidative effect of mulberry leaf extract[4,6−8] and reported that it is due to abundant antioxidative flavonoid components of mulberry leaves. Our previous study reported that the major component for antioxidative activity of low-density lipoprotein (LDL) in mulberry leaves is quercetin 3-(6-malonylglucoside), which is a flavonol glucoside.[4] Enkhmaa et al.[9] reported that quercetin 3-(6-malonylglucoside) prevents arterial sclerosis based on their animal experiment using LDL-receptor-deficient mice, and Katsube et al.[10] reported that quercetin 3-(6-malonylglucoside) improves the hyperglycemic state by reducing the oxidative stress in the liver. Therefore, antioxidative components of mulberry leaves, especially quercetin 3-(6-malonylglucoside), are considered very important for health improvement.

For the purpose of product development of mulberry leaves and improvement of its beneficial effects for human health, manipulation of the quantity of the functional components in the leaves via breeding would be useful. The mulberries cultivated for silk-worm feed and human food in Japan are mainly 'Ichinose' and 'Shinichinose'. These cultivars have not been developed for food use with the idea of maximizing the functional ingredients, because they were originally selected and bred for generating silk-worm feed. However, elucidation of such active substances will allow for the breeding to focus on the functional components. Several crop cultivars, such as rice,[11] soybean,[12] potato,[13] and rapeseed,[14] have already been bred to maximize their respective functional components: γ-aminobutyric acid, isoflavone, lipoxygenase, anthocyanin, carotenoid pigment, and linolenic acid. The number of reports on mulberry for edible use is substantially less than those for silk-worm feed. Most reports on flavonol in mulberry leaves pertain to *Morus alba* L.[4,6−8,15] In Japan, although *M. alba* L., *Morus kagayamae* Koidz., and *Morus multicaulis* Perr. are bred as the three major cultivars, the differences in their contents have not been studied. Also, the varietal differences have not been clarified, although it is the most important point in choosing parents for cross-breeding.

The purpose of the present study was to examine the varietal differences in flavonol content in mulberry to support the

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targeted breeding of mulberry and the possibility of cultivating the production of mulberry with high levels of quercetin 3-(6-malonylglucoside) by crossbreeding. We also discuss the year-to-year variation in flavonol content, because it is likely to be influenced by cultivation and environmental conditions.

MATERIALS AND METHODS

Plant Material. Mulberry (*Morus* spp.) plants collected and stored at the National Institute of Agrobiological Sciences (NIAS), Owashi, Tsukuba city, Ibaraki prefecture, Japan, were used. A total of 176 cultivars were sampled on August 1, 2007, August 5, 2008, and August 11, 2009, from which 59 cultivars were studied for year-to-year variation in their flavonol content over 3 years. Two trees were used of each cultivar. The youngest open leaves, where leaflets had completely opened but not attained a maximum size, and the following two leaves on the longest branch were sampled. Genetic distance, characteristics of morphology, and characteristics of cultivation were examined among 12 species. However, we focused on three species commonly cultivated in Japan, namely, *M. alba* L., *M. kagayamae* Koidz., and *M. multicaulis* Perr. The number of cultivars in each species was as follows: 2 *Morus nigriformis* Koidz., 8 *Morus notabilis* C.K.Schn., 42 *Morus bombysis* Koidz., 3 *Morus rotundiloba* Koidz., 1 *Morus acidosa* Griff., 4 *M. kagayamae* Koidz., 1 *Morus tiliaefolia* Makino., 1 *Morus mesozygia* Stapf., 1 *Morus microphylla* Buckl., 58 *Morus multicaulis* Perr., 48 *M. alba* L., 3 *Morus atropurpurea* Roxb., and 1 unknown.

Mode of Inheritance and Quercetin 3-(6-Malonylglucoside) Content in Crossed Offspring. The mode of inheritance of quercetin 3-(6-malonylglucoside) was investigated using the current year seedlings of crossed offspring grown at the Experimental Field of Shimane Agricultural Technology Center (Izumo city, Shimane prefecture, Japan) between the years 2008 and 2010. The scions were collected from the mulberry field at NIAS in January of each year. The collected cultivars were those containing quercetin 3-(6-malonylglucoside): 'Yonbaiseisou', 'Kokusou 21' (diploid), 'Kokusou 21' (tetraploid), 'Seijuurou', 'Jikunashi', 'Kokusou 20', 'Kanadasansou-A', 'Tanakaoushuu', 'Itouwase', 'Aizujyujima', and 'Negoyatakasuke', and those containing no quercetin 3-(6-malonylglucoside): 'Shounai-wase', 'Sousukewase', and 'Popberry'. The scions cut into pieces with three or four buds were grafted on the seedling stocks. They were heated at 27 °C with high humidity in a NC-350S incubator (NK System, Inc., Osaka, Japan). A total of 10 grafted seedlings were planted in a 27 × 30 cm plastic pot and cultivated in an air-conditioned room. They were crossed artificially after bloom, and the seeds preliminarily germinated at 30 °C over 3 or 4 days were planted in a 7.5 cm poly pot. The seedlings with four true leaves open were planted in the nursery with a density as furrow of 80 cm and spacing between planting of 50 cm. Cultivation and fertilization management was performed according to the established method. The youngest open leaves, where the leaflets have completely opened but not attained their maximum size, and the following two leaves on the longest branch were sampled once every year from the first 10 days of September to the last 10 days of October.

Extraction Procedure. Sampled leaves were dried in a MOV-212F convection drying oven (Sanyo Electric, Inc., Osaka, Japan) at 60 °C for 36−48 h. Dry matter samples were ground into a fine powder using an IFG-700G food mill (Iwatani, Inc., Tokyo, Japan). To extract the flavonols, 100 mg of dry mulberry leaf powder was suspended in 10 mL of 60% (v/v) ethanol aqueous solution and stirred for 3 h at 30 °C in a LTI-600 SD incubator (Tokyo Rikakikai, Co., Tokyo, Japan).[4] After centrifugation at 10000*g* for 5 min, the extracted solution was filtered through a 0.45 μm filter (Advantec MFS, Inc., Tokyo, Japan) and used for quantitative determination of flavonols.

Analysis. Flavonols in mulberry leaves were analyzed by an Alliance HPLC system (Waters Corporation, Milford, MA), equipped with an Alliance separations module 2695, a photodiode array detector 2996, an ODS Wakosil-II 5C18 RS column (4.6 × 250 mm) (Wako Chemicals, Inc., Osaka, Japan), and an ultraviolet (UV) detector. The column temperature, solvent, and flow rate was 40 °C, acetonitrile/0.1% formic acid (20:80), and 1 mL/min, respectively. The wavelength for the detection of flavonol was 350 nm. The standard flavonols quercetin 3-(6-malonylglucoside), kaempferol 3-(6-rhamnosylglucoside), and kaempferol 3-(6-malonylglucoside) were purified from the mulberry leaves according to the method by Katsube et al.[4] Rutin was obtained from Wako Chemicals, Inc. (Osaka, Japan), and quercetin 3-(6-acetylglucoside), isoquercitrin, and astragalin were obtained from Funakoshi, Inc. (Tokyo, Japan). The concentrations were analyzed by setting the standard concentration at 100 μM.

Statistical Analysis. Statistical analysis of the data was performed using version 9.0 JMP statistical analysis software (SAS Institute, Japan). Results were expressed as the mean ± standard error (SE). The data were tested by one-way analysis of variation (ANOVA) followed by Tukey's test for multiple comparisons. A significant difference was determined when $p < 0.05$.

RESULTS AND DISCUSSION

Year-to-Year Variations of the Flavonol Content. Figure 1 shows the total flavonol content in leaves of 59 mulberry cultivars in 2007, 2008, and 2009. The means of total flavonol content of 59 mulberry cultivars differed significantly between year 2007 and other years, with yearly averages of 1203 mg/100 g of dry weight (2007), 855 mg/100 g of dry weight (2008), and 871 mg/100 g of dry weight (2009). The mean total flavonol content was the highest in 2007 with a wide range from 550 to 1800 mg/100 g of dry weight. In contrast, in 2008 and 2009, the range of the mean was not so wide compared to 2007. Figure 2 shows the scatter diagrams of the content of the major flavonol quercetin 3-(6-malonylglucoside) in each mulberry cultivar for 2007, 2008, and 2009. The correlation coefficients of quercetin 3-(6-malonylglucoside) content from year-to-year were 0.91, 0.84, and 0.87 between 2007 and 2008, between 2008 and 2009, and between 2009 and 2007, respectively. The multiple correlation coefficient was 0.88 among the 3 years. These results indicate that the relative quercetin 3-(6-malonylglucoside) content is constant, irrespective of the year of harvest. This tendency is observed for both the total flavonol content and the amounts of other components. Table 1 shows the means of the proportions of flavonol glycosides in four representative mulberry cultivars for 3 years. The proportion of each flavonol glycoside was relatively constant in each cultivar with very little yearly fluctuation. Annual variation in yield and quality of crops is generally caused by climate conditions. Also, it is known that the content of functional components is sensitive to differences in the cultural environment. The flavonol synthesis occurred through

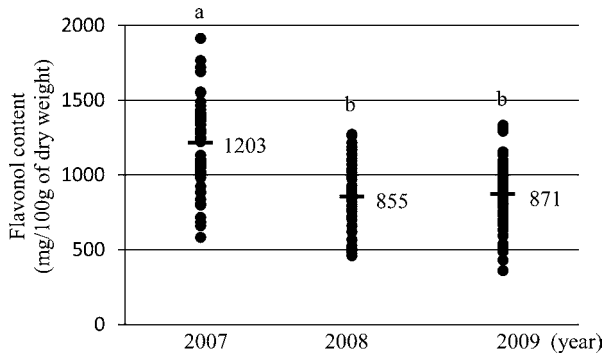

Figure 1. Year-to-year variations of flavonol content per dry weight of leaves. Horizontal short bars and numbers in the figure indicate the means of 59 cultivars for each year. Data were analyzed by one-way ANOVA followed by Tukey's test. There was a significant difference between a and b ($p < 0.05$).

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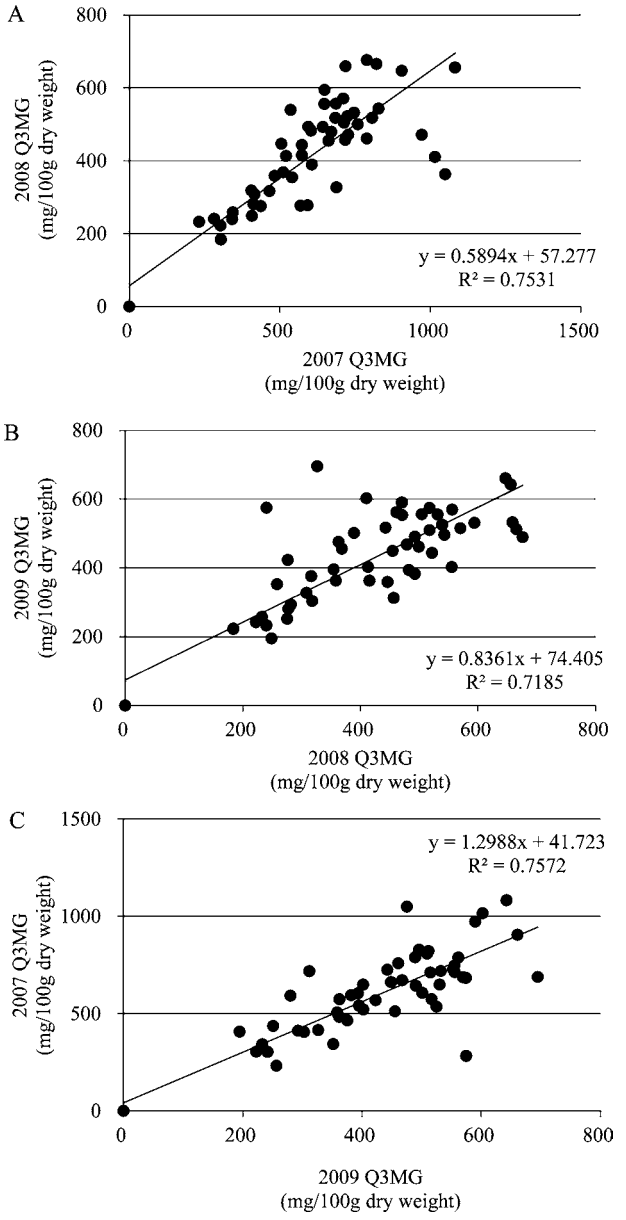

Figure 2. Correlation of quercetin 3-(6-malonylglucoside) (Q3MG) content in each cultivar between years (A) 2007 and 2008, (B) 2008 and 2009, and (C) 2007 and 2009. A circle indicates each mulberry cultivar. The multiple correlation coefficient was 0.88. Each year-to-year correlation was 0.91 (2007 versus 2008), 0.84 (2008 versus 2009), and 0.87 (2007 versus 2009).

the flavonoid pathway in a similar fashion as anthocyanin and proanthocyanidin. Fujita et al.[20] reported that flavonol and anthocyanin biosyntheses were regulated by different systems in grape berry skins because of their different responses to light and plant hormones. The annual variation of flavonol content in mulberry leaves would also be influenced by the amount of solar radiation that the plant receives. Future research should involve studies into other cultural conditions that influence the flavonol content in mulberry leaves.

Varietal Differences of the Flavonol Content. A total of 176 mulberry cultivars were investigated for 1−3 years. The flavonol content for each year was corrected because it varied between year 2007 and other years. Because the ratio of the total flavonol content and the composition of flavonol glycoside between cultivars of each year were relatively constant, they were used as indices. The mean total flavonol content of 59 cultivars in 2007 was used as the standard value (1203 mg/100 g of dry weight), and the values for 2008 (855 mg) and 2009 (871 mg) were corrected to compare to the value of 2007. First, the mean of 2007 was divided by those of 2008 and 2009. Using the resulting values of 1.41 and 1.38, each flavonol glycoside of 2008 and 2009 was calculated by multiplication of each value. The seven flavonol glycosides contained in mulberry leaves were rutin, isoquercitrin, kaempferol 3-(6-rhamnosylglucoside), quercetin 3-(6-malonylglucoside), quercetin 3-(6-acetylglucoside), astragalin, and kaempferol 3-(6-malonylglucoside). Quercetin 3-(6-malonylglucoside) was found in the highest abundance in 176 mulberry cultivars as represented by the species *M. bombysis* Koidz., *M. alba* L., and *M. multicaulis* Perr. (Table 1 and Figure 3). There have been some reports regarding flavonol contained in mulberry leaves. Onogi et al.[16] detected isoquercitrin, quercetin 3-(6-acetylglucoside), astragalin, and kaempferol 3-(6-acetylglucoside). Kim et al.[7] detected quercetin 3-glucoside, quercetin 3-(6-acetylglucoside), rutin, and quercetin. Naowaratwattana et al.[6] detected rutin, isoquercitrin, kaempferol 3-(6-rhamnosylglucoside), quercetin 3-(6-malonylglucoside), astragalin, kaempferol 3-(6-malonylglucoside), quercetin dicoumaryl glycoside, and kaempferol dicoumaryl glycoside. Choi et al.[8] detected quercetin, kaempferol, and astragalin. All of these researchers used *M. alba* L. Thabti et al.[15] stated that their *M. alba* and *Morus rubra* contained kaempferol 3-(6-malonylglucoside), astragalin, kaempferol 7-glucoside, kaempferol 3-(6-rhamnosylglucoside), quercetin 3-(6-malonylglucoside), quercetin 3-glucoside 7-rhamnoside, quercetin 3-glucoside 7-glucoside, and quercetin 3,7-diglucoside. Differences of flavonols that they detected are believed to be related to differences of the extraction methods. The flavonol components found in our study were similar to those by Naowaratwattana et al.,[6] except for quercetin dicoumaryl glycoside and kaempferol dicoumaryl glycoside.

Table 1. Relative Proportions of Flavonol Glycosides in Four Representative Mulberry Cultivars[a]

cultivar	rutin (%)	isoquercitrin (%)	K3RG (%)	Q3MG (%)	astragalin (%)	Q3AG (%)	K3MG (%)
Ichinose	13.6 ± 0.7	3.9 ± 0.1	3.0 ± 0.2	53.5 ± 1.4	2.9 ± 0.2	0	23 ± 1.5
Itouwase	32.4 ± 3.3	11 ± 3.5	11.5 ± 3.2	29.1 ± 7.0	3.2 ± 0.5	0	12.7 ± 2.8
Popberry	45.7 ± 1.4	25.5 ± 0.8	23.5 ± 1.1	0	5.4 ± 0.5	0	0
Keguwa	24.4 ± 2.2	31 ± 1.3	0.6 ± 0.3	24.8 ± 0.6	1.3 ± 0.1	15.7 ± 1.3	2.1 ± 0.1

[a]Abbreviations: K3RG, kaempferol 3-(6-rhamnosylglucoside); Q3MG, quercetin 3-(6-malonylglucoside); Q3AG, quercetin 3-(6-acetylglucoside); and K3MG, kaempferol 3-(6-malonylglucoside). All mulberry cultivars are classified into four groups: Ichinose (largest Q3MG proportions; 155/176 cultivars), Itouwase (small Q3MG proportions; 6/176 cultivars), Popberry (no Q3MG; 14/176 cultivars), and Kegwa (uniquely contains Q3AG; 1/176 cultivars). Data represent the mean ± SE over 3 years.

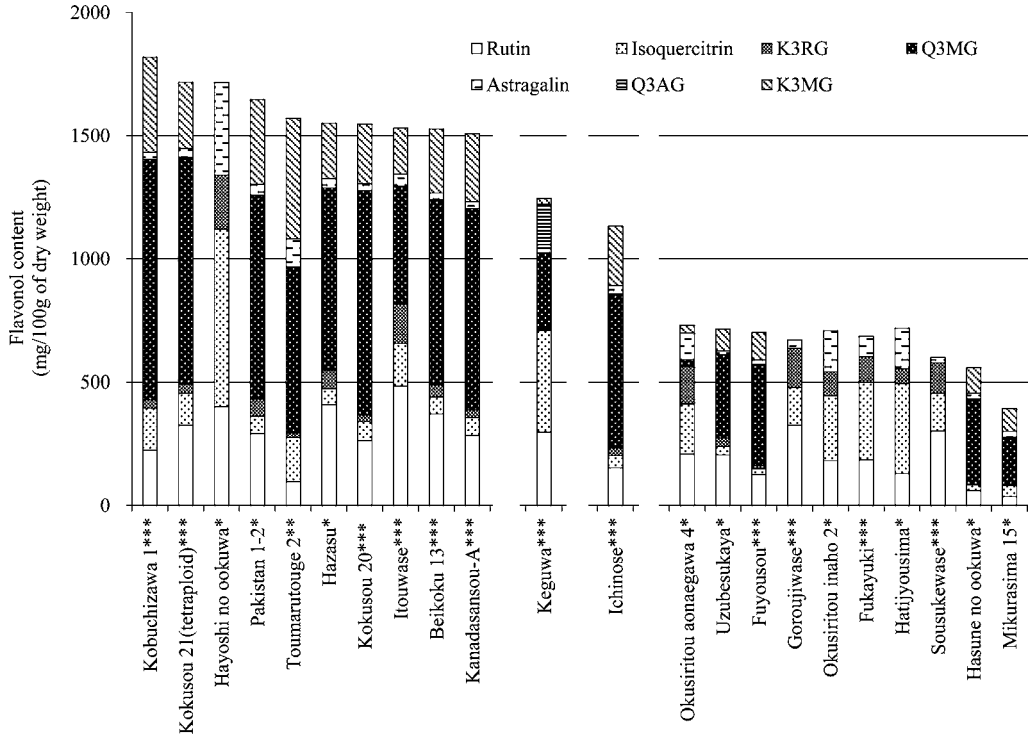

Figure 3. Flavonol content of mulberry cultivars. The cultivars shown are the 10 containing the highest and the 10 containing the lowest amounts of total flavonols among the 176 cultivars examined. In the center, 'Keguwa' is shown as having an unique component and 'Ichinose' is shown as the most commonly seen cultivar. Cultivars were analyzed for 1−3 years. (*) tested for only 1 year, (**) mean of 2 years, and (***) mean of 3 years. Abbreviations: K3RG, kaempferol 3-(6-rhamnosylglucoside); Q3MG, quercetin 3-(6-malonylglucoside); Q3AG, quercetin 3-(6-acetylglucoside); and K3MG, kaempferol 3-(6-malonylglucoside).

Figure 3 shows the total flavonol content of the 10 highest and 10 lowest cultivars in order among the 176 cultivars and 'Ichinose', which is mainly cultivated in Japan. The amounts of flavonol were widely different among the mulberry cultivars, being highest in 'Kobutizawa 1' (1819 mg/100 g of dry weight) and lowest in 'Mikurasima 15' (393 mg/100 g of dry weight), showing approximately 20% of the former. The flavonol content of 73 cultivars was higher than that of 'Ichinose'. There were also significant differences in the amount of quercetin 3-(6-malonylglucoside) among cultivars; for example, 'Kobutizawa 1' (1082 mg/100 g of dry weight) was 2-fold higher than 'Ichinose' (607 mg/100 g of dry weight).

The composition of flavonol glycoside in mulberry leaves depended upon the cultivar (Figure 3), where about 90% contained flavonol glycosides, including rutin, isoquercitrin, kaempferol 3-(6-rhamnosylglucoside), quercetin 3-(6-malonylglucoside), astragalin, and kaempferol 3-(6-malonylglucoside), in which quercetin 3-(6-malonylglucoside) showed the highest abundance. The flavonol glycosides from 176 mulberry cultivars of 12 species were classified into the following four groups: (1) Ichinose-type group [containing multiple components except for quercetin 3-(6-acetylglucoside)], 161 cultivars; (2) Popberry-type group [containing multiple components except for quercetin 3-(6-malonylglucoside), kaempferol 3-(6-malonylglucoside), and quercetin 3-(6-acetylglucoside)], 14 cultivars; (3) Keguwa-type group [containing quercetin 3-(6-acetylglucoside)], 1 cultivar; and (4) Itouwase-type group [containing multiple components including a small amount of quercetin 3-(6-malonylglucoside) but no quercetin 3-(6-acetylglucoside)]. We identified two distinct characteristics regarding the composition of mulberry cultivars. Quercetin 3-(6-acetylglucoside) was contained only in 'Keguwa'. The other cultivars were divided into two groups: cultivars that contained both quercetin 3-(6-malonylglucoside) and kaempferol 3-(6-malonylglucoside) and cultivars that did not. The abundant flavonols were rutin (45−61%) in eight cultivars without quercetin 3-(6-malonylglucoside) and kaempferol 3-(6-malonylglucoside) and isoquercitrin (39−55%) in six cultivars.

Table 2 shows the examined species, the number of cultivars, and those found to contain no quercetin 3-(6-malonylglucoside). A total of 5 of the tested 12 species contained no quercetin 3-(6-malonylglucoside). The cultivars without quercetin 3-(6-malonylglucoside) included 8 cultivars of *M. bombysis* Koidz. (of 42 tested), 2 cultivars of *M. rotundiloba*

Table 2. Cultivars without Quercetin 3-(6-Malonylglucoside) (Q3MG) Identified in Mulberry Species

	number of cultivars	number of Q3MG-free cultivars
M. nigriformis Koidz.	2	0
M. notabilis C.K.Schn.	8	0
M. bombysis Koidz.	42	8
M. rotundiloba Koidz.	3	2
M. acidosa Griff.	1	0
M. kagayamae Koidz.	4	1
M. tiliaefolia Makino.	1	0
M. mesozygia Stapf.	1	0
M. microphylla Buckl.	1	0
M. multicaulis Perr.	58	2
M. alba L.	49	1
M. atropurpurea Roxb.	3	0

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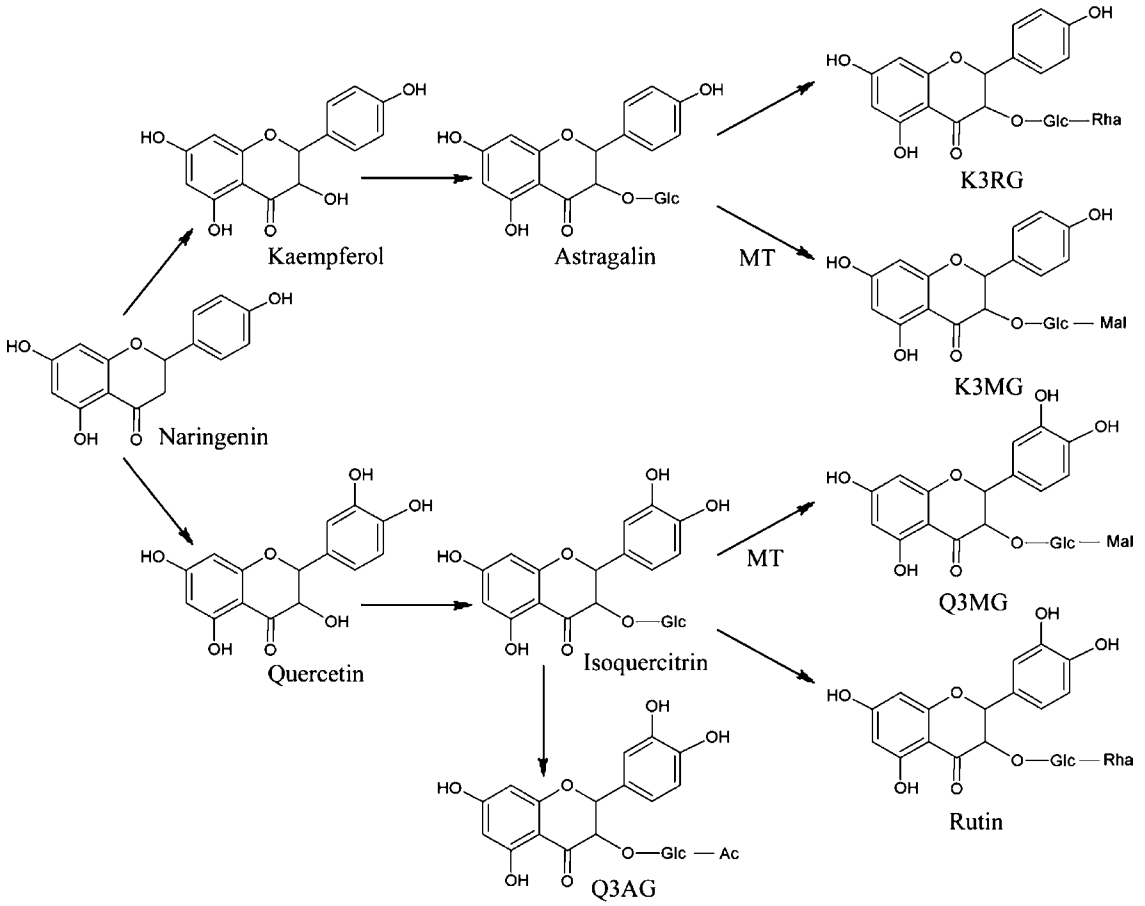

Figure 4. Proposed metabolic pathway of flavonol glycosides in mulberry in our study. Abbreviations: MT, malonyltransferase; K3RG, kaempferol 3-(6-rhamnosylglucoside); Q3MG, quercetin 3-(6-malonylglucoside); Q3AG, quercetin 3-(6-acetylglucoside); and K3MG, kaempferol 3-(6-malonylglucoside).

Koidz. (of 3 tested), 1 cultivar of *M. kagayamae* Koidz. (of 4 tested), 2 cultivars of *M. multicaulis* Perr. (of 58 tested), and 1 cultivar of *M .alba* L. (of 49 tested). The ratio of cultivars without quercetin 3-(6-malonylglucoside) was low in *M. multicaulis* Perr. and *M. alba* L. The flavonoid biosynthetic pathway and its regulation have been studied in plants.[17,18] Figure 4 shows the presumed flavonol pathway in mulberry leaves. Generally the aglycones synthesized from naringenin are kaempferol, quercetin, and myricetin. Kaempferol and quercetin glycoside are contained in mulberry, while myricetin glycoside is not. Only 'Keguwa' contained quercetin 3-(6-acetylglucoside), while the other cultivars did not. Thus, 'Keguwa' is considered to specifically contain acetyltransferase activity. Wild *M. tiliaefolia* Makino var. Keguwa obtained in Sakurae-cho, Gotsu city, Shimane prefecture, Japan, also contained quercetin 3-(6-acetylglucoside) based on our analysis. On the other hand, there were several cultivars that did not produce the main mulberry flavonol, quercetin 3-(6-malonylglucoside). These cultivars included neither quercetin 3-(6-malonylglucoside) nor kaempferol 3-(6-malonylglucoside). It was believed that a malonyltransferase was not involved in the flavonol synthesis in these cultivars. Many cultivars without quercetin 3-(6-malonylglucoside) belong to the Japanese local species *M. bombysis* Koidz., while a few cultivars belong to the exotic species *M. multicaulis* Perr. and *M. alba* L. The minor species, *M. rotundiloba* Koidz. and *M. kagayamae* Koidz., included cultivars without quercetin 3-(6-malonylglucoside) at a high ratio of $^{2}/_{3}$ and $^{1}/_{4}$, respectively. This suggests that deletion of malonyltransferase results in a local specificity. Sharma et al.[19] classified 21 mulberry species into four groups by amplified fragment length polymorphism (AFLP) analysis: *M. alba* L. and *M. bombysis* Koidz. are in the same group because they are close in genetic distance, while *M. tiliaefolia* Makino. is in a different group because it is genetically distant. It was considered that *M. multicaulis* Perr., *M. alba* L., and *M. bombysis* Koidz. can easily cross with each other because of their close genetic distance, but *M. tiliaefolia* Makino. is difficult to cross with other species. On the basis of their genetic background, we considered that *M. bombysis* Koidz. has many species that contain quercetin 3-(6-malonylglucoside), while *M. tiliaefolia* Makino. is the only species that specifically contains quercetin 3-(6-acetylglucoside). Kim et al.[7] detected quercetin 3-(6-acetylglucoside) in *M. alba* L., although it was not detected in Japanese *M. alba* L in our study. This is possibly due to geographic isolation: genetic diversity on a continent versus specificity on an island country.

The quercetin 3-(6-malonylglucoside) content in all 176 cultivars ranged between 30 and 62%, except for 14 cultivars that had none, and comparatively, 'Shukutsu no ookuwa' had 5% and 'Okushiritou aonaegawa' had 4%. The histogram (Figure 5) indicates the truncated normal distribution, which shows 60% as the upper limit. This result suggests that the hereditary limit of quercetin 3-(6-malonylglucoside) synthesis is about 60%; therefore, an increase of quercetin 3-(6-malonylglucoside) content would not be expected as a result

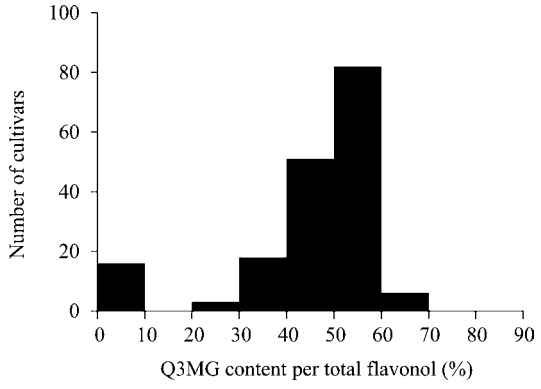

Figure 5. Frequency distribution of the percent of quercetin 3-(6-malonylglucoside) (Q3MG) content per total flavonol content.

of the increasing quercetin 3-(6-malonylglucoside) ratio to total flavonol via breeding.

Figure 6 shows the relationship between the total flavonol and quercetin 3-(6-malonylglucoside) contents of each cultivar.

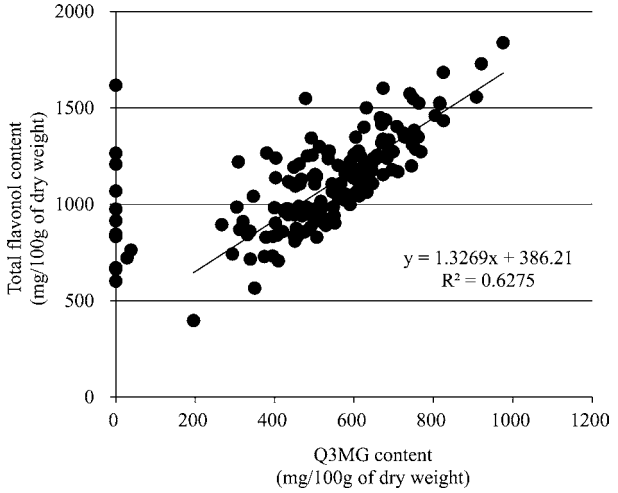

Figure 6. Correlation between the total flavonol and quercetin 3-(6-malonylglucoside) (Q3MG) contents for each cultivar. The regression line includes all points, except for those cultivars that were Q3MG-free. Each cultivar is represented by one black circle.

A positive correlation (R^2 = 0.6275) was found between the total flavonol and quercetin 3-(6-malonylglucoside) contents of each cultivar. A regression line and a correlation efficient were calculated for all cultivars, except for those that had no quercetin 3-(6-malonylglucoside). This finding suggests that the increase of the quercetin 3-(6-malonylglucoside) content was related to the increase of the total flavonol content. No relationship was recognized between the total flavonol content and the mulberry species (data not shown).

Mode of Inheritance and Content Rate of Quercetin 3-(6-Malonylglucoside). We focused on the presence of quercetin 3-(6-malonylglucoside) in mulberry leaves, because our purpose was to breed mulberry, which maximizes this particular functional component. The mode of inheritance of quercetin 3-(6-malonylglucoside), the breeding target, was examined with respect to the species, the total flavonol content, and the presence or absence of various specific flavonols. The appearance of cultivars without quercetin 3-(6-malonylglucoside) was examined using seedlings of crossed offspring from cultivars with quercetin 3-(6-malonylglucoside) and those without quercetin 3-(6-malonylglucoside) (Figure 3). The rate of no quercetin 3-(6-malonylglucoside) appearance was 100% in offspring, resulting from crossing between cultivars with and without quercetin 3-(6-malonylglucoside), and it was 50% in subsequent offspring. Furthermore, the appearance ratio was 11% in the crossing between 'Kokusou 20' (diploid) and 'Kokusou 21' (tetraploid), which is close to 12.5%, the appearance ratio of a recessive homozygote. Table 3 shows the assumable genotype of the parents from the appearance ratio of the seedling. We concluded that the malonyltransferase gene that is involved in quercetin 3-(6-malonylglucoside) synthesis was accompanied by Mendelian dominant inheritance. 'Kokusou 20' and 'Kokusou 21' were assumed to have a heteroallele for malonyltransferase, because the quercetin 3-(6-malonylglucoside) content of both cultivars was high (Figure 3). Therefore, dominance of the homozygote was not necessarily required to yield a high content of quercetin 3-(6-malonylglucoside). Figure 7 shows the frequency distribution of quercetin 3-(6-malonylglucoside) content in the seedling of crossbred offspring between 'Kanadasansou-A' and 'Tanakaoushuu'. The histogram shows a normal distribution. An individual was obtained with a higher content of quercetin 3-(6-malonylglucoside) than both parents. Similar results were obtained with other combinations.

To date, there have been no reports of breeding studies that aim to elucidate the functional components contained in mulberry leaves. 'Tsuruta' was found to contain a high DNJ content,[3] and 'Souki' (cultivar accession number 16963) was registered as a functional food material by Soka University, although these cultivars were originally bred for use in sericulture. In this study, many cultivars were identified that had higher flavonol concentrations than the commonly cultivated 'Ichinose'. However, for breeding purposes, the crossing parents must be carefully considered. For example, many cultivars are dioecious; 'Kobutizawa 1', which contained the highest quercetin 3-(6-malonylglucoside) concentration (975 mg/100 g of dry weight), is triploid and, thus, was identified as the cause of breeding inefficiency by Koyama et al.[21] Some cultivars, such as 'Tanakaoushuu', have a lot of lateral branches, which is an unfavorable characteristic for cultivation because of the difficulty of regermination. The male cultivar 'Kokusou 21' (tetraploid) [quercetin 3-(6-malonylglucoside) concentration of 920 mg/100 g of dry weight] and the female cultivar 'Kokusou 20' are suitable crossing parents because they provide a high yield and it is easy to obtain the seedlings by crossing with a tetraploid and diploid. This result suggests that crossbreeding is effective to acquire cultivars with higher concentrations of functional components. Presently, we are conducting breeding for purposes of acquiring cultivars with a high concentration of quercetin 3-(6-malonylglucoside).

In summary, the content, composition, and proportion of flavonols in mulberry leaves varied widely. The presence of flavonol, quercetin 3-(6-malonylglucoside), was the most abundant and valuable, although it was not found in all cultivars. Malonyltransferase, an enzyme involved in quercetin 3-(6-malonylglucoside) synthesis, was acquired according to Mendelian inheritance. Our findings suggest that crossbreeding is effective for acquiring cultivars with a higher content of quercetin 3-(6-malonylglucoside).

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Table 3. Mode of Inheritance of Quercetin 3-(6-Malonylglucoside) (Q3MG)[a]

cross combination					
♀		♂			
cultivar	genotype of Q3MG	cultivar	genotype of Q3MG	number of seeds bred	number of Q3MG-free
Yonbaiseisou	MM	Kokusou 21	Mm	282	2 (1%)
Seijuro	MM	Kokusou 21	Mm	54	0
Jikunashi	MM	Kokusou 21	Mm	74	0
Kokusou 20	Mm	Kokusou 21 (4×)[b]	MMmm	225	25 (11%)
Kanadasansou-A	MM	Kokusou 21 (4×)	MMmm	155	0
Kokusou 20	Mm	Tanakaoushuu	MM	132	0
Kanadasansou-A	MM	Tanakaoushuu	MM	132	0
Jikunashi	MM	Shounaiwase[c]	mm	62	0
Itouwase	Mm	Shounaiwase[c]	mm	53	30 (57%)
Shounaiwase[c]	mm	Aizujushima	Mm	55	34 (62%)
Shounaiwase[c]	mm	Nekoyatakasuke	Mm	44	20 (45%)
Popberry[c]	mm	Nekoyatakasuke	Mm	56	23 (41%)
Shounaiwase[c]	mm	Shounaiwase[c]	mm	58	58 (100%)
Popberry[c]	mm	Shounaiwase[c]	mm	10	10 (100%)

[a]Genotype was assumed on the basis of the presence or absence of malonyltransferase as dominant (M) or recessive (m) based on the resulting ratio of Q3MG-free offspring. [b]4× = tetraploid. [c]Q3MG-free cultivars.

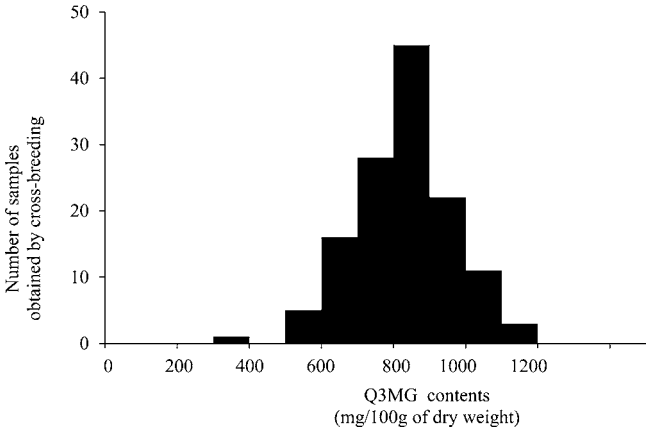

Figure 7. Frequency distribution of quercetin 3-(6-malonylglucoside) (Q3MG) content in the offspring obtained by crossing 'Kanadasansou-A' (Q3MG content of 607 mg/100 g of dry weight) and 'Tanakaoushuu' (Q3MG content of 599 mg/100 g of dry weight).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Table of flavonol content of mulberry cultivar. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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