

Research Article

Flavonoid Productivity Optimized for Green and Red Forms of *Perilla frutescens* via Environmental Control Technologies in Plant Factory

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Received 21 June 2018; Revised 9 November 2018; Accepted 27 November 2018; Published 20 December 2018

Academic Editor: Encarna Aguayo

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Perilla frutescens (Lamiaceae) is a dietary staple in Asia. It is an abundant source of flavonoids that are bioactively beneficial to human health and fitness. The current popularity of plant-based consumption is being driven by the healthful benefits of bioactive nutrition, and the concentration of bioactive agents found in raw plant materials is an important factor in the assessment of food quality. To test the feasibility of promoting flavonoid productivity in *perilla* plants via environmental treatment, plant factory technology was applied to *perilla* plant cultivation. Apigenin (AG) and luteolin (LT) are two of the most potent anticarcinogenic flavonoids in *perilla*, and these are also found in many vegetables and fruits. Quantitative analysis of AG and LT was conducted on plants cultivated under nine environmental forms of treatment imposed by three levels of light intensity (100, 200, and 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) combined with three levels of nutrient-solution concentration (1.0, 2.0, and 3.0 $\text{dS}\cdot\text{m}^{-1}$) for hydroculture. The contents of AG in green and red *perilla* plant were increased by high nutrient-solution levels under the same light intensity. In green *perilla*, the highest concentration of AG (8.50 $\mu\text{g}\cdot\text{g}^{-1}$) was obtained under treatment of the highest level of nutrient-solution (3.0 $\text{dS}\cdot\text{m}^{-1}$) and 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of light intensity, whereas in red *perilla*, the highest concentration of AG (6.38 $\mu\text{g}\cdot\text{g}^{-1}$) was achieved from the highest levels of both of these forms of treatment (300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and 3.0 $\text{dS}\cdot\text{m}^{-1}$). The increase in AG content per plant between the lowest and the highest levels was recorded by 6.4-fold and 8.6-fold in green and red *perilla*, respectively. The behavior of LT concentration differed between green and red forms of *perilla*. LT concentration in red *perilla* was enhanced under nutrient deficiency (1.0 $\text{dS}\cdot\text{m}^{-1}$) and affected by light intensity. Different responses were observed in the accumulations of AG and LT in red and green *perilla* during treatments, and this phenomenon was discussed in terms of biosynthetic pathways that involve the expressions of phenylpropanoids and anthocyanins. The total yield of flavonoids (AG and LT) was improved with the optimization of those forms of treatment, with the best total yields: 33.9 $\text{mg}\cdot\text{plant}^{-1}$ in green *Perilla*; 10.0 $\text{mg}\cdot\text{plant}^{-1}$ in red *perilla*, and a 4.9-fold and a 5.4-fold increase was recorded in green and red *perilla*, respectively. This study revealed that flavone biosynthesis and accumulation in *perilla* plants could be optimized via environmental control technologies, and this approach could be applicable to leafy vegetables with bioactive nutrition to produce a stable industrial supply of high flavonoid content.

1. Introduction

Secondary metabolites (SMs) in plants are an important part of food nutrition, and their content could be used to determine food quality. SMs include terpenoids, phenylpropanoids, flavonoids, and alkaloids that are essential food ingredients that determine flavor, color, and taste in grains, vegetables, and fruits [1]. In addition, since the beneficial

bioactivities to human health from compounds such as antioxidants have become well known, many SMs are being recognized as having medicinal effects on both the human body and mind [2, 3].

Recently, the intake of foods rich in bioactive SMs has gained in popularity worldwide. However, maintaining stable supplies of raw plant materials that can be used to produce foods high in nutritional value remains problematic

due to the lack of an effective method to control the SM content in plant cultivation. Both the production and accumulation of SMs are sensitive to environmental conditions [4, 5]. Generally, plants grow rapidly in less stressful environments and produce better yields in biomass. On the other hand, both biotic and abiotic factors of stress induce the formation of SMs, such as phenolic compounds [6]. Meanwhile, a few attempts have been made to evaluate the quality of plants grown under different environments by detailing the stable expressions of the secondary metabolisms and concentrations of bioactive SMs that are expected to be included in plant-based foods as an additional health promoting component [7].

A plant factory is a food production system that can supply stable plant materials year-round with no influence from climate change [8], which has proven to be effective for medicinal plant production since the environmental conditions can be fully controlled. Owing to high levels of bacterial and insect control, no pesticides are needed for the plants produced in these systems. Therefore, plant factories can produce safe food with a desired level of quality when the optimal environmental condition can be determined for a particular crop. Regulating environmental factors such as light, temperature, and water has a significant impact on plant metabolic and synthetic pathways and enhances the SM content. It is well known that concentrations of SMs in plant are varying with its growth conditions. Providing high stressful environment to plants may promote accumulation of SMs; however, higher stress levels usually meanwhile result in lower yields. In the practical production in plant factories, it is greatly important to keep a balance between biomass yield and SMs concentration to maximize economic benefits. Therefore, quantifying the optimal stress level from a single environmental factor or from an interaction of multiple environmental factors becomes crucial for actual production of medicinal plants in a commercial plant factory. Unlike plant production in outdoor condition, a plant factory can precisely tune its environmental factors such as light intensity, light spectrum, nutrient solution level, temperature, air flow rate, etc. without limitation of locations. If the treatments and their effects on SMs synthesis are quantified, this technique can be directly applied into commercial plant factories all over the world not only theoretically but also practically.

Perilla frutescens (Lamiaceae) is widely used as a culinary herb in Asia, particularly in Japan. The color and flavor of *perilla* are familiar parts of traditional Japanese cuisine. In Japan, the extract of *perilla* is used to add color and flavor to pickled vegetables and Japanese plums. In Japan, Korea, and India, people commonly use *perilla* leaves in the preparation of raw fish and shell-fish to reduce the odor, and it is added to grilled red meat to add flavor [9]. As a medicinal herb for crude drugs, the extract of *perilla* is used for clinical applications, being listed in the pharmacopoeia in Japan [10], Republic of Korea [11], and in People's Republic of China [12].

Apigenin (AG) and luteolin (LT) are the main flavonoids present in *perilla* and are also contained in many vegetables and fruits: celery, parsley, and onions for AG; red peppers,

lettuce, berries, and onions for LT. AG, and LT are known as antioxidant, anti-inflammatory, and anticarcinogenic agents and, as such, they have attracted researchers' attention due to their potential as chemopreventive agents [1, 13–15]. Because of that, AG and LT have been candidate compounds for many pharmaceuticals or nutraceuticals from plant-derived dietary agents for the development of new therapies [1, 13–15]. For medical use, however, it must be stated that many flavonoids including AG and LT have hermetic effects that have the potential to make them toxic, so that dose adjustment is necessary to achieve safety and efficacy [15]. Thus, the concentrations of AG and LT in plant-based foods are important and must be controlled by meeting the challenges of technology that can supply these raw materials in good quality.

In a previous study, we found that rosmarinic acid (RA), a major phenylpropanoid compound in herbs of Lamiaceae family, is highly increased in *P. frutescens* grown under uptake stress created by a nutrient-limited condition combined with high light intensity (LI) in plant factories, while maintaining a constant concentration of perillaldehyde (PA), a main terpenoid found in *perilla* essential oils [16]. Given the differences that exist between terpenoids and phenylpropanoids with respect to biosynthetic pathways and accumulation, there likely are no synchronous increases in PA and RA. However, since the biosyntheses of flavonoids share the biosynthetic pathway with phenylpropanoids, it remains unclear how flavonoids might react to an environment that affects the production of phenylpropanoids. This should be clarified in order to establish quality control for raw plant materials according to the concentrations of compounds that are a rich source of food nutrition. Therefore, in the present study, we investigated the quantitative production of flavonoids in a *perilla* plant via the gradual introduction of stresses commonly encountered in cultivation and compared the quantitative expressions of AG and LT. In a plant factory with artificial lighting, *perilla* plants were cultivated under nine different levels of environmental treatment created by a combination of light intensity and nutrient concentration in hydroponics. Quantitative chemical analysis via liquid chromatography-mass spectrometry (LC-MS) was conducted to measure the concentrations of AG and LT as two major flavonoids in *perilla* plants grown under different growth conditions.

2. Materials and Methods

2.1. Perilla Materials and Growth Conditions. Green *perilla* (*P. frutescens* var. *crispa* f. *viridis* Makino, Takii Seed Co., Ltd., Kyoto, Japan) and red *perilla* (*P. frutescens* (L.) Britton. var. *acuta* Kudo f. *crispa* Makino, 0657-79TS, National Institute of Biomedical Innovation, Osaka, Japan) seeds were sown in rockwool cubes (125 cm³) in a cultivation room. The LI was set to 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (the unit of $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ represents photosynthetic photon flux density on the surface of cultivation containers) with a photoperiod of 16 h per day provided by cool white fluorescent lamps (FHF32 EX-N-H, Panasonic, Co., Ltd., Japan), and the plants were irrigated with a nutrient solution (Otsuka hydroponic composition,

OAT Agrio Co., Ltd., Tokyo, Japan) (Otsuka formula as described by Lu et al. [16]). The electrical conductivity (EC) and pH of the nutrient solution were adjusted to 1.2 dS·m⁻¹ and 6.0, respectively. Air temperature, relative humidity, and CO₂ concentration were set to 25/20°C (light/dark periods), 60–80%, and 400 μmol·mol⁻¹, respectively.

2.2. Treatments. Three weeks after sowing, red *perilla* and green *perilla* seedlings were transplanted into a walk-in type plant factory (2.9 m × 2.0 m × 2.3 m in LWH) and subjected to three LI levels (100, 200, and 300 μmol·m⁻²·s⁻¹) with a photoperiod of 16 h per day supplied by cool white fluorescent lamps and three EC levels (1.0, 2.0, and 3.0 dS·m⁻¹) for five weeks. The LI was measured at the surface of the rockwool cubes using a light meter (LI-250A; Li-Cor Inc., Lincoln, NE, USA) before placing the plants. The experiment was set up in a 3 × 3 full factorial in split plot design with LI as the main plot and EC levels as subplot, and each treatment contained 18 plants. Three nutrient solution tanks (EC of 1.0, 2.0, and 3.0 dS·m⁻¹ for each) were prepared beside the cultivation system. The plants were irrigated every 2 days from the bottom using fresh nutrient solution from each tank. The overflowed nutrient solution was discarded after each rockwool cube was saturated. Air temperature, relative humidity, and CO₂ concentration were set at 23/20°C (light/dark periods), 60–80%, and 1000 μmol·mol⁻¹, respectively.

2.3. Extraction. Extraction from *perilla* leaves was conducted according to Japanese Pharmacopoeia [10] and methods described in a previous article [16]. The leaves were sampled and dried at 30°C for 2 weeks. The dried *perilla* leaves were ground to powder and filtered through a sieve. A 10.00–10.50 mg sample was weighed accurately and transferred to a 1.5 mL tube. Methanol (1 mL) was added, mixed for 10 min at 2,000 rpm and 15°C using an Eppendorf ThermoMixer (Hamburg, Germany), and centrifuged for 5 min. To the residue, methanol (1 mL × 2) was added, and the same extract manner was performed twice. The extracts (about 3 mL) were combined and transferred to a 5 mL volumetric flask and diluted with methanol to a 5 mL total volume. The solution was filtered through Agilent 0.2 μm nylon syringe filters (Agilent Technologies Inc., Palo Alto, CA, USA) to prepare the samples for LC-MS.

2.4. Contents of AG and LT. LC-MS analysis of AG and LT was conducted according to a method described by Nishimura et al. [17] with some modifications. A Shimadzu LC-20A prominence system (HPLC) with a SIL-20AC auto-sampler and a LCMS-2020 mass spectrometer (MS) equipped with an electrospray ionization (ESI) source operating in negative mode were used for identification and quantification of AG and LT by chromatographic data processed using LabSolutions software (Shimadzu, Kyoto, Japan). The HPLC conditions for AG and LT were XBridge BEH C18 column (3.5 μm, 2.1 × 150 mm, Waters, MA, USA); temperature, 40°C; flow rate, 0.2 mL/min; run time, 15 min; mobile phase, 30% acetonitrile/0.1% formic acid;

and injection volume, 1 μL. The eluent was passed to the electrospray source. A capillary voltage of 3.5 kV was used in the negative ion mode. Nitrogen was used as drying gas with a flow rate of 15 L·min⁻¹ and as nebulizing gas with a flow rate of 1.5 L·min⁻¹. The desolvation line temperature was set at 250°C. The ion trap was operated in full-scan mode from *m/z* 50 to 1000 and selected-ion monitoring (SIM) mode with *m/z* 269 for a molecular ion [M–H]⁻ of AG and *m/z* 285 for a molecular ion [M–H]⁻ of LT. The standards of AG and LT were dissolved in a little of THF and then diluted with methanol to prepare the standard solutions before use for identification and quantification. AG and LT contents per leaf dry weight (hereafter, AG and LT concentration) were estimated by dividing the AG and LT contents in samples by sample weight.

2.5. Chemicals. Methanol (LC-MS grade) and water (LC-MS grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The AG and LT standards were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Acetonitrile (HPLC grade) was obtained from Sigma-Aldrich Japan (Tokyo, Japan). Formic acid was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

2.6. Statistical Analysis. All measurements were repeated three times for each sample. Five to six plants were sampled from each treatment to determine concentrations. The means of the treatment data were subjected to analysis of variance for comparison via Tukey's test using SPSS statistical software (IBM SPSS Statistics, Version 25.0. Armonk, NY: IBM Corp.). A *P* value <0.05 was considered significant.

3. Results and Discussion

3.1. Concentrations of AG and LT in Green and Red Perilla. Concentrations of AG and LT in green *perilla* are shown in Figures 1(a) and 1(b), respectively, as the content per unit of leaf dry weight. The concentration of AG was decreased under an EC of 1.0 dS·m⁻¹ using the same LI, and it tended to increase with increases in the EC under LIs of 100 and 200 μmol·m⁻²·s⁻¹ (Figure 1(a)). The concentration of AG was the lowest (2.95 μg·g⁻¹) under an EC of 1.0 dS·m⁻¹ and a LI of 200 μmol·m⁻²·s⁻¹, but it was increased to the highest level (8.50 μg·g⁻¹) of concentration (2.9-fold increase) under an EC of 3.0 dS·m⁻¹ and LI of 200 μmol·m⁻²·s⁻¹. LI had little effect on the AG concentration under the same EC, with the exception of a comparison between LIs of 200 and 300 μmol·m⁻²·s⁻¹ under an EC of 3.0 dS·m⁻¹. The resultant LT concentration showed no significant differences as a result of treatment and was affected neither by LI nor by EC (Figure 1(b)).

Concentrations of AG and LT in red *perilla* are shown in Figures 1(c) and 1(d), respectively, as the content per unit of leaf dry weight. The concentrations of AG tended to decrease with decreases in EC under the same LI, but LI showed little effect when EC treatment was unchanged. The lowest values (3.43 μg·g⁻¹) were obtained under the lowest levels of EC and LI, and the highest values (6.38 μg·g⁻¹) were obtained under

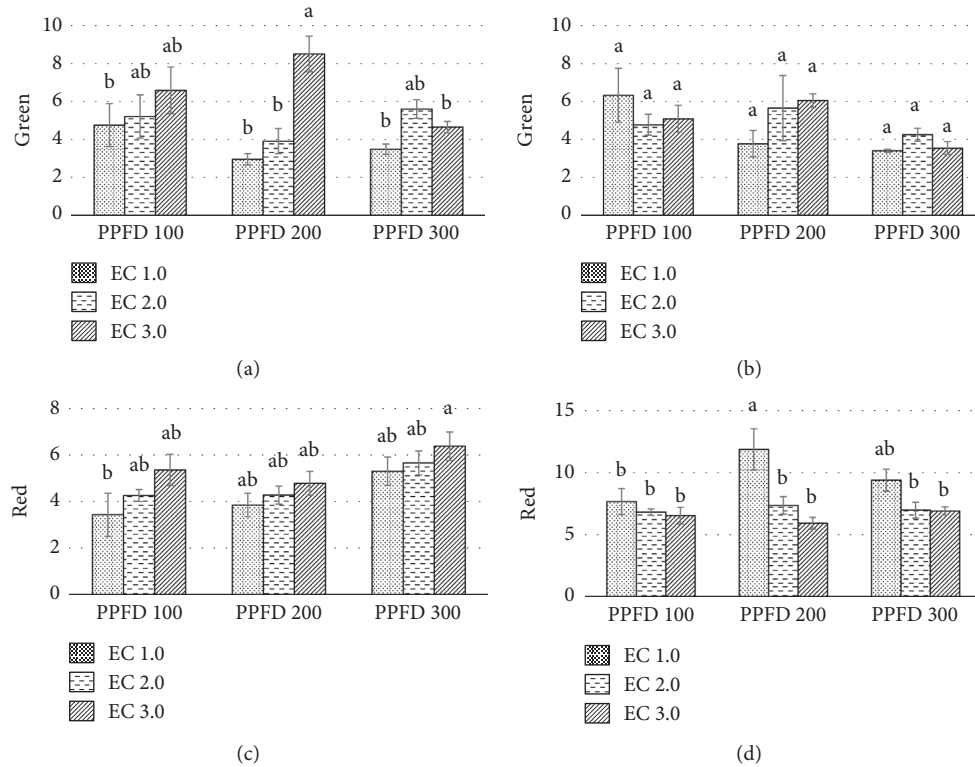


FIGURE 1: Content per unit of leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in *perilla* plants after 5 weeks of cultivation under different LI and EC treatments. (a) AG content per leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in green; (b) LT content per leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in green; (c) AG content per leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in red; (d) LT content per leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in red. Values are mean \pm standard error ($n = 5 - 6$). Different letters indicate significant differences between at $P < 0.05$, as determined by Tukey's test.

the highest levels of EC and LI (1.9-fold increase). LT concentrations showed small differences after treatments, with the noted exception of treatments between 100 and 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ under an EC of 1.0 $\text{dS}\cdot\text{m}^{-1}$. LT concentrations tended to increase with decreases in EC and were significantly increased by the lowest level of EC (1.0 $\text{dS}\cdot\text{m}^{-1}$) with a LI of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The concentration of LT was the lowest ($5.92 \mu\text{g}\cdot\text{g}^{-1}$) under an EC of 3.0 $\text{dS}\cdot\text{m}^{-1}$ and LI of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, but it was increased to the highest level ($11.9 \mu\text{g}\cdot\text{g}^{-1}$) of concentration (2.0-fold increase) under an EC of 1.0 $\text{dS}\cdot\text{m}^{-1}$ and LI of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

3.2. AG and LT Content per Plant in Green and Red *Perilla*.

The contents of AG and LT per plant in green *perilla* are shown in Figures 2(a) and 2(b), respectively. The highest EC (3.0 $\text{dS}\cdot\text{m}^{-1}$) yielded the higher AG and LT content per plant under the same LI. The lowest levels of AG and LT content per plant were recorded under an EC of 1.0 $\text{dS}\cdot\text{m}^{-1}$ and LI of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, but those levels were increased to the highest levels (6.4-fold and 3.7-fold increases in AG and LT, respectively) under an EC of 1.0 $\text{dS}\cdot\text{m}^{-1}$ and LI of 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The AG and LT content per plant depended on growth conditions that were determined by the level of either low EC or low LI.

The contents of AG and LT per plant in red *perilla* are shown in Figures 2(c) and 2(d), respectively. Unlike green

perilla plants, the highest LI (300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) yielded the higher AG content per plant under the same EC in red *perilla*. The higher level of AG content per plant was obtained under an EC level of 2.0 or 3.0 $\text{dS}\cdot\text{m}^{-1}$ combined with the highest LI (300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). All other treatments obtained significantly lower levels of AG content per plant compared with the above treatments. The lowest AG content per plant ($0.57 \mu\text{g}\cdot\text{plant}^{-1}$) obtained under an EC level of 1.0 $\text{dS}\cdot\text{m}^{-1}$ and LI of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ was increased to the highest ($4.90 \mu\text{g}\cdot\text{plant}^{-1}$) AG content per plant (8.6-fold increase) under an EC of 3.0 $\text{dS}\cdot\text{m}^{-1}$ and LI of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. LT content per plant tended to increase with increases in LI under EC levels of 2.0 and 3.0 $\text{dS}\cdot\text{m}^{-1}$. LT content per plant was the lowest ($1.30 \mu\text{g}\cdot\text{plant}^{-1}$) under an EC of 1.0 $\text{dS}\cdot\text{m}^{-1}$ and LI of 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and the highest value ($5.19 \mu\text{g}\cdot\text{plant}^{-1}$) was obtained (4.0-fold increase) under an EC of 3.0 $\text{dS}\cdot\text{m}^{-1}$ with LI of 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

3.3. Flavonoid Production in Green and Red *Perilla*.

Flavonoid biosynthesis has been studied extensively in different plant species [18]. Both AG and LT have a flavone in their chemical structure, and enzymes that work as key biocatalysts to synthesize the core structure of flavonoids such as flavone, flavonol, flavanone, and anthocyanidin. RA is an ester of two C6-C3 molecules called phenylpropanoid,

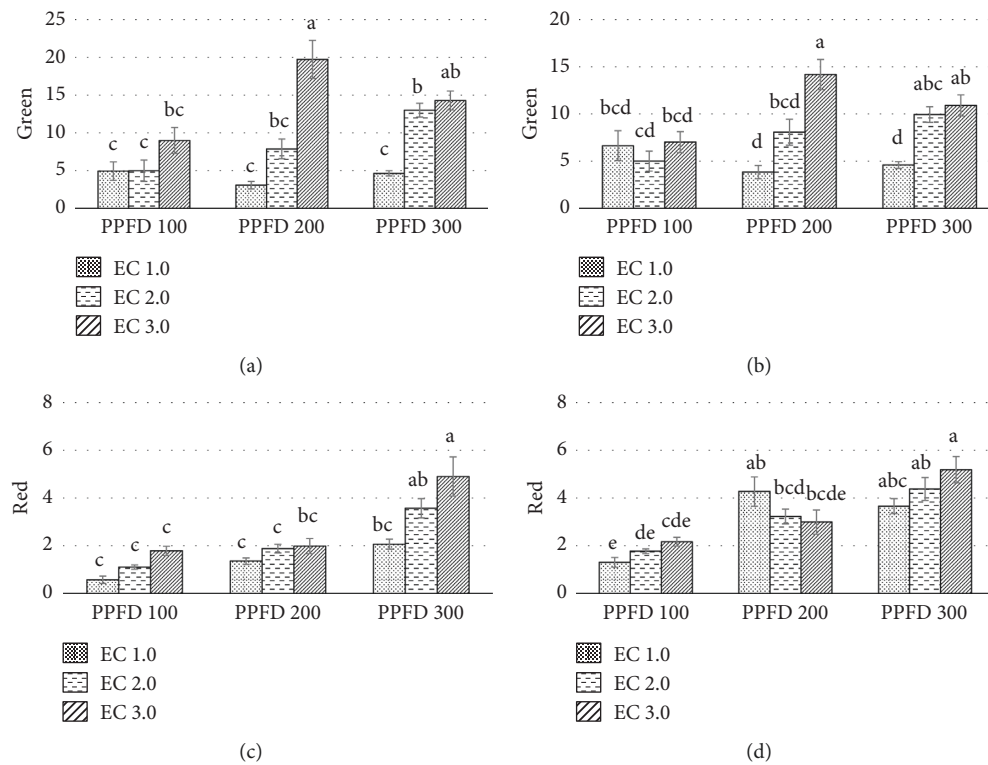


FIGURE 2: Content per plant in *perilla* plants after 5 weeks of cultivation under different LI and EC treatments. (a) AG content per plant ($\mu\text{g}\cdot\text{plant}^{-1}$) in green; (b) LT content per plant ($\mu\text{g}\cdot\text{plant}^{-1}$) in green; (c) AG content per plant ($\mu\text{g}\cdot\text{plant}^{-1}$) in red; (d) LT content per plant ($\mu\text{g}\cdot\text{plant}^{-1}$) in red. Values are mean \pm standard error ($n = 5 - 6$). Different letters indicate significant differences between at $P < 0.05$, as determined by Tukey's test.

and enzymes involved in the biosynthesis of RA have also been identified in *Coleus blumei* L. [19]. Recently, gene expression in *P. frutescens* associated with the biosynthetic pathways of flavonoids and phenylpropanoids has been revealed in studies using transcriptome analysis [20]. Thus, based on the knowledge above, we have proposed the biosynthetic pathways relevant to RA, AG, and LT, which are present in *perilla*, as shown in Figure 3. The pathway to flavones, AG and LT, includes the general biosynthesis of phenylpropanoids derived from L-phenylalanine to produce 4-coumaroyl-CoA, which is a crucial precursor for the biosynthesis of flavones and RA [19]. The substrate 4-coumaroyl-CoA is one of two used for esterification, during which it reacts with 4-hydroxyphenyllactic acid in a step that is important in the biosynthesis of RA. That fact suggests that producing molecules of AG, LT, and RA requires the use of an equivalent of 4-coumaroyl-CoA molecules as a biosynthetic precursor and also suggests the need to activate the enzymes involved in the phenylpropanoid pathway.

Our results showed that the effect of EC levels on the accumulation of AG and LT was quite different. The concentration of AG was highest for an EC of $3.0 \text{ dS}\cdot\text{m}^{-1}$, whereas that of the LT was highest for an EC of $1.0 \text{ dS}\cdot\text{m}^{-1}$. In addition, the AG concentration was lowest under an EC of $1.0 \text{ dS}\cdot\text{m}^{-1}$, whereas that of the LT was lowest under an EC of $3.0 \text{ dS}\cdot\text{m}^{-1}$. These opposition responses between AG and LT could reflect their competitive relationship in the sharing of

naringenin, which resides in the connection between the pathways to AG and anthocyanins (Figure 3). In our previous report, the concentration of RA was the highest under nutrient-limited conditions (EC of $1.0 \text{ dS}\cdot\text{m}^{-1}$) and decreased with the highest EC level (EC of $3.0 \text{ dS}\cdot\text{m}^{-1}$) in green and red *perilla* [16]. Interestingly, the similar responses between LT and RA were observed in red *perilla*, so that the LT concentration was enhanced in red *perilla* grown under nutrient-limited stress.

The effect of EC on LT accumulation differed between green and red *perilla*. In green *perilla*, the results of LT concentration showed neither a change nor a trend, but the LT content per plant had a wide range of figures depending on the environment. The yield of LT was promoted under high levels of both EC and LI in accordance with the biomass yield (Figures 1(b) and 2(b)). In red *perilla*, however, the concentration of LT was enhanced under an EC of $1.0 \text{ dS}\cdot\text{m}^{-1}$, which stood in opposition to the biomass trend (Figures 1(d) and 2(d)). This suggests that the LT metabolism in red *perilla* overcame the negative effect of EC during growth when LI reached at least $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, which caused an increase in the LT yield against a decrease in the biomass yield but only at this level of LI. With LI values of 100 and $300 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the yield of LT showed a clear dependence on the biomass yield.

We compared AG and LT accumulation in green and red *perilla* (Figures 4 and 5). In red *perilla*, LT accumulation was always higher than that of AG, whereas in green *perilla*

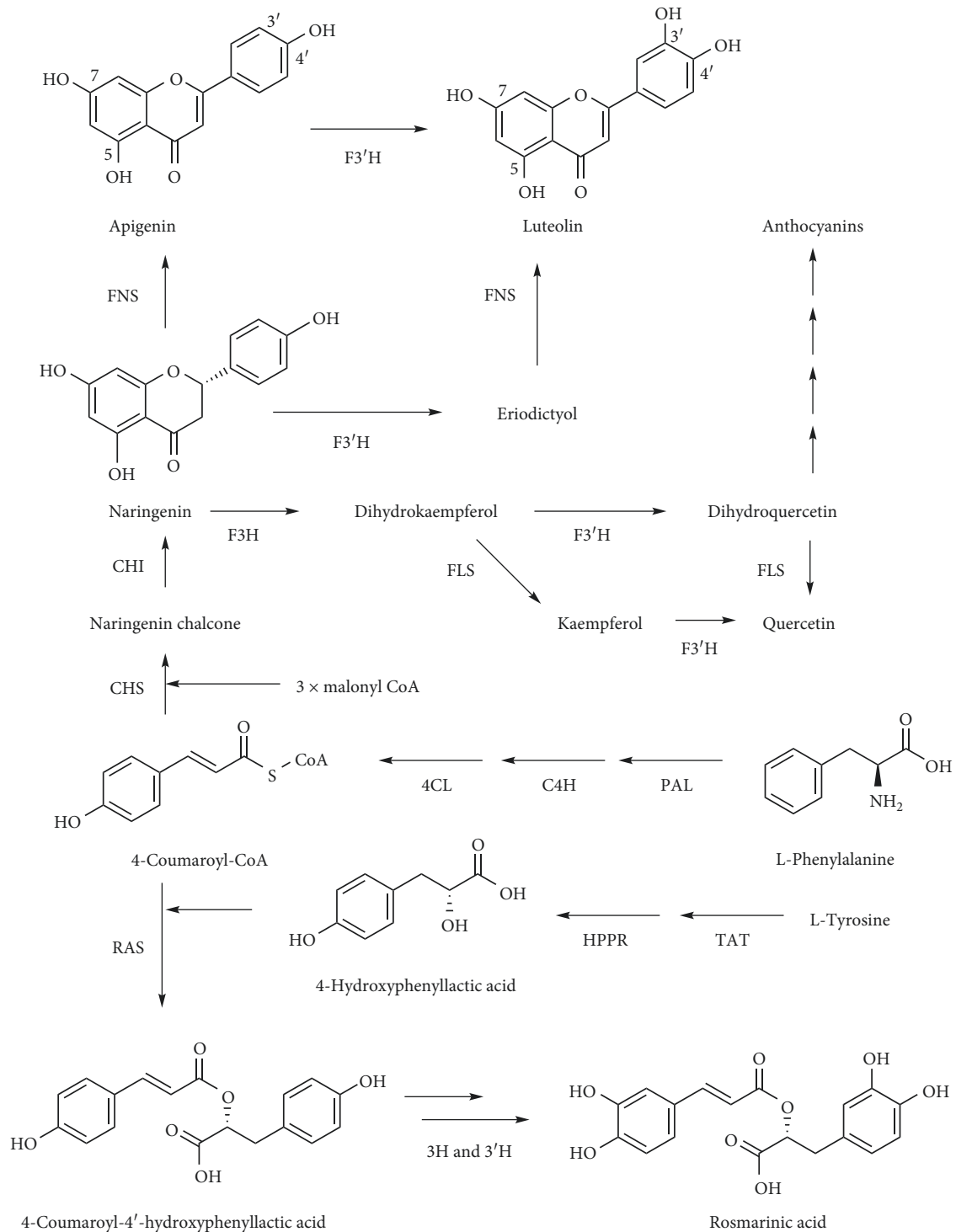


FIGURE 3: Proposed biosynthetic pathways for apigenin, luteolin, rosmarinic acid, and anthocyanins in *perilla* plants. Core enzymes involved in the biosynthesis of phenylpropanoid, flavone, and anthocyanin skeletons are shown. F3'H, flavonone 3'-hydroxylase; FNS, flavone synthase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; CHI, chalcone isomerase; CHS, chalcone synthase; PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumaric acid: CoA ligase; TAT, tyrosine aminotransferase; HPPR, hydroxyphenylpyruvate reductase; RAS, hydroxycinnamoyl-CoA: hydroxyphenyllactate hydroxycinnamoyl transferase; 3H and 3'H, hydroxycinnamoyl-hydroxyphenyllactate 3- and 3'-hydroxylases.

AG was higher than LT except for three treatments (Figure 4). LT molecules have a hydroxyl group at the 3' position on the flavone skeleton, and a crucial enzyme that introduces the 3'-hydroxyl group on the corresponding

precursor has been identified as flavone 3'-hydroxylase (F3'H) (Figure 3). Because red *perilla* plants manifest higher expression levels of genes encoding F3'H compared with levels expressed in green plants and because F3'H is

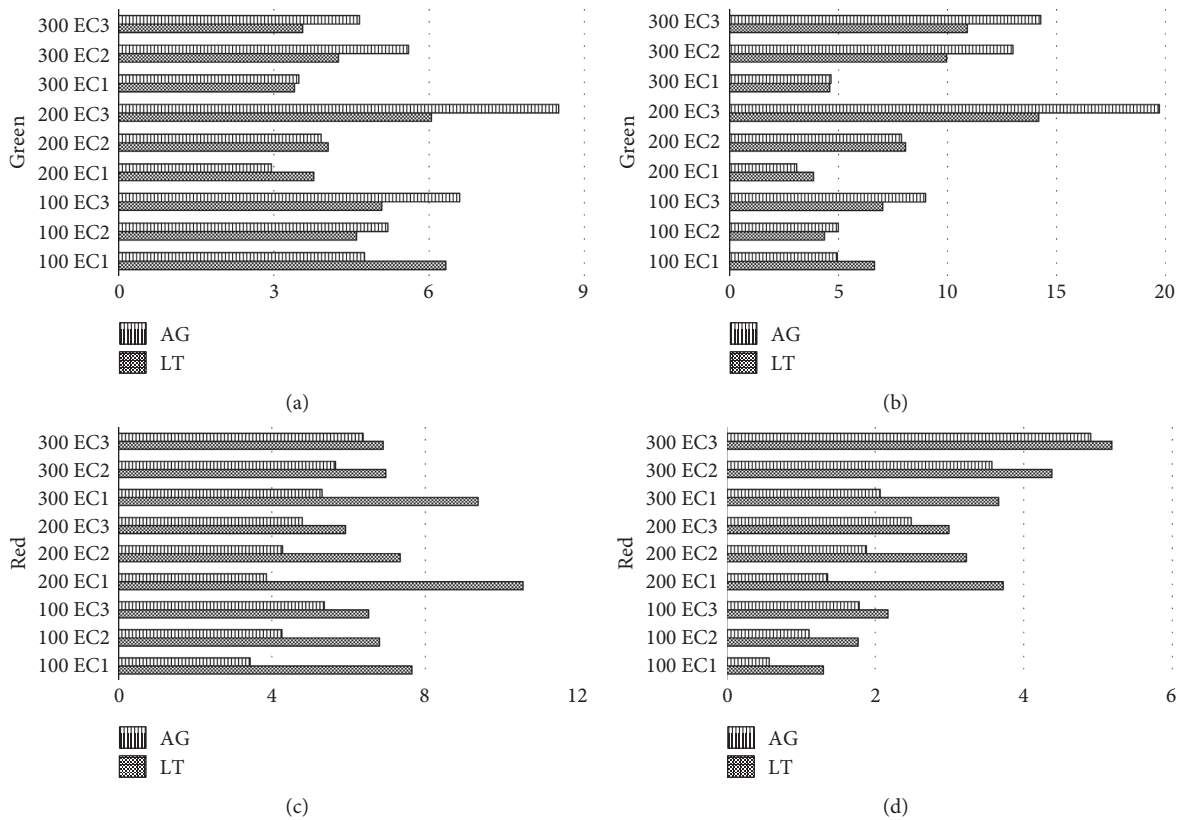


FIGURE 4: Comparative content of apigenin and luteolin in *perilla* plants after 5 weeks of cultivation under different LI and EC treatments. (a) AG vs LT content per leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in green; (b) AG vs LT content per plant ($\text{mg}\cdot\text{plant}^{-1}$) in green; (c) AG vs LT content per leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in red; (d) AG vs LT content per plant ($\text{mg}\cdot\text{plant}^{-1}$) in red.

one of the most important catalysts in anthocyanin synthesis in *Arabidopsis* [20], the accumulation of LT that was higher than that of AG in red *perilla*, presumably caused by the higher transformation activity of F3'H in anthocyanin biosynthesis in red plants compared with that in green plants. To clarify this interaction, we tried to trace biosynthetic precursors: naringenin chalcone, naringenin, kaempferol, quercetin, and eriodictyol by quantitative analysis using LC-MS, but none of them detected from extract of *perilla* dry leaves. It means that those molecules were not accumulated enough in a detective level on their relative pathway. Then, we thought that those precursors are transformed quickly and used for biosynthesis of AG, LT, and anthocyanins.

The total concentrations of AG and LT in green *perilla* were the lowest under a LI of $200\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and an EC of $1.0\ \text{dS}\cdot\text{m}^{-1}$, but these were increased to the highest levels (2.2-fold increase) of concentration under LI of $200\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and an EC of $3.0\ \text{dS}\cdot\text{m}^{-1}$ (Figure 5(a)). In red *perilla*, however, concentrations were the lowest under LI of $200\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and an EC of $3.0\ \text{dS}\cdot\text{m}^{-1}$ and increased to the highest levels (1.4-fold increase) under LI of $300\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and an EC of $1.0\ \text{dS}\cdot\text{m}^{-1}$ (Figure 5(c)). In other words, the range of total concentration was narrower in red plants compared with green plants, which indicated that red plants could withstand a greater environmental impact on flavone accumulation, which could result in

greater uniformity of food quality. The total yield of AG and LT in green *perilla* was the lowest under LI of $200\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and an EC of $1.0\ \text{dS}\cdot\text{m}^{-1}$ and increased to the highest yield (a 4.9-fold increase) under LI of $200\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and an EC of $3.0\ \text{dS}\cdot\text{m}^{-1}$ (Figure 5(b)). For red *perilla*, the total yield of AG and LT was the lowest under LI of $100\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and an EC of $1.0\ \text{dS}\cdot\text{m}^{-1}$ and increased to the highest yield (a 5.4-fold increase) under LI of $300\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and an EC of $3.0\ \text{dS}\cdot\text{m}^{-1}$ (Figure 5(d)). Because green plants obtained larger biomass yields than red plants, they had the best total yield of AG and LT ($33.9\ \text{mg}\cdot\text{plant}^{-1}$; LI: $200\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; EC: $3.0\ \text{dS}\cdot\text{m}^{-1}$) that were triple more than that of red plants ($10.0\ \text{mg}\cdot\text{plant}^{-1}$; LI: $300\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; EC: $3.0\ \text{dS}\cdot\text{m}^{-1}$) under application of the best treatments for both (Figures 5(b) and 5(d)), in which the total concentrations of AG and LT in green and red plants were almost the same, $14.6\ \mu\text{g}\cdot\text{g}^{-1}$ and $13.3\ \mu\text{g}\cdot\text{g}^{-1}$, respectively (Figures 5(a) and 5(c)).

4. Conclusions

We examined the bioactive flavonoids that appear in green and red forms of *perilla* plants. The results indicated that LI and EC were the factors that most affected flavonoid productivity in both concentration and yield of apigenin and luteolin. Therefore, environmental treatments that were regulated by those factors had a remarkable impact on

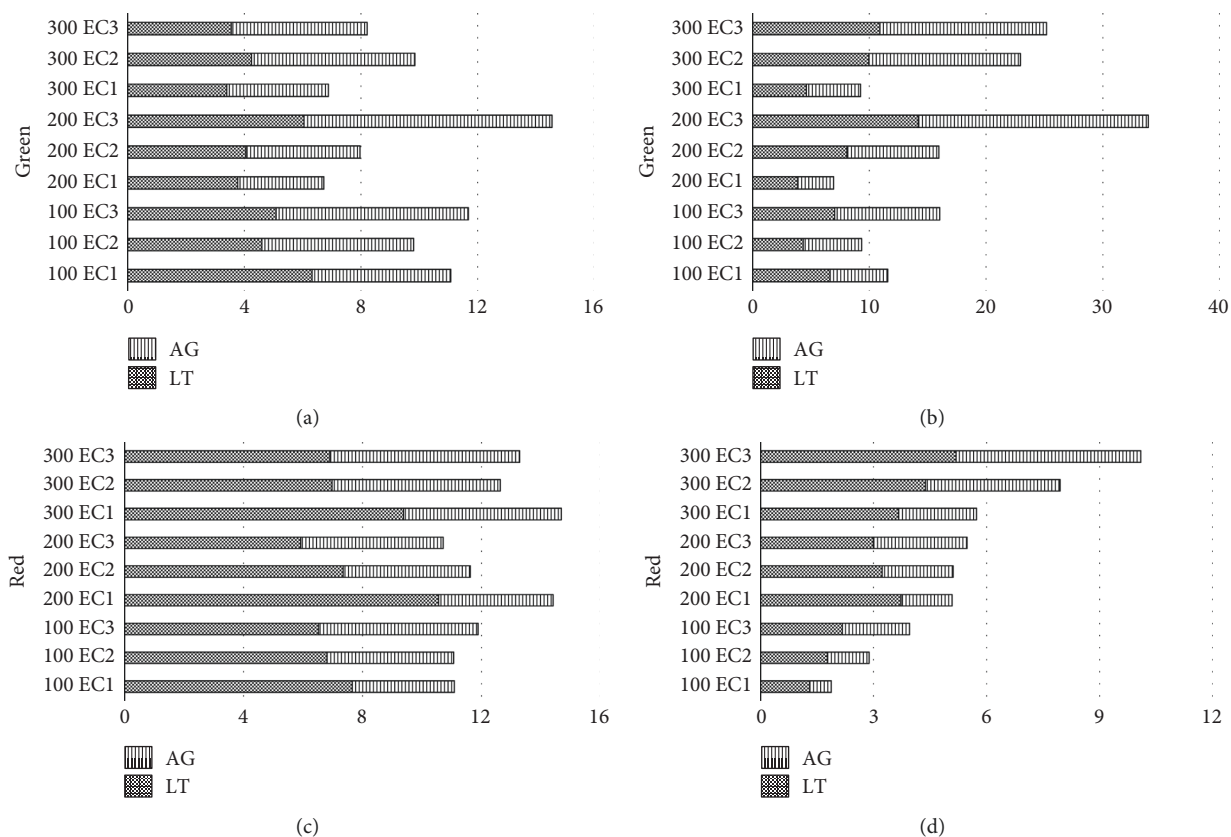


FIGURE 5: Total content of apigenin and luteolin (apigenin + luteolin) in *perilla* plants after 5 weeks of cultivation under different LI and EC treatments. (a) AG and LT content per leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in green; (b) AG and LT content per plant ($\text{mg}\cdot\text{plant}^{-1}$) in green; (c) AG and LT content per leaf dry weight ($\mu\text{g}\cdot\text{g}^{-1}$) in red; (d) AG and LT content per plant ($\text{mg}\cdot\text{plant}^{-1}$) in red.

flavone biosynthesis and accumulation, and their use is proposed as an effective method for the cultivation of green and red *perilla* plants. These findings could be applicable to an optimization of cultivation control in the production of foods composed of leafy vegetables containing bioactive compounds.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

This work was supported by the Japan Food Chemical Research Foundation and Phytochemical Plant Molecular Sciences, Chiba University. We thank Ms. Chayanit Tip-payadarapanich (Kasetsart University) for technical assistance in cultivation. We also thank Dr. Kazuki Saito and Dr.

Mami Yamazaki (Chiba University) for encouraging us to examine flavones in *perilla*.

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