Metabolomic Elucidation of the Effect of Sucrose on the Secondary Metabolite Profiles in *Melissa officinalis* by Ultraprformance Liquid Chromatography–Mass Spectrometry

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ABSTRACT: Sucrose induces flavonoid accumulation in plants as a defense mechanism against various stresses. However, the relationship between the biosynthesis of flavonoids as secondary metabolites and sucrose levels remains unknown. To understand the change in flavonoid biosynthesis by sucrose, we conducted secondary metabolite profiling in *Melissa officinalis* treated with different levels of sucrose using ultraperformance liquid chromatography–quadrupole time-of-flight mass spectrometry. The partial least squares-discriminant and hierarchical clustering analyses showed significant differences in secondary metabolite profiles in *M. officinalis* at 50, 150, and 300 mM sucrose levels. The levels of 3 flavonoids such as quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside, 6-methoxymaradendrin 3-O-acetate, and 3-hydroxyxoumarin and 19 flavonoids including 6-methoxymaradendrin 3-O-acetate, aureusidin, iridin, flavonol 3-O-(6-O-malonyl-β-D-glucoside) quercetin 3-O-glucoside, and rutin increased at 150 and 300 mM sucrose, respectively, compared to 50 mM sucrose, indicating that the flavonoids were accumulated in *M. officinalis* by a higher concentration of sucrose. This is the first investigation of the change in individual flavonoids as secondary metabolites in *M. officinalis* by varying sucrose levels, and the results demonstrate that the sucrose causes the accumulation of certain flavonoids as a defense mechanism against osmotic stress.

INTRODUCTION

Plants contain a variety of primary and secondary metabolites, which are the intermediate or end products of cellular processes. Secondary metabolites including flavonoids play an important role in various biochemistry and physiological processes in plants. Their levels are considered important because they are used in obtaining valuable information such as the physiological state; they reflect specific biochemical processes in plants as metabolite levels serve as the ultimate response of biological systems to various genetic or environmental changes.

Metabolomics, the study of chemical processes involving the entire metabolome of an organism, is a useful tool in determining metabolites in response to such changes. Various analytical tools have been used for metabolite profiling of plants, including gas chromatography/mass spectrometry, liquid chromatography–mass spectrometry (LC–MS), and nuclear magnetic resonance. *LC–MS* is the most commonly used in secondary metabolite profiling of plants because it offers high selectivity and sensitivity and allows the analysis of nonvolatile, unstable, and high-molecular-weight compounds without derivatization.

*Melissa officinalis*, a perennial herb distributed throughout East Asia, has been well known as a traditional medicine used in treating human disorders such as headache, digestion disorder, Alzheimer’s disease, and cancer. Various secondary metabolites in *M. officinalis* are known to be responsible for antioxidative, antibacterial, anti-inflammatory, antifungal, and antitumor activities. Thus, many studies have manipulated the metabolism of *M. officinalis* to produce target secondary metabolites that can be used as valuable substances.

Sucrose can function as the hormone-like signaling molecule and control various metabolisms and growth in plants. It is an important factor affecting the synthesis of the secondary metabolites pathway including flavonoid. Secondary metabolites are well known to accumulate during stressful conditions.
| ESI     | exact mass | mass error (ppm) | matched metabolite from database                                      | PubChem ID  |
|---------|------------|------------------|-----------------------------------------------------------------------|-------------|
| negative | 242.080    | 19.531           | lumichrome                                                            | 5326566     |
| negative | 174.100    | 11.809           | N\(^{-}\)-ethyl-L-glutamine                                            | 439378      |
| negative | 258.062    | 4.088            | streptamine phosphate                                                 | 439394      |
| negative | 276.025    | 2.137            | 2-carboxy-o-arabinitol 1-phosphate                                     | 129417      |
| negative | 232.016    | 0.655            | N-phosphohypotauracocaine                                              | 16019959    |
| negative | 192.063    | 10.309           | valiolone                                                             | 443630      |
| negative | 634.132    | 2.383            | actinorhodine                                                          | 441143      |
| negative | 105.033    | 6.208            | cyanoprazine                                                          | 73172       |
| negative | 588.127    | 5.164            | kolalavannone                                                          | 155169      |
| negative | 184.999    | 15.551           | l-serine O-sulfate                                                    | 164701      |
| negative | 278.115    | 9.500            | isohelenol                                                            | 155586      |
| negative | 332.074    | 19.246           | 1-O-galloyl-β-D-glucose                                               | 124021      |
| negative | 331.082    | 3.990            | malvidin                                                              | 159287      |
| negative | 504.169    | 7.928            | cellocitrin                                                           | 5287993     |
| negative | 128.047    | 0.992            | 2-hydroxy-cis-hex-2,4-dienoate                                        | 11953951    |
| negative | 317.066    | 7.140            | petunidin                                                             | 73386       |
| negative | 110.037    | 8.061            | catechol                                                              | 289         |
| negative | 514.115    | 1.270            | MK 571                                                                | 5281888     |
| negative | 650.252    | 14.857           | BQ_518                                                               | 443291      |
| negative | 292.121    | 17.599           | INF271                                                               | 443080      |
| negative | 856.254    | 4.466            | 7-hydroxyproadiminic A                                                | 441176      |
| negative | 342.110    | 14.691           | 3-(4-methoxyphenyl)-5,6,7-trimethoxy-4H-1-benzopyran-4-one             | 248269      |
| negative | 674.221    | 18.819           | premethranycin A2’                                                   | 443797      |
| negative | 326.121    | 8.104            | robinobiose                                                          | 441428      |
| negative | 490.171    | 19.730           | RMS-268770                                                           | 56928083    |
| negative | 344.108    | 12.008           | TRAM-34                                                              | 656734      |
| negative | 328.102    | 16.053           | 7-hydroxy-6-methyl-8-ribityl lumazine                                   | 440869      |
| negative | 518.159    | 0.550            | esmeralacid                                                           | 443632      |
| negative | 216.027    | 4.060            | 5-carboxymethyl-2-hydroxymaconate                                     | 54675765    |
| negative | 192.047    | 6.535            | 6-(allythio)purine                                                   | 3633259     |
| negative | 133.038    | 11.861           | l-aspartate                                                           | 5960        |
| negative | 405.100    | 5.991            | cefaloglycin                                                          | 19150       |
| negative | 356.098    | 17.147           | Bay K-8644                                                           | 2303        |
| negative | 168.019    | 12.028           | butanoylphosphate                                                   | 266         |
| negative | 307.069    | 14.055           | narcilasine                                                           | 72376       |
| negative | 150.032    | 1.013            | α-oxo-benzeneacetic acid                                             | 11915       |
| negative | 471.150    | 4.355            | 10-formylidihydrofolate                                               | 135398690   |
| negative | 194.063    | 1.680            | 6-(isopropythio)purine                                              | 3698120     |
| negative | 273.086    | 10.110           | brugine                                                              | 443298      |
| negative | 296.105    | 18.929           | calophyllin B                                                       | 5281624     |
| negative | 109.900    | 6.524            | calcium chloride anhydrous                                           | 5284359     |
| negative | 296.092    | 19.494           | 2,3,9,10-tetrahydroxyberberine                                        | 443768      |
| negative | 332.069    | 10.829           | hyposylone                                                           | 442747      |
| negative | 310.121    | 18.357           | 7-hydroxy-3-(4-methoxyphenyl)-4-propyl-2H-1-benzopyran-2-one          | 5357444     |
| negative | 306.060    | 18.751           | isoprothiolane sulphoxide                                           | 93275       |
| negative | 312.106    | 11.416           | 6-O-(β-D-xylopyranosyl)-β-D-glucopyranose                             | 443248      |
| negative | 814.211    | 3.276            | victorin C                                                           | 21549934    |
| negative | 506.100    | 7.917            | cassiamin C                                                          | 442728      |
| negative | 392.199    | 8.060            | abyssinomine VI                                                      | 5281219     |
| negative | 610.153    | 1.052            | rutin                                                                | 5280805     |
| negative | 372.100    | 0.836            | oholoensin-A                                                        | 442531      |
| negative | 594.159    | 10.092           | vitexin 2'-O-β-D-glucoside                                           | 5280641     |
| negative | 464.096    | 9.183            | querceatin 3-O-glucoside                                             | 5280804     |
| negative | 294.021    | 15.387           | 4-[2,2-dichloro-1-(4-methoxyphenyl)ethenyl]phenol                     | 156639      |
| negative | 448.101    | 7.367            | isooreniitin                                                          | 114776      |
| negative | 360.085    | 15.746           | thymonin                                                             | 442662      |
| negative | 575.058    | 16.657           | isopentenyldenosine-5′-triphasate                                     | 23724748    |
| negative | 522.137    | 7.259            | iridin                                                               | 5281777     |
| negative | 342.074    | 17.721           | dihydroxymethylsterigmatocystein                                     | 5280636     |
| negative | 516.127    | 7.769            | 1,3-dicaffeoylquinic acid                                            | 6474640     |
| negative | 580.312    | 11.523           | hordatine B                                                          | 72193633    |
| ESI | exact mass | mass error (ppm) | matched metabolite from database | PubChem ID |
|-----|------------|-----------------|---------------------------------|------------|
| negative | 870.218 | 14.415 | irresinin I | 11953907 |
| negative | 714.466 | 11.184 | (2′,3′)-deoxyxymycol 2′-α-L-fucoside | 23724611 |
| negative | 487.120 | 5.943 | luciferyl sulfate | 11953812 |
| negative | 486.116 | 12.242 | aspulvinone H | 54675755 |
| negative | 463.074 | 3.945 | N6-(1,2-dicarboxyethyl)-AMP | 447145 |
| negative | 286.048 | 2.426 | aureusidin | 5281220 |
| negative | 136.039 | 17.392 | hypoxanthine | 135398638 |
| negative | 198.038 | 3.888 | 2,4-dinitrophenylhydrazine | 440898 |
| negative | 374.058 | 7.988 | glutocochlearin | 5281135 |
| negative | 374.058 | 11.945 | luciferyl sulfate | 11953812 |
| negative | 364.095 | 19.154 | justicidin B | 442988 |
| negative | 750.140 | 16.841 | UDP-N-acetylmuramoyl-L-alanine | 3037124 |
| positive | 305.939 | 2.050 | 3-iodo-4-hydroxyphenylpyruvate | 440184 |
| positive | 542.121 | 11.040 | isochamaejasmin | 390361 |
| positive | 184.023 | 3.201 | 5-hydroxyisourate | 250388 |
| positive | 543.110 | 11.677 | CMP-3-deoxy-D-manno-octulosonate | 445888 |
| positive | 292.132 | 11.326 | SB 206553 | 5163 |
| positive | 146.069 | 6.737 | D-glutamine | 44288 |
| positive | 115.063 | 14.931 | proline | 145742 |
| positive | 129.043 | 1.863 | 5-oxoproline | 7405 |
| positive | 303.137 | 0.083 | 2,4-dinitro-1-(3-nitrophenoxy)benzene | 221812 |
| positive | 117.079 | 9.054 | L-valine | 6287 |
| positive | 324.106 | 4.923 | D-fructofuranose 1,2′,3′-dianhydride | 440332 |
| positive | 307.084 | 6.429 | glutathione | 124886 |
| positive | 334.057 | 1.241 | nicotinamide D-ribose-1-phosphate | 14180 |
| positive | 450.116 | 4.305 | neoastilbin | 442437 |
| positive | 305.028 | 13.271 | 2,4-dinitro-1-(3-nitrophenoxy)benzene | 221812 |
| positive | 303.007 | 18.316 | 2-[(3,5-dichlorophenyl)carbamoyl]oxy)-2-methyl-3-butenolic acid | 119359 |
| positive | 900.168 | 18.543 | N-methylanthraniloyl-CoA | 24883420 |
| positive | 162.032 | 2.679 | umbelliferone | 5281426 |
| positive | 540.163 | 14.055 | cleistanthin A | 442833 |
| positive | 621.109 | 14.679 | cyanidin 3-O-3′,6′-O-dimalonylgalactoside | 23724697 |
| positive | 360.085 | 3.860 | 6-methoxyxamendrin 3-O-acetate | 442415 |
| positive | 522.110 | 1.595 | cefoselis | 5748845 |
| positive | 610.132 | 0.389 | 3-hydroxycoumarin | 13650 |
| positive | 341.054 | 18.994 | gallicatechin (4αR-8αS)-epigallocatechin | 442682 |

33188 https://dx.doi.org/10.1021/acsomega.0c04745
ACS Omega 2020, 5, 33186–33195
conditions because of defense mechanisms in plants. For example, flavonoids accumulate in the presence of sucrose as defense mechanisms against osmotic stress in plants. In these studies, analysis of gene expression or total flavonoid levels revealed that sucrose induces the upregulation of flavonoid biosynthesis. However, to our knowledge, there is no study on the relationship between flavonoid biosynthesis and sucrose levels through metabolite profiles, especially the individual levels of flavonoids.

In this study, the secondary metabolite profile changes in *M. officinalis* were analyzed in response to different levels of sucrose. To accomplish this, we used ultraperformance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF MS), and the metabolite profiles were statistically analyzed using partial least squares-discriminant analysis (PLS-DA) and hierarchical clustering analysis (HCA). These results can be used in understanding the alteration in metabolisms based on the sucrose level and give clues on the molecular breeding of plants for overproducing high-value metabolites.

### RESULTS AND DISCUSSION

Identification of Secondary Metabolites from *M. officinalis*. To analyze the changes in the profile of secondary metabolites...
metabolites of *M. officinalis* in response to sucrose, *M. officinalis* leaves treated with 50, 150, or 300 mM sucrose were extracted with MeOH and analyzed using UPLC-Q-TOF MS. More than 20,000 peaks of the negative electrospray ionization mode (ESI⁻) and positive electrospray ionization ions (ESI⁺) were detected, and 169 metabolites were identified using XCMS in all the 15 samples obtained from five biological replicates of each condition group (Table 1), indicating that our results were more accurate and less biased than the previous reports that showed metabolic changes under the stressful conditions with 6 unidentified secondary metabolites using LC–MS/MS, 30 identified secondary metabolites using UPLC-Q-TOF MS, and 95 identified metabolites using LC–MS/MS. These metabolites were found to be major intermediates in the secondary metabolisms of plants, including the biosynthesis of carotenoids (e.g., (2'S)-deoxyxymycol 2'-α-L-fucose), phenylpropanoids (e.g., 1-O-galloyl-β-D-glucose, justicidin B, 1-acetoxyiresinol, 3-hydroxycoumarin, cleistanthin A, and umbelliferone), flavones, and flavonoids (e.g., scullcapflavone II, flavonol 3-O-(6-O-malonyl-β-D-glucose)), cyanidin 3-O-(6''-glucosyl-2''-xylosylgalactoside), iridin, isoorientin, kolaflavone, thymolin, quercetin 3-O-glucoside, quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside, rutin, vitexin 2''-O-β-D-glucoside, malvidin, and petunidin).

The secondary metabolites identified in this study are well known to have beneficial health effects. For example, rutin, lithospermic acid, moxalactam, isoorientin, 5′-methoxyhydroxycarpin-D, oxyzanethine, 1,3-dicafeoylquinic acid, isohelenol, lappaconitine, phytolaccoside B, iridin, and scullcapflavone II are known to possess various physiological activities such as antioxidative, antibacterial, hepatoprotective, anti-HIV-1, antifungal, antimutagenic, and anti-inflammatory activities. Specifically, malvidin, a primary plant pigment, inhibits human leukemia cells by arresting the G1/M phase and then inducing apoptosis. Lithospermic acid can be used in diabetic retinopathy and mesenteric ischemia reperfusion injury because of its antioxidative, hepatoprotective, and anti-inflammatory effects.

**PLS-DA of the Sucrose Effect on Secondary Metabolite Profiles.** To statistically compare changes in the profile of secondary metabolites of *M. officinalis* in response to different levels of sucrose, principal component analysis (PCA) was performed using SIMCA-P+. Because the metabolite profiles of the groups were slightly discriminated by PCA, with 0.52 of $R^2$X and 0.33 of $Q^2$ (data not shown), PLS-DA was employed to obtain better separations between the groups. Among the three groups treated with sucrose levels of 50, 150, and 300 mM, the metabolite profiles were clearly separated by partial least squares 1 (PLS1) and 2 (PLS2) in the score plot of PLS-DA (Figure 1). The model generated explained variation values, such as 0.52 of $R^2$X and 0.97 of $R^2$Y, and a predictive capability value, such as 0.87 of cumulative $Q^2$, indicating a good model. Our previous study on the change in flavonoid levels in lemon balm by sucrose also showed that six

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**Table 2. Top 20 Identified Metabolites with High Absolute Loadings on PLS1 and PLS2 as Determined by PLS-DA**

| Metabolite | Loading |
|------------|---------|
| PLS1 | |
| anisatin | 0.113 |
| 2,2′-(1-phenyl-1H-1,2,4-triazole-3,5-diyI)bisphenol | 0.107 |
| quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside | 0.107 |
| isohelenol | 0.102 |
| i-aspartate | 0.092 |
| thalidamine | 0.091 |
| aspidovnine H | 0.091 |
| 2,2-bis(4-hydroxyphenyl)hexafluoropropane | 0.091 |
| allicin | 0.090 |
| 6-O-(β-D-xylopyranosyl)-β-D-glucopyranose | 0.090 |
| isopentenyldenosine-5′-triphosphate | -0.115 |
| 6-methoxyaromadendrin 3-O-acetate | -0.115 |
| BMS-268770 | -0.115 |
| 2,4-dinitrophenylhydrazine | -0.115 |
| gentamicin C1 | -0.115 |
| iridin | -0.118 |
| 3-hydroxycoumarin | -0.118 |
| celoselis | -0.119 |
| lithospermic acid | -0.119 |
| dihydromethylstergmatocystin | -0.123 |

| Metabolite | Loading |
|------------|---------|
| 10-formyldihydrof Late | 0.197 |
| 2,4-dinitrophenylhydrazine | 0.159 |
| 2-carboxy-β-arabinitol 1-phosphate | 0.157 |
| 2-methyl-3-hydroxy-5-formylpyridine-4-carboxylate | 0.112 |
| 5′-methoxyhydroxycarpin-D | 0.138 |
| 7-hydroxy-6-methyl-8-ribityl lumazine | 0.134 |
| allicin | 0.132 |
| 6-oxo-benzenoacetic acid | 0.130 |
| Ba²⁺ | 0.120 |
| butanoylphosphate | 0.115 |
| CMP-3-deoxy-β-D-manno-octulosonate | -0.107 |
| DG(14:1(9Z)/22:5(4Z,7Z,10Z,13Z,16Z))/0:0 | -0.107 |
| 5-oxoproline | -0.109 |
| 1-dehydro-9-fluoro-11-oxotestololactone | -0.115 |
| cassiamin C | -0.121 |
| cellotriose | -0.121 |
| victorin C | -0.130 |
| rutin | -0.132 |
| thalidamine | -0.132 |
| quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside | -0.141 |
metabolites were significantly different between 50, 150, and 300 mM sucrose. However, the present results may be considered more accurate and reliable because only six secondary metabolites (i.e., 435.13, 523.129, 540.063, 573.200, 615.714, and 617.153) were used in the previous study without identification. In the permutation test, all points with a negative intercept, indicating that the PLS-DA model (Figure S2) was used in the previous study without identification. In the permutation test, all points with a negative intercept, indicating that the PLS-DA model (Figure S2) was used in the previous study without identification.

The loading scores of the selected 20 metabolites, which represented the magnitude of the contribution of each metabolite to PLS, are listed in Table 2. Of the identified 169 metabolites in this study, 40 metabolites including anisatin, quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside, iso-3-nepenthenol, and 1-aspartate contributed positively to PLS1. However, 129 metabolites such as lissopermic acid, iridin, 3-hydroxycoumarin, and 6-methoxyaromadendrin 3-O-acetate contributed negatively to PLS1. Seventy-nine metabolites including 10-formylidihydrofolate, 2,4-dinitrophenyldihydrazine, and allicin contributed positively to PLS2, while 90 metabolites such as quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside, rutin, thalidasine, and victorin C contributed negatively to PLS2.

In variable importance in projection (VIP) analysis, VIP values greater than 1 are considered important. In this study, 78 metabolites such as quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucoside, rutin, umbelliferone, and cleistanthin A were shown to have VIP values greater than 1. Of these 78 metabolites, 68 metabolites were critical metabolites for discriminating flavonoid classes (Table 3). These results suggested that the flavonoids were critical metabolites for discriminating between the groups.

### Table 3. VIP Scores of the 78 Metabolites with a VIP >1.0 That Strongly Contributed to the PLS-DA Model

| metabolite | VIP |
|------------|-----|
| catechol   | 1.990 |
| scullcapflavone II | 1.982 |
| lissopermic acid | 1.973 |
| N-methylanthraniloyl-CoA | 1.700 |
| iridin | 1.688 |
| galloatechin-(4α→8)-epigalloatechin | 1.688 |
| 2-(4-(3,5-dichlorophenyl)carbamoyl)oxy)-2-methyl-3-butoenoic acid | 1.518 |
| PC(22:6(4Z,Z,7Z,10Z,13Z,16Z,19Z)/16:1(9Z)/0:0) | 1.516 |
| dihydroxymethylsterigmatocystin | 1.511 |
| vinitin 2'-O-β-D-glucoside | 1.499 |
| evodiamine | 1.443 |
| lophocerine | 1.432 |
| CMP-3-deoxy-α-manno-octulosonate | 1.338 |
| aureusidin | 1.116 |
| INF271 | 1.115 |
| BQ518 | 1.113 |
| tauroursodeoxycholic acid | 1.110 |
| isohelenol | 1.104 |
| hypoxanthine | 1.101 |
| isopentenyladenosine-5′-triphosphate | 1.093 |
| 2′-(1-phenyl-1,2,4-triazole-3,5-diy1)bisphenol | 1.082 |
| PC(20:3(S,Z,Z,11Z)/16:1(9Z)/0:0) | 1.075 |
| UDP-N-acetylmuramoyl-i-alanine | 1.065 |
| PE(20:3(S,Z,Z,11Z)/14:0/0:0) | 1.060 |
| celtotriose | 1.057 |
| BMS-268770 | 1.056 |
| 3-iodo-4-hydroxyphenylpyruvate | 1.056 |
| 6-(isopropylthio)purine | 1.053 |
| carmine | 1.053 |
| butanoylphosphate | 1.046 |
| DG(14:1(9Z)/22:5(4Z,Z,7Z,10Z,13Z,16Z)/0:0) | 1.045 |
| anisatin | 1.043 |
| 4-[2,2-dichloro-1-(4-methoxyphenyl)ethyl]phenol | 1.042 |
| PI(20:4(S,Z,11Z,14Z,17Z)/18:1(11Z)) | 1.040 |
| isoorientin | 1.034 |
| quercetin 3-O-glucoside | 1.017 |
| cefaloglycin | 1.015 |
| avodaride | 1.013 |
| justicidin B | 1.009 |
sucre levels, 50, 150, and 300 mM. The secondary metabolite profile of 150 mM sucrose was closer to that of 300 mM sucrose than to that of 50 mM sucrose. These results are similar to those obtained in a previous study on primary metabolite profiles in M. officinalis with 64 metabolites. This comparison indicates that the effect of sucrose level on primary metabolite profiles may be associated with the secondary metabolite profiles in M. officinalis. Moreover, the clustering of secondary metabolite profiles between sucrose levels was enabled by certain individual metabolites. For example, L-serine O-sulfate, thalidasin, spirilloxanthin, and quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucose increased in 50 mM. However, the levels of proline, glutathione, isoorientin scullcapflavone II, flavonol 3-O-(6-O-malonyl-β-D-glucoside), luciferyl sulfate, cassiamin C, and rutin were much higher in 300 mM sucrose than in 50 and 150 mM sucrose.

**Comparison of Individual Flavonoid Levels with 50, 150, and 300 mM Sucrose.** Most studies have reported only total flavonoid abundances to reveal the relationship between sucrose levels and contents of total flavonoids or the phenylpropanoid pathway without identifying or comparing individual flavonoid abundances. In this study, we identified individual secondary metabolites and determined the changes in each flavonoid, anthocyanin, and phenylpropanoid levels depending on sucrose levels.

To compare the changes in flavonoid level between the groups, one-way analysis of variance with the post hoc Tukey’s honestly significant difference test was conducted using Statistica ($p > 0.05$). The abundance of three flavonoids such as quercetin 3-O-β-D-glucosyl-(1→2)-β-D-glucose, 6-methoxyaromadendrin 3-O-acetate, and 3-hydroxycoumarin increased with 150 mM sucrose compared to those with 50 mM sucrose. However, compared to those with 50 mM sucrose, the abundances of most flavonoids such as 6-methoxyaromadendrin 3-O-acetate, 3-hydroxycoumarin, aurones, thymonin, rutin, justicidin B, isoorientin, quercetin 3-O-glucoside, umbelliferone, iridin, scullcapflavone II, cleistanthin A, flavonol 3-O-(6-O-malonyl-β-D-glucoside), isochamaemisin, gallocatechin-(α→8)-epigallocatechin, vitexin 2″-O-β-glucoside, kolaflavonone, kaempferide, and neoastilbin were significantly increased with 300 mM (Figure 3). These results showed that flavonoids accumulated depending on the sucrose level, indicating that sucrose induced the production of more flavonoids via the phenylpropanoid pathway.

Similar to these results, previous studies have reported that rutin accumulates in *Fagopyrum esculentum* Moench in response to sucrose and quercetin 3-O-glucoside accumulates in *Arabidopsis* under abiotic and oxidative stress. Our results showed that the types of accumulating flavonoids in *M. officinalis* differed depending on sucrose levels, and active flavonoid biosynthesis served as a defense mechanism against osmotic stress, suggesting that the biosynthetic pathway of flavonoids was regulated by the sucrose signaling pathway. Previously, it was observed at the messenger RNA level that sucrose caused the accumulation of anthocyanins and the upregulation of anthocyanin synthesis. However, our results showed that anthocyanins (e.g., malvidin and petunidin) did not accumulate under a high sucrose level at both 150 and 300 mM. This is possibly because anthocyanins other than malvidin and petunidin were not identified in this study, and malvidin and petunidin could not represent the behaviors of all other anthocyanins under a high sucrose level. The precise prediction and speculation of the secondary metabolism and
change in individual secondary metabolites of *M. officinalis* in response to the concentrations of sucrose should be supported and verified by further experiments.

### CONCLUSIONS

This is the first report to investigate the change in secondary metabolite profiles in *M. officinalis* depending on the sucrose level using UPLC-Q-TOF MS. One hundred and sixty-nine metabolites were identified using XCMS; these metabolites were major intermediates in the secondary metabolism of plants such as the biosynthesis of carotenoids, phenylpropanoids, flavones, and flavonols, which serves as a defense mechanism against stress in plants. PLS-DA and HCA results showed a significant difference in secondary metabolite profiles in *M. officinalis* between 50, 150, and 300 mM sucrose. In contrast to that with 50 mM sucrose, 32 secondary metabolites such as 6-methoxyaromadendrin 3-O-acetate and 3-hydroxyycoumarin accumulated in 150 mM, and 76 metabolites such as aureusidin, thymonin, quercetin 3-O-glucoside, and rutin increased in 300 mM. Accumulation of different types of flavonoids was observed depending on the sucrose level, suggesting that the accumulation of these flavonoids acts as a defense mechanism against osmotic stress. This study demonstrated that secondary metabolite profiles could be a useful tool for investigating the change in certain secondary metabolites and secondary metabolism in plants under osmotic stress and provide clues for manipulating plant metabolisms to produce target flavonoids, which have various properties such as antitumoral, antioxidant, antifungal, and antibacterial activities.

### MATERIALS AND METHODS

#### Plant Growth Conditions

*M. officinalis* was prepared as previously described. Briefly, *M. officinalis* was cultivated in 4 g/L Murashige and Skoog medium (0.025 mg/L of CoCl₂·6H₂O, 0.025 mg/L of CuSO₄·5H₂O, 36.70 mg/L of FeNaEDTA, 6.20 mg/L of H₃BO₃, 0.83 mg/L of KI, 16.9 mg/L of MnSO₄·H₂O, 0.25 mg/L of Na₂MoO₄·2H₂O, 8.60 mg/L of ZnSO₄·7H₂O, 332.02 mg/L of CaCl₂, 170.00 mg/L of KH₂PO₄, 1900.00 mg/L of KNO₃, 180.54 mg/L of MgSO₄, and 1650.00 mg/L of NH₄NO₃) containing 50 mM sucrose and 7 g/L agar at pH 5.7 after 2 cm-long explants with two leaves were transferred to the culture and test media with three different concentrations of sucrose, 50 (control), 150, or 300 mM, for examining the effects of sucrose concentration on flavonoid accumulation in *M. officinalis*. The leaves were incubated at 25 °C for 20 days (15:9 h light–dark cycle). The leaves of *M. officinalis* were harvested and quickly frozen in liquid nitrogen to quench cellular metabolism, and the frozen samples were stored at −80 °C.

#### Metabolite Extraction and UPLC-Q-TOF MS Analysis

Fifty milligrams of ground *M. officinalis* leaves were extracted with 0.5 mL of cold methanol (high-performance liquid chromatography grade, Merck, Darmstadt, Germany). The methanol extract was diluted with 50 μL and was thoroughly vortexed, after which it was centrifuged at 14,000 g for 5 min. The supernatant was filtered using a 0.45 μm syringe filter (hydrophilic poly(tetrafluoroethylene), Advantec, Dublin, OH). The metabolite extract was stored at −20 °C before UPLC-Q-TOF MS analysis.

Metabolite extract was analyzed by UPLC-Q-TOF MS. The UPLC analysis was performed using a Waters ACQUITY UPLC system (Waters, Milford, MA) equipped with a Waters ACQUITY BEH C18 column (100 × 2.1 mm, 1.7 μm). The mobile phase consisted of solvent A, 0.1% (w/v) formic acid in distilled water, and solvent B, 0.1% (w/v) formic acid in acetonitrile. The UPLC was eluted first with a linear gradient from 10 to 100% of solvent B (0−7.0 min) and then eluted isocratically with 100% of solvent B (7.0−8.0 min). The flow rate was 0.3 mL/min, and the injection volume was 5 μL. The column and autosampler were maintained at 35 and 15 °C, respectively. Mass spectrometry was performed using a Q-TOF platform.
micromass detector (Waters, Manchester, UK). The conditions of the Q-TOF mass spectrometer in the negative electrospray ionization (ESI) mode were 2800 V of capillary voltage, 35 V of sample cone voltage, 1.0 V of extraction cone voltage, 250 °C of desolvation temperature, 100 °C of source temperature, and 500 L/h of desolvation gas flow rate. The positive ESI was under the same conditions, except for an extraction cone voltage of 2.0 V. The ESI mass spectra were acquired over m/z 100–1500. Leucine-enkephalin was used as a reference ion by the LockSpray interface to measure mass more accurately and reproducibly.

**Data Processing and Statistical Analysis.** Acquired data were analyzed using Waters MassLynx (version 4.1). The noise elimination level was set at 6.0 with 10 masses per retention time being collected. Before further processing, lock spray scans were removed because lock spray peaks disrupted the detection and analysis of actual signals from samples (Figure S1A,B). UPLC-Q-TOF MS data were preprocessed using XCMS with signal-to-noise ratios as described in the literature (Table S1).43,44 Mass and retention time windows were set at 0.05 Da and 0.20 min, respectively. After normalization by log transformation, the processed data were further analyzed using PLS-DA and HCA with the Euclidean distance coefficient and average linkage methods. SIMCA-P+ (version 14.1, Umetrics AB, Umea, Sweden) was used for PLS-DA,36 and MeV (MultiExperiment Viewer; Dana-Farber Cancer Institute, Boston, MA) was used for HCA.55 Statistica (version 7.1; StatSoft, Tulsa, OK) was used for the univariate analysis.

**GUIDED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04745.

Chromatograms for the same samples before and after removal of lock spray scans as examples; validation of the PLS-DA model using the 100 permutation test; and number of peaks detected, peak groups, IP clusters, and predictions in negative and positive modes (PDF)

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**Character Contributions**

K.H.K. and D.L. conceived and designed the project. S.K. and H.L. collected the samples. S.K., J.K., and N.K. performed the experiments. S.K., J.K., N.K., D.Y.L., and D.L. acquired the metabolomics data. S.K., D.L., H.L., D.Y.L., and K.H.K. analyzed the data. S.K., D.Y.L., and K.H.K. wrote the manuscript.

**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by the Mid-career Researcher Program and Young Researcher Program from the National Research Foundation of Korea (NRF-2020R1A2B5B0202631 and NRF-2020R1G1A100826811, respectively). S.K. acknowledges the support of the Research Grant of Jeonju University in 2019. Facility support at the Korea University Food Safety Hall by the Institute of Biomedical Science and Food Safety is also acknowledged.

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