**Title page**

Footnotes

Abstract

Key words

**INTRODUCTION**

In the first paragraph of the introduction, include the theoretical or conceptual basis for your work in a context accessible to the diverse botanical readership that AJB attracts.

Medicinal plants have played an important role in the traditional medicines of many indigenous populations for thousands of years (Shang et al., 2010). Due to this widespread usage, modern research techniques are being applied to identify the specific compounds responsible for these medicinal properties, and better characterize their method of action. Biotechnology as mass production system for medicines: <https://link.springer.com/article/10.1007/s11627-007-9055-4>

Part of the mint family Lamiaceae, the *Scutellaria* genus contains multiple species of plants renowned for their medicinal properties. *S. barbata* is commonly used in traditional Eastern medicines to treat swelling and inflammation, and multiple reports have recently been published describing its effectiveness in cancer treatments (Shang et al., 2010; Q. Wang et al., 2018). *S. lateriflora* is used in traditional Native American treatments as a nerve tonic and sedative, with recent studies demonstrating its anticonvulsant and anxiolytic properties (Awad et al., 2003; Zhang et al., 2009). Perhaps the most well-known species is *S. baicalensis* due to its extensive use in traditional Eastern remedies. More commonly called Huang Qin in Chinese medicine, the roots of *S. baicalensis* are prescribed to treat a variety of ailments, including edema, dysentery, pneumonia, jaundice, and more (T. Zhao et al., 2019). In clinical studies, *S. baicalensis* root extracts have been found to exhibit neuroprotective, antibacterial, antitumor, antioxidant, and other beneficial health effects (Tao et al., 2018; Venkatarame Gowda Saralamma et al., 2017; Zhu et al., 2016).

These beneficial effects can be largely attributed to the bioactive phytochemicals which these *Scutellaria* species accumulate in high concentrations (Q. Zhao, Chen, et al., 2016). 4’-hydroxyflavones, which include apigenin and its derivatives, have been isolated from multiple *Scutellaria* species, but are also widely distributed throughout multiple families in the plant kingdom. 4’-deoxyflavones however, which includes chrysin and its derivatives, have been demonstrated to be mostly specific to the *Scutellaria* genus (Q. Zhao, Zhang, et al., 2016). Only several species of plants outside of the *Scutellaria* genus have been found to be capable of synthesizing this class of flavones (Kato et al., 1992; V. M. Rao et al., 2009; Y. K. Rao et al., 2002). The specific biological activities of the majority of both these classes of flavones have been studied (Lin et al., 2012; Salehi et al., 2019; Shang et al., 2010). In addition, the accumulation patterns of these flavones have been characterized for several more well-known *Scutellaria* species, such as the previously mentioned *S. barbata*, *S. lateriflora*, and *S. baicalensis* (Cole et al., 2008; L. Wang et al., 2020).

In *S. baicalensis*, an organ-specific accumulation pattern can be observed, where 4’-hydroxyflavones accumulate in the aerial parts at higher concentrations than in the roots, and 4’-deoxyflavones accumulate in the roots at higher concentrations than in the aerial parts. The biosynthetic pathway responsible for this organ-specific accumulation of flavones has been largely elucidated (Q. Zhao, Zhang, et al., 2016). More explanation – specific genes responsible for organ-specificity. In addition, a reference genome for *S. baicalensis* was published in 2019 (Q. Zhao et al., 2019).

Although the phytochemical profile of *S. baicalensis* has been well-characterized, the *Scutellaria* genus contains more than 470 species, distributed nearly worldwide (Yoonkyung & Kim, 2020). Because phytochemical profiles for most of these species are not well-studied, the stability of the flavonoid biosynthetic pathway across the genus is largely unknown. To better characterize flavonoid diversity in *Scutellaria*, we performed a phylogenetic-based survey of 14 flavonoids in 75 species of *Scutellaria*. One phenol was also quantified. A subset of 13 species was then selected for additional chemical and genome size analysis. Our results indicate a relationship between phylogeny and phytochemical profile, and we identified several species which could serve as targets for biotechnology improvement.

**MATERIALS AND METHODS**

***Chloroplast genome sequencing and phylogenetic tree construction –***

Ask Yoonkyung

***Planting and growing conditions for fresh samples –***

Viable seeds were obtained for several species, including *S. altissima*, *S. arenicola*, *S. baicalensis*, *S. barbata*, *S. havanensis*, *S. leonardii*, and *S. racemosa*. To improve germination rate, seeds were incubated in a 100 uM solution of gibberellic acid for 1 hour with gentle shaking. Soil/water/light?

Need KR growing info – collected from field?

***Phytochemical extraction and quantification –***

*Herbarium tissue samples –*

14 flavonoids and one phenol were extracted and quantified from the aerial tissues of herbarium vouchers of 67 unique *Scutellaria* species. The flavonoids quantified were apigenin, apigenin-7-glucuronide (apigeninG), scutellarein, scutellarin, hispidulin, hispidulin-7-glucuronide (hispidulinG), chrysin, chrysin-7-glucuronide (chrysinG), baicalein, baicalin, oroxylin A, oroxyloside, wogonin, and wogonoside. The phenol quantified was acetoside. Tissue samples were weighed with an analytical balance, and an extraction buffer of 80% High Performance Liquid Chromatography (HPLC) grade methanol added to each so that the following ratio was achieved: 10 mg tissue / 1 mL solvent. Samples were sonicated for 1 hour at room temperature. The extraction buffer from each sample was withdrawn and further diluted by adding 80% HPLC grade methanol so that the following ratio was achieved: 1 mg tissue / 1 mL solvent. To remove any remaining tissue particles, the diluted extraction buffer was centrifuged at 15,000 rpm for 5 minutes and ran through a syringe filter with a pore size between 0.2 and 0.45 µM. Phytochemical concentrations in this final solution were quantified with a Thermo Scientific UltiMate 3000 HPLC system. Phytochemicals were separated with an 3 x 100 mm Acclaim RSLC 120 C18 column, and eluted by a mixture of 0.1% formic acid (A) and 100% acetonitrile (B) with the following gradient: -8 to 0 min, 5% B; 2 min, 25% B; 2 to 6 min, 25% B; 9 min, 50% B; 9 to 11 min, 50% B; 15 min, 95% B; and 15 to 23 min, 95% B. A flowrate of 0.5 mL/min was used and the column oven temperature set to 40°C. Calibration mixes of 0.1, 0.5, 1, 5, 10, 25, 50, and 100 µM were used to convert peak areas to concentrations in µM. Preparation of calibration mixes + source of phytochemicals. So that rough comparisons could be made between herbarium and fresh samples, all measured phytochemical concentrations were divided by 2 to represent a ratio of 0.5 mg tissue / 1 mL solvent.

*Fresh tissue samples –*

The same 15 phytochemicals were extracted and quantified from the root, stem, and leaf tissues of 13 *Scutellaria* species. Tissue samples for each organ were collected in triplicate from mature plants. Root tissue samples were gently washed to remove soil. The phytochemical extraction and quantification method closely follows that used for the herbarium samples. However, the initial extraction buffer was added to achieve a ratio of 30 mg tissue / 1 mL solvent instead of 10 mg tissue / 1 mL solvent, and then after sonication, diluted to achieve a ratio of 5 mg tissue / 1 mL solvent instead of 1 mg tissue / 1 mL solvent.

***Phylogeny-phytochemical comparison by multiple factor analysis –***

Comparisons between phylogenetic clade and phytochemical profile were made using a multiple factor analysis-based approach. This analysis used HPLC data for the 15 phytochemicals extracted from aerial tissue samples of 75 *Scutellaria* species. 67 of these tissue samples were herbarium vouchers, and the remaining 8 were fresh tissue. To reduce bias resulting from mixing data from herbarium and fresh tissue samples, all phytochemical concentrations were converted into a binary format where only the presence or absence of a phytochemical was considered. The multiple factor analysis was conducted in R with the “MFA” function from the FactoMineR package (ver. 2.3). Following the biosynthetic pathway for *S. baicalensis* described by Zhao et al. (2016), phytochemicals were split into two group – aerial-specific flavonoids, root-specific flavonoids, and phenols. Aerial-specific flavonoids included apigenin, apigeninG, scutellarein, scutellarin, hispidulin, and hispidulinG. Root-specific flavonoids included chrysin, chrysinG, baicalein, baicalin, oroxylin A, oroxyloside, wogonin, and wogonoside. Phenols included acetoside only. 95% confidence ellipses were drawn from the covariance matrix calculated with the “covMcd” function from the robustbase package (ver. 0.93-6).

***Estimation of genome size with flow cytometry –***

Ten species of *Scutellaria* were collected from the field and transplanted in the greenhouses of the Sungshin Women’s University (Seoul, Korea) and the University of Florida (Florida, USA). Voucher specimens were deposited in each herbarium of the university (Table #). We requested seeds of *Solanum lycopersicum L*. ‘Stupické polní rané’ (2C = 1.96 pg), and *Glycine max Merr.* ‘Polanka’ (2C = 2.50 pg) from Dr. Jaroslav Doležel (Institute of Experimental Botany, Olomouc, Czech Republic) who suggested them as size-standards for flow cytometry (Doležel et al., 2007). Leaves of these plants were used as size standards for genome-size estimation. In each estimation, a standard sample was selected based on the previously reported genome size information in *Scutellaria* (Lee and Kim, 2017).

The genome size of each plant was estimated using flow cytometry as described in Doležel et al. (2007). Fresh leaves from a standard plant and a sample for estimation (each ca. 5 mm²) were co-chopped using a razor blade in a petri dish with DAPI Prep DNA Staining Solution (SONY, Biotechnology Inc., USA) and incubated two minutes for staining. Cell sorter SH800 (SONY, Biotechnology Inc., USA) was used to measure the fluorescence of the stained cells and ensure each sample measured more than 5,000 particles. Each analysis was repeated three or more times using different leaves or different individuals, and their average and standard deviation were calculated. The 2C-value was estimated based on the relative counts between G1 (growth 1 stage on the cell division) peak from a standard plant and that from a sample for the estimation. The genome size (bp) was estimated based on relative rate between 1C-value and number of bases: 1C (pg) DNA = 0.978 × 109 bp (Doležel et al., 2007).

**RESULTS**

***Phylogeny of selected species as revealed by chloroplast genome sequences –***

***Aerial tissue phytochemical diversity and comparison with phylogeny –***

**10.1007/s11306-010-0269-9**

We applied high performance liquid chromatography (HPLC) to analyze the concentrations of 14 flavonoids and 1 phenol from aerial tissue samples of 75 species of *Scutellaria* (Figure 2A). 50 of the 75 species were included in the phylogenetic tree of Figure 1. 67 of the tissue samples were from herbarium vouchers with one replicate, and the remaining eight from fresh tissue. So that rough comparisons could be made between data from herbarium and fresh tissue samples, concentrations for herbarium samples were calculated at 0.5 mg tissue / 1 mL solvent, and concentrations for fresh samples at 5 mg tissue / 1 mL solvent.

The most commonly occurring phytochemical was chrysinG, which we detected in 49 of the 75 species at an average concentration of 8.330 µM. As its non-glycosylated form, chrysin, is a precursor for all 4’-deoxyflavones we quantified, this supports the role of chrysin as an important … metabolic intersection/decision? Pct of species accumulating downstream products, with chrysin/chrysinG detected. Rarity and low abundance of chrysin – very quickly converted into downstream products. We detected chrysin in only 31 species at an average concentration of 3.987 µM. This reflects the lower stability/higher reactivity of chrysin as compared to its glycosylated counterpart, and could possibly indicate that in most species, chrysin, once synthesized, is very quickly converted into other downstream 4’-deoxyflavones. A similar observation can be made regarding apigenin, a precursor for all 4’-hydroxyflavones we quantified. We detected apigenin in 22 species at an average concentration of 2.150 µM. However, we detected the glycosylated form of apigenin, apigeninG, in 42 species at an average concentration of 4.246 µM. The rarest flavonoid we quantified was hispidulinG, which was detected in only one species, *S. coerulea*.

In general, we detected the glycosylated form of each phytochemical (chrysinG, wogonoside, apigeninG, baicalin, scutellarin, and oroxyloside) more often and in greater amounts, than its non-glycosylated counterpart (chrysin, wogonin, apigenin, baicalein, scutellarein, and oroxylinA). This reinforces … that the glycosylated form is a more stable, storage form. The exception was hispidulinG and hispidulin, as hispidulin was detected in 27 species, but hispidulinG in only one. Out of the five most commonly occurring phytochemicals, four were 4’-deoxyflavones (chrysinG, wogonoside, baicalin, and wognonin), and only one was a 4’-hydroxyflavone (apigeninG). This result suggests that at least in aerial tissues, the 4’-deoxyflavone biosynthetic pathway is more well conserved across the *Scutellaria* genus than the 4’-hydroxyflavone pathway. Comment on *S. baicalensis* organ-specific pathway*.*  However, it is also possible that the 4’-deoxyflavones we quantified are more stable than the 4’-hydroxyflavones, and as a result, were less likely to be degraded over time in the herbarium vouchers.

Clade-by-clade analysis – identify any flavonoids which are differentially accumulated between clades

Grouping of species by flavonoid profile (MFA).

***Organ-specific phytochemical diversity –***

From the initial set of 75 species selected for aerial tissue phytochemical profiling, we identified 13 species with interesting accumulation patterns to investigate further. Comment on morphology of selected species? (Fig 3). At least one species was selected from each of the five clades previously identified (Fig 1). The selected species were grown fresh, and tissue samples taken in triplicate from the roots, stems, and leaves of mature plants. We applied HPLC to quantify concentrations of the same 15 phytochemicals as in our aerial tissue analysis. Phytochemical concentrations are expressed at a ratio of 5 mg tissue / 1 mL solvent.

Based on our root-specific phytochemical profiling results, the 4’-deoxyflavone pathway appears to be very well conserved across all of the species we selected. We detected wogonin, oroxylinA, baicalein, or their glycosylated forms in the majority of root samples from the 13 species. A notable exception was *S. insignis*, which accumulated only wogonin and wogonoside at relatively low concentrations – exact #. Interestingly, although chrysin serves as a precursor for all the 4’-deoxyflavonoids we quantified, we only detected chrysin in the root tissues of 2 species, and its glycosylated form, chrysinG, in 7. This rarity in detection is possibly a result of chrysin rapidly being converted into downstream products before it accumulates to a level detectable by our HPLC method.

The absence of 4’-hydroxyflavones in the root tissues of all but 3 species (*S. barbata*, *S. arenicola*, and *S. leonardii*) indicates the specificity of the 4’-hydroxyflavone biosynthetic pathway to the aerial tissues of the plant, in line with that described for *S. baicalensis* (Q. Zhao, Zhang, et al., 2016). Of the three species we found to contain 4’-hydroxyflavones in their root tissues, *S. arenicola* accumulated the highest amount, with ... uM of scutellarin. Interestingly, we detected a much smaller concentration of scutellarin in the stems of *S. arenicola*, and none in its leaves. This accumulation pattern suggests that the biosynthetic enzymes responsible for scutellarin production are active in the roots of *S. arenicola*, and scutellarin is not just simply being transported from the aerial tissues.

Although root-specific phytochemical profiles were relatively consistent across the 13 species we selected, aerial tissue-specific profiles were much more varied. We detected a mix of 4’-deoxyflavones and 4’-hydroxyflavones in the leaves of 8 species. Two species accumulated exclusively 4’-deoxyflavones in their leaves, and 3 accumulated exclusively 4’-hydroxyflavones in their leaves. General comments about species – havanensis with high accumulation, insignis with low

Our detection of 4’-deoxyflavones in the leaves of the majority of the species we selected indicates that the 4’-deoxyflavone pathway is not root-specific to the same degree that the 4’-hydroxyflavone pathway is aerial tissue-specific. However, it is possible that 4’-deoxyflavones being synthesized in root tissues are simply being transported to aerial tissues. This is supported by flavonoid profiles displayed in the stems of several species, such as *S. altissima* and *S. tournefortii*. The stems of these species accumulate a mix of 4’-hydroxyflavones from the leaves of the plant, and 4’-deoxyflavones from the roots. Although 4’-deoxyflavones are possibly being shuttled to aerial tissues from the roots of these species, our failure to detect 4’-hydroflavones in the roots indicates that 4’-hydroxyflavones produced in aerial tissues are not being shuttled in a similar fashion. This 4’-deoxyflavone shuttling hypothesis can explain the accumulation patterns of *S. altissima* and *S. tournefortii*, which contain lower concentrations of 4’-deoxyflavones in their aerial tissues relative to their roots. However, for a species such as *S. racemosa*, which we found to accumulate the 4’-deoxyflavones oroxylinA and oroxyloside at concentrations # times higher in its leaves as compared to in its roots, it is likely that at least some biosynthetic enzymes in the 4’-deoxyflavone pathway are active in the aerial parts of the plant.

Interestingly in *S. altissima* and *S. tournefortii*, we detected much greater concentrations of chrysin and chrysinG in the leaves of the plants than we did in the roots. We observed a similar pattern in several other species as well, including *S. baicalensis*, *S. havanensis*, *S. leonardii*, and *S. pekenensis var. alpina*. Possible explanations – downstream 4’-deoxyflavone biosynthetic enzymes are not active in the aerial parts of these species. Thus, instead of being quickly converted into downstream products as occurs in the roots, chrysin is able to accumulate to much higher concentrations.

In *S. baicalensis*, we were able to detect several 4’-deoxyflavones in our leaf tissue samples. This finding directly contrasts with the organ-specific flavonoid pathway proposed for *S. baicalensis* by Q. Zhao, Zhang, et al. (2016). However, in our stem tissue samples, we detected a mix of both

This variation in accumulation pattern indicates that 4’-deoxyflavone pathway is not exclusive to the root tissues ... Pathway doesn’t match with observed profiles for *S. baicalensis* – phytochemicals could be transported between tissues. However, in some species (e.g. *S. strigillosa*), we observed that concentrations of 4’-deoxyflavones are greater in the leaves of the plant than they are in the roots. This is evidence supporting the activity of the 4’-doexyflavone biosynthetic pathway in the aerial tissues.

Stems – mixing between leaf and root flavonoids

Proposed aerial flavonoids are mostly specific to aerial tissues, but root flavonoids are non-specific

***Genome size estimations –***

**DISCUSSION**

Include a summary of conclusions and a take-home message for the generally informed reader in the DISCUSSION.

Differences with *S. baicalensis* biosynthetic pathway and pattern of accumulation

Chrysin not detectable in roots despite being a precursor for all 4’-deoxyflavones – possible metabolon?

4’-deoxyflavones are more well-conserved – serve important core roles in plant growth/signaling

4’-hydroxyflavones are less well-conserved – serve accessory roles, specific to environmental conditions (e.g. attracting specific pollinators, pest or pathogen defense)

**Acknowledgments**

**Author contributions**

**Data availability statement**

Github? - need to clean up

Chloroplast genome sequences?

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**Tables**

Table 1: Genome sizes as estimated by flow cytometry, and a summary of previously published genome sizes and chromosome numbers.

**Appendices**

Table S1: Table w/ voucher #s for all herbarium samples

Table S2: .csv of all flavonoid profiling results

**Figure Legends**

Figure 1: Maximum likelihood phylogenetic tree inferred from 3 chloroplast genomic regions for 51 species of *Scutellaria* and 1 outgroup. To facilitate downstream analysis, the tree was subdivided into 5 color coded clades.

Figure 2: Aerial flavonoid concentrations measured with High Performance Liquid Chromatography (HPLC) for 75 species of *Scutellaria*. (A) Heatmap of collected data. Colored circles next to species names indicate phylogenetic clade, as shown in Figure 1. An empty circle indicates that the species was not included in the tree from Figure 1. Data for fresh tissue samples is shown at 5000 ppm (5 mg sample / 1 mL solvent). Data for herbarium tissue samples is shown at 500 ppm (0.5 mg sample / 1 mL solvent). (B) Multiple Factor Analysis (MFA) results generated from binary version of flavonoid data. (C) Variable representations from MFA.

Figure 3: Representative images of 13 species of *Scutellaria* selected for additional flavonoid and genome size profiling. Scale bar in bottom left of images represents a length of 5 cm. Images without a scale bar did not have a ruler included in the image. Colored circles next to species names indicate the clade which the species belongs to.

Figure 4: Organ-specific flavonoid data collected from 13 *Scutellaria* species via High Performance Liquid Chromatography (HPLC). Species are ordered based on phylogenetic relationship determined from chloroplast genome data, shown in Figure 1. Colored circles next to species names indicate phylogenetic clade, as shown in Figure 1. An empty circle indicates that the species was not included in the phylogenetic tree. Flavonoids are ordered based on proposed flavonoid pathway for *S. baicalensis*.