Food Sci. Biotechnol. 25(4): 1035-1041 (2016)
DOI 10.1007/s10068-016-0167-7

Food Science
and
Biotechnology

Comprehensive metabolic profiles of mulberry fruit (*Morus alba* Linnaeus) according to maturation stage

Kyung-Min Lee, Taek-Joo Oh, So-Hyun Kim, Hye-Youn Kim, Hyunmi Chung, Daniel Seungwook Min[1], Joong-Hyuck Auh[2], Hong Jin Lee[2], Jaehwi Lee, and Hyung-Kyoon Choi*

College of Pharmacy, Chung-Ang University, Seoul 06974, Korea
[1]*Taejon Christian International School, Daejeon 34035, Korea*
[2]*Department of Food Science and Technology, Chung-Ang University, Anseong, Gyeonggi 17546, Korea*

Received May 2, 2016
Revised June 13, 2016
Accepted June 13, 2016
Published online August 31, 2016

*Corresponding Author
Tel: +82-2-820-5605
Fax: +82-2-812-3912
E-mail: hykychoi@cau.ac.kr

pISSN 1226-7708
eISSN 2092-6456

Abstract In this study, comprehensive metabolic profiles of mulberry fruits (*Morus alba* Linnaeus*)* at various maturation stages were determined using GC-MS and HPLC. In total, 48 compounds, including 3 alcohols, 16 amino acids, 7 organic acids, 2 sugars, 4 phenolics, 2 terpenes, 3 vitamins, 9 fatty acids, and 2 cyanidins were identified in the mulberry samples. Levels of chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid, ascorbic acid, and δ-tocopherol, and total fatty acid content were significantly higher in the semi-matured mulberry fruits. Furthermore, levels of glycerol, citrate, fructose, glucose, 3-*O*-glucoside, and cyanidin-3-*O*-rutinoside were significantly higher at the fully matured stage than at the other stages. Twelve biosynthetic pathways were suggested as major pathways involved in mulberry fruit maturation. The information obtained in this study will provide a basis for future investigations toward quality control or metabolic engineering for development of mulberry fruits possessing commercially valuable characteristics.

Keywords: gas chromatography-mass spectrometry, maturation stage, metabolic profile, mulberry fruit

Introduction

The mulberry (*Morus alba* Linnaeus), which belongs to the family Moraceae, is a fast-growing deciduous tree that is widely cultivated in the subtropical regions of Asia, Africa, and the Americas (1). The interest in the mulberry fruit as a functional food has increased because of its potential bioactivities, including antitumorigenic (2,3), neuroprotective (4), immunomodulating (5), antioxidant (6,7), antidiabetic (8), and hypolipidemic (9) activities. It was reported that those bioactivities were related to anthocyanins (2,6), flavonoids (3,8), polysaccharides (5), chlorogenic acids (6,8), and phenolics (7) in mulberry fruits, and most of the compounds were investigated by a targeted analysis approach. Especially, a study sought to elucidate the contributing compounds involved in free-radical scavenging activity of mulberry fruits at various stages of maturity using targeted HPLC, which resulted in the identification of only two chlorogenic acids and two anthocyanins (6).

Recently, GC-MS-based metabolic profiling has been applied to various plants, and it is a powerful tool for the investigation of plant metabolic responses (10,11). However, no study has provided comprehensive metabolic profiling of mulberry fruits according to various maturation stages. It is known that comprehensive metabolic profiling is the key to plant metabolic engineering (12). Thus, the understanding of comprehensive metabolic profiles of mulberry fruits at different maturation stags is important for the development of mulberry fruits with commercially desirable characteristics.

Our main hypothesis was that metabolic profiles of mulberry fruits differ during the process of maturation. In the present study, comprehensive metabolic profiles, including fatty acids and anthocyanins of mulberry fruits at various maturation stages were investigated by GC-MS and HPLC based metabolic profiling. The main objective of this study was to identify changes in metabolic profiles of mulberry fruits according to their stage of maturity, and to provide a basis for future investigations toward quality control or metabolic engineering for development of mulberry fruits possessing commercially valuable characteristics.

Materials and Methods

Plant materials Mulberry fruits (*Morus alba* L.) at different stages of maturation were harvested in between the month of August and September, 2013 at a local farm in Buan-gun, Jeollabuk-do, Republic of Korea, which lies between 35°43' North latitude and 126°44' East

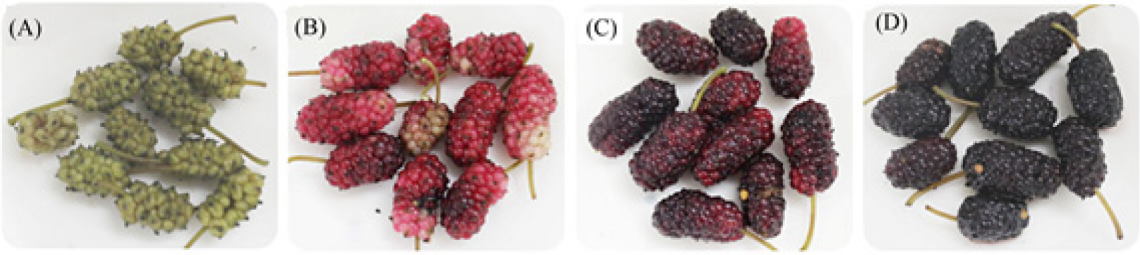

Fig. 1. Characteristics of mulberry fruits at different stages of maturation. (A) Immature, (B) Semi-mature, (C) Mature, and (D) Fully mature

longitude. After harvesting, the fruits were immediately washed with distilled water, freeze-dried, ground into powder using mortar and pestle, and stored at −70°C. A voucher specimen was deposited at the College of Pharmacy, Chung-Ang University in the Republic of Korea (CAUR 20131030-20131070). Those samples were classified into four groups based on their stage of maturity: immature, semi-mature, mature, and fully mature as presented in Fig. 1. The color values of mulberry fruits during maturity were measured by a spectrophotometer equipped with a pulsed xenon arc lamp (CM-3500d; Minolta, Osaka, Japan) to provide objective criteria for sample selection. Three individual fruit (biological replication) samples in each maturation stage group were used for color analysis. The values were obtained using the Commission Internationale de l'Eclairange method and those were shown in Table 1. L* represented the lightness value, positive and negative a* values were measures of redness and greenness, respectively, and positive and negative b* values were measures of yellowness and blueness, respectively. The highest values of L* and b* were in immature mulberry fruits (62.66±0.02 and 24.76±0.04, mean±SEM, respectively), and the values decreased as maturation proceeded. The value of a* was highest in matured mulberry fruits (17.88±0.07), followed by that in fully matured fruits. Using the colorimetric data analysis, mulberry fruit samples from various maturation stages were selected for further GC-MS analysis.

Table 1. Color values of mulberry fruits at different stages of maturation

Sample	L*	a*	b*
IM[1)]	62.66±0.02[a2)]	-0.42±0.03[d]	24.76±0.04[a]
SM	44.98±0.04[b]	15.57±0.03[b]	10.32±0.02[b]
M	35.10±0.10[c]	17.88±0.07[a]	6.87±0.04[c]
FM	29.50±0.01[d]	12.66±0.08[c]	4.63±0.11[d]

[1)]IM, Immature; SM, Semi-mature; M, Mature; FM, Fully-mature
[2)]Data represent the means±SEM (n=3). All values in different groups within the same row were significantly different at p<0.05 (Tukey's test).

Comprehensive metabolite analysis For comprehensive metabolite profiling, 30 mg of each sample at the various stages of maturation was transferred into a GC vial (Agilent Technologies, Santa Clara, CA, USA) and extracted with 1 mL of methanol and hexane. After sonication for 40 min at room temperature, the supernatant was collected from each extracted sample and filtered through a 0.2-μm PTFE Syringe filter (Whatman, Maidstone, UK). A trimethylsilylation (TMS) derivatization reaction in each methanol extract was performed before GC-MS analysis. Briefly, 100 μL of each sample solution was transferred into a clear GC vial and dried with nitrogen gas flow. A 30 μL aliquot of methoxylamine hydrochloride (20,000 μg/mL), 50 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane, and 10 μL of 2-chloronaphthalene were added to the dried content in the vials. The derivatized samples were incubated for 60 min at 60°C. Finally, each sample was extracted with hexane and methanol and was used to extract various compounds including fat-soluble and low polar compounds and to detect non-polar compounds more clearly for GC-MS analysis.

For the analysis of global metabolites, a GC system (model 7890A; Agilent Technologies) equipped with a mass selective detector (model 5975C; Agilent Technologies) was used. In brief, a 1-μL injection volume was injected into a split/splitless inlet at 250°C. Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. Electron impact mode with ionization energy of 70 eV and the split ratio of 1:10 was used. The data were obtained in the full scan mode with the mass range of 50-600 Da. The auxiliary, ion source, and quadrupole temperatures were maintained at 280, 230, and 150°C, respectively. The oven temperature for global metabolite analysis was 70°C programmed to 150°C (5°C/min), then to 250°C (3°C/min), and then to 320°C (10°C/min; hold 3 min).

The metabolites were identified by comparison of mass spectra with those of NIST-Wiley Mass Spectra Library and commercially available standards. The automated mass spectral deconvolution and identification system (AMDIS, http://chemdata.nist.gov/mass-spc/amdis/) with an online peak-filtering algorithm (SpectConnect, http://spectconnect.mit.edu) was used to analyze the acquired full scan datasets. Normalization to an internal standard (2-chloro-naphthalene) peak area was performed to calculate relative amounts of each metabolite. The values of metabolites were reported as means±SD of total 6 replicates (3 biological and 2 technical replicates).

FAME analysis Fatty acid composition of lipids from mulberry fruits at the different stages of maturation was evaluated by fatty acid methyl esters (FAMEs) as described previously with minor modification (13). Freeze-dried mulberry fruit samples (10 mg) were weighed in glass vials with polytetrafluoroethylene (PTFE)-lined caps (Agilent Technologies). For solubilization and transesterification, 0.4 mL of

chloroform/methanol (2:1, v/v) and 0.6 mL of 1.25 M methanolic hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were added to the vials and incubated for 1 h at 85°C. After the transesterification reaction, 1 mL of hexane was added to the vials and incubated for 1 h at room temperature in order to extract the resulting FAMEs. Before GC-MS analysis, 10 μL of 2-chloronaphthalene (Tokyo Chemical Industry, Tokyo, Japan) as an internal standard (100 mg/L) was added to 90 μL of the upper phase, and the mixture was vortexed.

FAMEs analysis was performed using a VF-WAXms column (30 m × 0.25 i.d., mm × 0.25 μm film thickness, Agilent Technologies) with a GC system (model 7890A; Agilent Technologies) equipped with a mass selective detector (model 5957C; Agilent Technologies). Mass spectra were acquired within the range of m/z 40-400 under full scan mode. The initial oven temperature of 100°C was held for 5 min before ramping to 215°C at 10°C min^{-1}, and then to 250°C at 7°C min^{-1}. The relative values of metabolite were reported as means±SD of total 6 replicates (3 biological and 2 technical replicates).

Anthocyanin analysis Fifty milligrams of each sample was transferred into a microfuge tube (Eppendorf, Hamburg, Germany) and extracted with 1 mL of methanol using sonication for 40 min at room temperature. The supernatant from the extracts were filtered through a 0.2-μm PTFE Syringe filter (Whatman). The extracted samples were analyzed using a HPLC system, equipped with an ultraviolet detector L-2400, an autosampler L-2200, pump L-2130 (Hitachi, Tokyo, Japan), and online-degasser ERC-3415 α (ERC, Tokyo, Japan). Column temperature was controlled with a metatherm™ column oven (Varian, Palo Alto, CA, USA). The separation was achieved on a Luna C18 column (25 cm × 4.6 mm i.d., 5 μm, Phenomenex Inc., Torrance, CA, USA). The chromatographic data were acquired and analyzed using EZchrom Elite software (Hitachi). The solvent system for determination of anthocyanin consisted of acetonitrile (A) and 0.1% aqueous formic acid (B). The gradient conditions were: 0-10 min, 10-15% A; 10-20 min, 15-28% A; 20-22 min, 28-40% A (at a flow rate of 0.8 mL min^{-1}); 22-24 min, 40-60% A; 24-30 min, 60-65% A; 30-35 min, 5% A (at a flow rate of 1 mL min^{-1}). The wavelength for detection was 520 nm. Ten microliters of the samples were analyzed in this investigation.

Each component analyzed by HPLC was quantified using the calibration curve constructed from each standard. The standard concentrations of cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside were respectively, 0.30 and 0.29 mg mL^{-1}. The accuracy of the method was evaluated by means of a recovery test. The relative values of anthocyanin were reported as means±SEM of total 10 replicates (5 biological and 2 technical replicates).

Data analysis and enrichment analysis The relative intensities and significant differences of assigned metabolite levels in each sample by GC-MS analysis were detected by one-way analysis of variance (ANOVA) using SPSS Statistics 21 software (IBM, Somers, NY, USA) followed by Tukey's significant-difference test. The level of statistical significance was set at $p<0.05$. A functional enrichment analysis with identified metabolic data for biological information was performed using MBRole (14). The interface used the ID conversion utility, which performed the analysis using the metabolites identified by KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/). *Arabidopsis thaliana* was selected as a background set. The results contain the list annotation over-represented in the input set with respect to the background set and metabolite-associated *p*-values.

Results and Discussion

Comprehensive metabolic profiles by maturation stage The relative intensities of the various metabolites in mulberry fruits are illustrated and listed in Fig. 2. The relative levels of mannitol, amino acids such as asparagine, aspartate, glutamate, glutamine, glycine, homoserine, isoleucine, lysine, norvaline, proline, serine, threonine, tryptophan, tyrosine, β-alanine, and γ-aminobutyrate, and organic acids such as 5-hydroxypipecolicate, fumarate, glycerate, malate, succinate, β-sitosterol, and β-tocopherol were significantly higher in immature mulberry fruits than the other stages. Similar results have been reported in other fruits. Higher levels of glutamine, isoleucine, tyrosine, and succinate were observed in the immature stage during the maturation of black raspberry (*Rubus coreanus* Miquel) fruits (15). Fumaric acid, γ-aminobutyratic acid, and glycine also characterized the immature stage in tomato (*Solanum lycopersicum*) fruits (16). Additionally, tryptophan, threonine, lysine, aspartate, proline, and serine exhibited high levels in the immature stage during maturation of medlar (*Mespilus germanica*) (17).

In semi-matured mulberry fruits, relative levels of aconitate, phenolics such as chlorogenic acid, cryptochlorogenic acid, and neochlorogenic acid, and vitamins such as ascorbic acid and δ-tocopherol significantly increased. Previous studies indicated that chlorogenic acid isomers and vitamins (ascorbic acid and tocopherols) are important antioxidants that protect against oxidative stress and oxygen scavengers that react rapidly with free radicals (18,19). In particular, chlorogenic acid isomers are strong free radical scavengers in prune fruits (*Prunus domestica* L.) (20). Coffee fruits (*Coffea pseudozanguebariae*) also showed the highest level of chlorogenic acid in the early stage of maturation (40% of the fructification time) (21). In addition, higher levels of phenolic acids were detected in semi-matured plum persimmon (*Diospyros lotus*) fruits (22). Although bioactivities of phenolics such as chlorogenic isomers have been reported in various studies, this is the first study to document the occurrence of chlorogenic acid, cryptochlorogenic acid, and neochlorogenic acid in mulberry fruits.

Fully matured mulberry fruits had significantly higher levels of citrate, sugars such as fructose and glucose, and anthocyanins (cyanidin 3-glucoside and cyanidin 3-rutinoside) than the other stages. Increased glucose and fructose levels were also observed

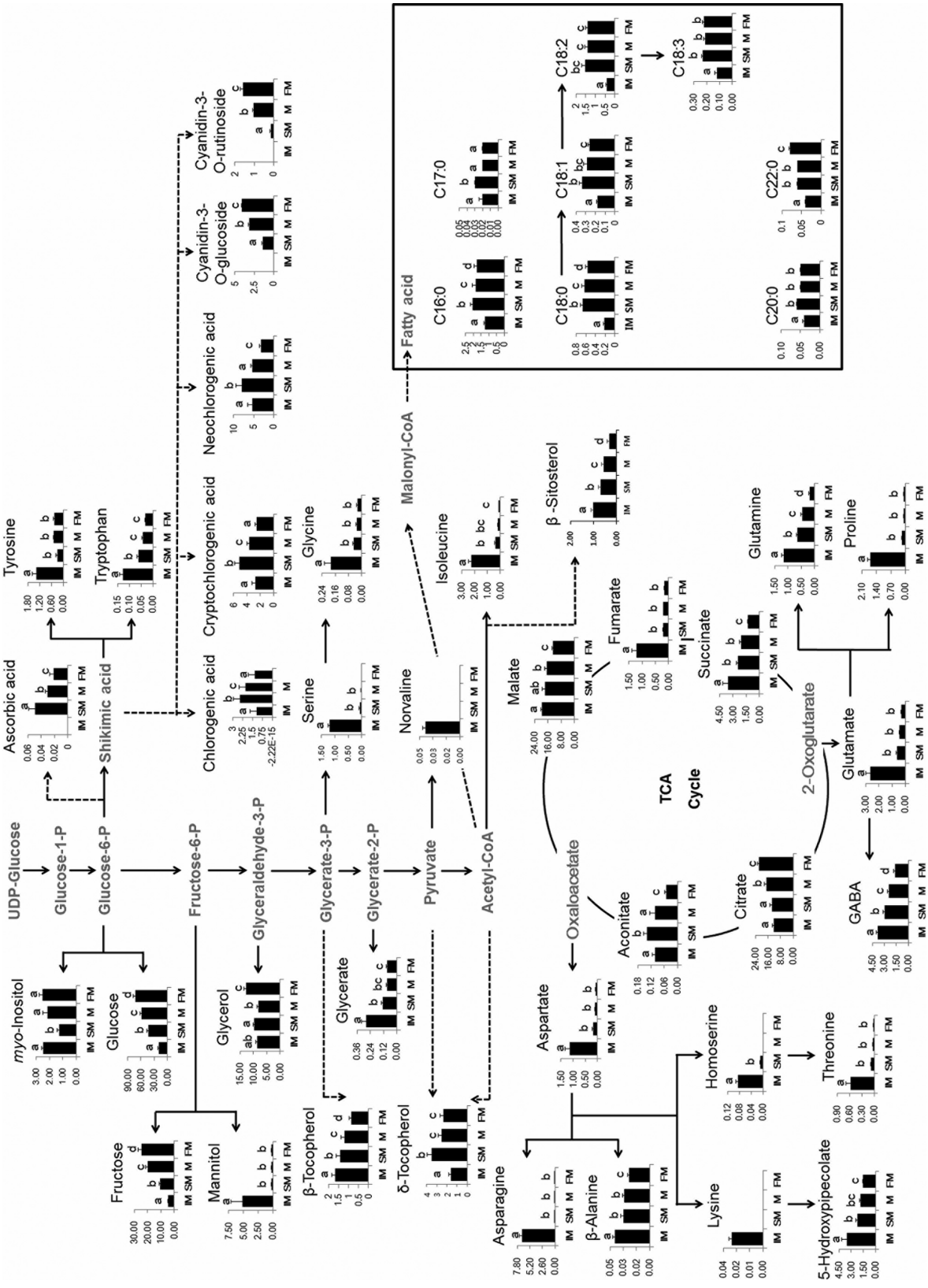

Fig. 2. Schematic diagram of the metabolic pathway and relative levels of the detected compounds in mulberry fruits according to maturation stages. It was modified by pathway presented in KEGG database (Kanehisa Laboratories, 2010). The intensities of each peak at the level of various compounds were scaled to 100% in those of fully matured stage samples. One-way analysis of variance was carried out to determine the statistical significance of differences between groups ($p<0.05$). Data are mean values with the error bars representing standard error of mean (SEM) values ($n=3$).

Table 2. Relative levels of anthocyanins during mulberry fruit development and ripening (mg/g of dry weight) analyzed by HPLC

Anthocyanin	IM	SM	M	FM
cyanidin-3-*O*-glucoside	ND[1)]	1.40±0.08[a2)]	3.17±0.09[b]	4.09±0.03[c]
cyanidin-3-*O*-rutinoside	ND	0.14±0.06[a]	1.03±0.06[b]	1.57±0.17[c]

[1)]ND, not detected

[2)]Different letters in the same row indicate a significant difference (p<0.05). Data represent the means±SEM of five biological replicates.

during grape and black raspberry maturation (15,23). As listed in Table 2, cyanidin-3-*O*-glucoside of fully-matured mulberry fruit exhibited the highest level with a value of 4.09 mg/g, followed by mature (3.17 mg/g), and immature (1.40 mg/g). Additionally, cyanidin-3-*O*-rutinoside of fully-matured mulberry fruit showed the highest level with a value of 1.57 mg/g, followed by mature (1.03 mg/g), and immature (0.14 mg/g). It has been reported that the anthocyanins (cyanidin-3-*O*-glucoside and cyanidin-3-rutinoside) are minor components in immature mulberry fruits but accumulate in mulberry fruits with maturation, and the cyanidins constituted more than 90% of anthocyanins in various mulberry cultivars (6). Higher levels of citric acid associated with hormones, phenylpropanoids, and flavonoid biosynthesis indicate possible contributions to the biosynthesis of anthocyanins. On the other hand, the levels of amino acids, such as glutamine, isoleucine, β-alanine, and γ-aminobutyrate; organic acids, such as 5-hydroxypipecolicate, glycerate, malate, and succinate; terpenes, such as β-sitosterol; and vitamins, such as β-tocopherol significantly declined with the progress of maturation. A comparison of the metabolite contents between mulberry fruits and other berries, including grapevine (*Vitis vinifera* L.) berries and kiwifruit (*Actinidia deliciosa*), reveals similar patterns in these fruits. The earlier stage of grapevine berry development is characterized by higher relative abundance of amino acids than observed in later stages (24). Amino acids, including glutamate, asparagine, glutamine, and γ-aminobutyrate also declined during the maturation of kiwifruit berries (25). Additionally, higher levels of the major soluble sugars, including fructose and glucose, were detected in the matured stage of plum persimmon (22).

Fatty acid profiles according to maturation stages of mulberry fruit are illustrated and listed in Fig. 2 and Table 3. The highest levels of total fatty acids were observed in the semi-matured mulberry fruits. In particular, relatively higher levels of saturated fatty acids such as palmitic acid, margaric acid, and stearic acid, monounsaturated fatty acids such as oleic acid, and polyunsaturated fatty acids such as linoleic acid were observed in the semi-matured mulberry fruits than in the other stages. The amount of these fatty acids decreased in the mulberry fruits as they matured. It is known that maturation or ripening activates pathways that generally influence the levels of pigments (typically carotenoids and flavonoids), sugars, acids, and aroma volatiles to make the organ more appealing (26,27). The lower levels of fatty acids observed in the fully matured mulberry fruits may be caused by the increased use of the fatty acids for biosynthesis of mulberry fruit volatiles or pigments.

Saturated fatty acids, such as stearic acid, have a neuroprotective effect (28), and monounsaturated fatty acids are also known to aid the prevention of memory loss and neuroprotection (29). Poly-unsaturated fatty acids are known to prevent oxidative stress and mitochondrial dysfunction, and to have high potential for being neuroprotective (30). Additionally, a monounsaturated fatty acid (oleic acid) and polyunsaturated fatty acid (linoleic acid) also exhibit antioxidant activities (31). In coriander (*Coriandrum sativum*) fruit, higher levels of saturated fatty acids such as palmitic acid, stearic acid, and arachidic acid, and polyunsaturated fatty acids, such as linoleic and α-linolenic acid were detected in the early stage (32). In mulberry fruits, most of the fatty acids were at higher levels at the semi-matured stage than in the other maturation stages, which indicates that the semi-matured mulberry fruits possess relatively higher potential antioxidant and neuroprotective activity.

Metabolic pathway analysis using enrichment analysis Commercial regulation of fruit maturation has currently gained much attention because of its importance in terms of improving fruit shelf life and quality (33). Therefore, understanding the biosynthetic pathways involved in maturation is necessary. The most relevant metabolic pathways involved in the maturation of mulberry fruits were examined by annotating the identified metabolites by MBRole enrichment analysis. Various metabolites overlapped in metabolic pathways (Table 4). Twelve pathways, including aminoacyl-tRNA biosynthesis, biosynthesis of plant hormones, biosynthesis of terpenoids and steroids, biosynthesis of unsaturated fatty acids, and biosynthesis of phenylpropanoids were shown to be primarily involved in mulberry fruit maturation (Table 4).

The following nine metabolites were associated with aminoacyl-tRNA biosynthesis (p=0.0000): asparagine, aspartic acid, glutamine, glycine, lysine, proline, serine, threonine, and tyrosine. The function of aminoacyl-rRNA is to transport amino acids to the ribosome for the biosynthesis of various proteins. Thus, these metabolites appeared to contribute to biosynthesize various proteins by aminoacyl-tRNA synthase in immature mulberry fruits in order to mature. Table 3 also lists the metabolites associated with biosynthesis of plant hormones (aconitic acid, aspartic acid, citric acid, fumaric acid, malic acid, and succinic acid: ethylene, and α-linolenic acid: jasmonic acid; p= 0.0001), which work in cycle with each other by affecting growth regulation and plant immunity (34, http://www.genome.jp/kegg-bin/show_pathway?map01070). In addition, biosynthesis of phenylpropanoids (aconitic acid, citric acid, caffeic acid, fumaric acid, malic acid, and succinic acid; p=0.0034), and biosynthesis of unsaturated fatty acids (arachidic acid, behenic acid, lignoceric acid,

Table 3. Relative levels of fatty acids in mulberry fruits according to various maturation stages

CX:Y	FA	Mass fragmentation (m/z)	RT	Relative FA profile			
				IM[3)]	SM	M	FM
C16:0	palmitic acid	**74**, 87, 143, 227, 270 (M^+)	15.19	1.25±0.07[1),a,2)]	2.03±0.15^b	1.84±0.21^c	1.76±0.18^d
C17:0	margaric acid	**74**, 87, 143, 241, 284 (M^+)	16.16	0.02±0.00^a	0.03±0.00^b	0.02±0.00^a	0.02±0.00^a
C18:0	stearic acid	**74**, 87, 143, 255, 298 (M^+)	17.14	0.21±0.02^a	0.66±0.05^b	0.62±0.06^c	0.56±0.05^d
C20:0	arachidic acid	43, **74**, 87, 143, 326 (M^+)	19.04	0.04±0.00^a	0.06±0.01^b	0.05±0.01^b	0.05±0.00^b
C22:0	behenic acid	43, **74**, 87, 143, 354 (M^+)	21.04	0.04±0.00^a	0.06±0.01^b	0.06±0.01^b	0.08±0.01^c
C24:0	lignoceric acid	55, **74**, 87, 143, 382 (M^+)	23.22	0.03±0.00^a	0.03±0.00^a	0.02±0.00^a	0.02±0.00^a
	ΣSFA[4)]			1.59±0.09^a	2.87±0.22^{bc}	2.61±0.24^{cd}	2.49±0.22^d
C18:1	oleic acid	**55**, 69, 97, 264, 296 (M^+)	17.34	0.18±0.01^a	0.34±0.03^b	0.29±0.03^{bc}	0.26±0.02^c
	ΣMUFA			0.18±0.01^a	0.34±0.03^b	0.29±0.03^{bc}	0.26±0.02^c
C18:2	linoleic acid	**67**, 81, 95, 109, 294 (M^+)	17.88	0.41±0.03^a	1.54±0.16^{bc}	1.39±0.15^c	1.39±0.11^c
C18:3	α-linolenic acid	65, **79**, 95, 109, 292 (M^+)	18.42	0.12±0.01^a	0.23±0.02^b	0.21±0.03^b	0.22±0.02^b
	ΣPUFA			0.54±0.03^a	1.77±0.17^{bc}	1.60±0.17^b	1.61±0.12^b
	Total FA			2.31±0.11^a	4.99±0.41^b	4.50±0.44^{bc}	4.37±0.35^c

[1)]Data represent the means±SD of three biological replicates.
[2)]The relative fatty acid profile of each metabolite was calculated as area ratio of the respective single fatty acid to the sum of all fatty acids. Different letters in the same row indicate a significant difference (p<0.05). Each base peak in the mass spectra is identified by bold type.
[3)]IM, Immature; SM, Semi-mature; M, Mature; FM, Fully-mature
[4)]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; M+, molecular ion peak

Table 4. List of selected pathways identified in enrichment analysis of metabolite roles

Interaction metabolite	p-value[1)]	Adjusted p-value[2)]	Pathway name	Hits
tyrosine, lysine, proline, glutamine, asparagine, glycine, threonine, serine, aspartic acid	0.0000	0.0000	Aminoacyl-tRNA biosynthesis	9
aspartic acid, citric acid, succinic acid, malic acid, α-linolenic acid, aconitic acid, fumaric acid	0.0000	0.0001	Biosynthesis of plant hormones	7
threonine, homoserine, 5-hydroxypipecolicate, glyceric acid, serine, aspartic acid, glycine	0.0000	0.0000	Glycine, serine and threonine metabolism	7
aconitic acid, malic acid, β-sitosterol, α-linolenic acid, succinic acid, fumaric acid, citric acid	0.0001	0.0006	Biosynthesis of terpenoids and steroids	7
stearic acid, palmitic acid, arachidic acid, linoleic acid, lignoceric acid, behenic acid, oleic acid	0.0000	0.0000	Biosynthesis of unsaturated fatty acids	7
fumaric acid, citric acid, caffeic acid, aconitic acid, malic acid, succinic acid	0.0008	0.0034	Biosynthesis of phenylpropanoids	6
asparagine, aspartic acid, succinic acid, γ-aminobutyric acid, fumaric acid, glutamine	0.0000	0.0000	Alanine, aspartate and glutamate metabolism	6
γ-aminobutyric acid, proline, fumaric acid, glutamine, aspartic acid	0.0023	0.0097	Arginine and proline metabolism	5
serine, tyrosine, glycine, aspartic acid, asparagine	0.0001	0.0006	Cyanoamino acid metabolism	5
aconitic acid, succinic acid, malic acid, citric acid, glyceric acid	0.0001	0.0007	Glyoxylate and dicarboxylate metabolism	5
citric acid, aconitic acid, succinic acid, fumaric acid, malic acid	0.0000	0.0000	Citrate cycle (TCA cycle)	5
glycine, aspartic acid, asparagine, glutamine	0.0002	0.0009	Nitrogen metabolism	4

[1)]p-value: statistically assessed against the background set.
[2)]Adjusted p-value: p-value corrected using the false discovery rate

linoleic acid, oleic acid, palmitic acid, and stearic acid, p=0.0000) were listed. This suggests that metabolites from various metabolic pathways involved in aminoacyl-tRNA biosynthesis, biosynthesis of plant hormones, biosynthesis of phenylpropanoids, and biosynthesis of unsaturated fatty acids were over-represented using pathway enrichment analysis.

It has been stated that the key to improving the success rate of plant metabolic engineering is to increase the understanding of the systems subject to engineering by comprehensive metabolic analyses (12). Thus, the understanding of comprehensive metabolic profiles

of mulberry fruits at different maturation stags is important for the development of mulberry fruit based-functional foods, herbal medicines, or cosmetics, and can be used as basic information for metabolic engineering of mulberry fruits possessing commercially valuable characteristics in the future. In the semi-matured stage, relatively higher levels of aconitate, chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid, ascorbic acid, δ-tocopherol, and total fatty acid contents were observed. In the fully matured stage, the relative levels of glycerol, citrate, fructose, glucose, and cyanidins increased, whereas those of glutamine, isoleucine, β-alanine, γ-aminobutyrate, 5-hydroxypipecolicate, glycerate, malate, succinate, and β-tocopherol decreased. Pathway enrichment analysis revealed that 12 pathways, including aminoacyl-tRNA biosynthesis, biosynthesis of plant hormones, biosynthesis of terpenoids and steroids, biosynthesis of unsaturated fatty acids, and biosynthesis of phenyl-propanoids primarily contributed to mulberry fruit maturation. These results provide basic information for metabolic engineering for improved characteristics and quality control of mulberry fruits as potential resources of functional foods, herbal medicines, or cosmetics.

Acknowledgments This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (NRF-2015R1A5A1008958), and by the Chung-Ang University Research Scholarship Grants in 2014.

Disclosure The authors declare no conflict of interest.

References

1. Ercisli S, Orhan E. Chemical composition of white (*Morus alba*), red (*Morus rubra*) and black (*Morus nigra*) mulberry fruits. Food Chem. 103: 1380-1384 (2007)
2. Huang HP, Shih YW, Chang YC, Hung CN, Wang CJ. Chemoinhibitory effect of mulberry anthocyanins on melanoma metastasis involved in the Ras/PI3K pathway. J. Agr. Food Chem. 56: 9286-9293 (2008)
3. Lotito SB, Frei B. Dietary flavonoids attenuate tumor necrosis factor α-induced adhesion molecule expression in human aortic endothelial cells structure-function relationships and activity after first pass metabolism. J. Biol. Chem. 281: 37102-37110 (2006)
4. Kim HG, Ju MS, Shim JS, Kim MC, Lee SH, Huh Y, Oh MS. Mulberry fruit protects dopaminergic neurons in toxin-induced Parkinson's disease models. Brit. J. Nutr. 104: 8-16 (2010)
5. Lee JS, Synytsya A, Kim HB, Choi DJ, Lee S, Lee J, Park YI. Purification, characterization and immunomodulating activity of a pectic polysaccharide isolated from Korean mulberry fruit Oddi (*Morus alba* L.). Int. Immunopharmacol. 17: 858-866 (2013)
6. Oki T, Kobayashi M, Nakamura T, Okuyama A, Masuda M, Shiratsuchi H, Suda I. Changes in radicalscavenging activity and components of mulberry fruit during maturation. J. Food Sci. 71: C18-C22 (2006)
7. Zhang W, Han F, He J, Duan C. HPLCDADESIMS/MS analysis and antioxidant activities of nonanthocyanin phenolics in mulberry (*Morus alba* L.). J. Food Sci. 73: C512-C518 (2008)
8. Hunyadi A, Martins A, Hsieh TJ, Seres A, Zupkó I. Chlorogenic acid and rutin play a major role in the *in vivo* anti-diabetic activity of *Morus alba* leaf extract on type II diabetic rats. PLoS ONE 7: e50619 (2012)
9. Yang X, Yang L, Zheng H. Hypolipidemic and antioxidant effects of mulberry (*Morus alba* L.) fruit in hyperlipidaemia rats. Food Chem. Toxicol. 48: 2374-2379 (2010)
10. Li J, Schauer N, Kopka J, Willmitzer L, Fernie AR. Gas chromatography mass spectrometry-based metabolite profiling in plants. Nat. Protoc. 1: 387-396 (2006)
11. Hill CB, Roessner U. Metabolic profiling of plants by GC-MS. pp. 1-23. In: The handbook of plant metabolomics. Weckwerth W, Kahl G (eds). Wiley-VCH Verlag GmbH & Co., Weinheim, Germany (2013)
12. Trethewey RN. Metabolite profiling as an aid to metabolic engineering in plants. Curr. Opin. Plant Biol. 7: 196-201 (2004)
13. Lee SY, Kim SH, Hyun SH, Suh HW, Hong SJ, Cho BK, Choi HK. Fatty acids and global metabolites profiling of *Dunaliella tertiolecta* by shifting culture conditions to nitrate deficiency and high light at different growth phases. Process Biochem. 49: 996-1004 (2014)
14. Chagoyen M, Pazos F. MBRole: Enrichment analysis of metabolomic data. Bioinformatics 27: 730-731 (2011)
15. Kim HS, Park SJ, Hyun SH Yang SO, Lee J, Auh JH, Choi HK. Biochemical monitoring of black raspberry (*Rubus coreanus* Miquel) fruits according to maturation stage by ^{1}H NMR using multiple solvent systems. Food Res. Int. 44: 1977-1987 (2011)
16. Oms-Oliu G, Hertog MLATM, Van de Poel B, Ampofo-Asiama J, Geeraerd AH, Nicolaï BM. Metabolic characterization of tomato fruit during preharvest development, ripening, and postharvest shelf-life. Postharvest Biol. Tec. 62: 7-16 (2011)
17. Glew RH, Ayaz FA, Sanz C, VanderJagt DJ, Huang HS, Chuang LT, Strnad M. Changes in sugars, organic acids and amino acids in medlar (*Mespilus germanica* L.) during fruit development and maturation. Food Chem. 83: 363-369 (2003)
18. Cort WM. Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid and their mode of action. J. Am. Oil Chem. Soc. 51: 321-325 (1974)
19. Sato Y, Itagaki S, Kurokawa T, Ogura J, Kobayashi M, Hirano T, Iseki K. *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. Int. J. Pharm. 403: 136-138 (2011)
20. Nakatani N, Kayano SI, Kikuzaki H, Sumino K, Katagiri K, Mitani T. Identification, quantitative determination, and antioxidative activities of chlorogenic acid isomers in prune (*Prunus domestica* L.). J. Agr. Food Chem. 48: 5512-5516 (2000)
21. Bertrand C, Noirot M, Doulbeau S, de Kochko A, Hamon S, Campa C. Chlorogenic acid content swap during fruit maturation in *Coffea pseudozanguebariae*: Qualitative comparison with leaves. Plant Sci. 165: 1355-1361 (2003)
22. Ayaz FA, Kadioglu A, Reunanen M. Changes in phenolic acid contents of *Diospyros lotus* L. during fruit development. J. Agr. Food Chem. 45: 2539-2541 (1997)
23. Ali K, Maltese F, Fortes AM, Pais MS, Choi YH, Verpoorte R. Monitoring biochemical changes during grape berry development in Portuguese cultivars by NMR spectroscopy. Food Chem. 124: 1760-1769 (2011)
24. Degu A, Hochberg U, Sikron N, Venturini L, Buson G, Ghan R, Fait A. Metabolite and transcript profiling of berry skin during fruit development elucidates differential regulation between cabernet sauvignon and shiraz cultivars at branching points in the polyphenol pathway. BMC Plant Biol. 14: 1-20 (2014)
25. Nardozza S, Boldingh HL, Osorio S, Höhne M, Wohlers M, Gleave AP, Clearwater MJ. Metabolic analysis of kiwifruit (*Actinidia deliciosa*) berries from extreme genotypes reveals hallmarks for fruit starch metabolism. J. Exp. Bot. 64: 5049-5063 (2013)
26. Giovannoni JJ. Genetic regulation of fruit development and ripening. Plant Cell 16: S170-S180 (2004)
27. Giovannoni JJ. Fruit ripening mutants yield insights into ripening control. Curr. Opin. Plant Biol. 10: 283-289 (2007)
28. Wang ZJ, Li GM, Tang WL, Yin M. Neuroprotective effects of stearic acid against toxicity of oxygen/glucose deprivation or glutamate on rat cortical or hippocampal slices. Acta Pharm. Sinic. 27: 145-150 (2006)
29. Capurso A, Panza F, Solfrizzi V, Capurso C, D'Introno A, Capurso S, Colacicco AM. The results of a study of cognitive decline in old age. Is there a case for this treatment in multiple sclerosis? pp. 97-107. In: Early indicators early treatments neuroprotection in multiple sclerosis, Monounsaturated fatty acids and neuroprotection. Hommes OR, Comi G (eds). Springer Milan, Milano, Italy (2004)
30. Joseph KD, Muralidhara M. Fish oil prophylaxis attenuates rotenone-induced oxidative impairments and mitochondrial dysfunctions in rat brain. Food Chem. Toxicol. 50: 1529-1537 (2012)
31. Henry GE, Momin RA, Nair MG, Dewitt DL. Antioxidant and cyclooxygenase activities of fatty acids found in food. J. Agr. Food Chem. 50: 2231-2234 (2002)
32. Msaada K, Hosni K, Taarit MB, Chahed T, Hammami M, Marzouk B. Changes in fatty acid composition of coriander (*Coriandrum sativum* L.) fruit during maturation. Ind. Crop. Prod. 29: 269-274 (2009)
33. Matas AJ, Gapper NE, Chung MY, Giovannoni JJ, Rose JK. Biology and genetic engineering of fruit maturation for enhanced quality and shelf-life. Curr. Opin. Biotech. 20: 197-203 (2009)
34. De Vleesschauwer D, Xu J, Höfte M. Making sense of hormone-mediated defense networking: From rice to Arabidopsis. Front Plant Sci. 5: 1-15 (2014)