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ORIGINAL ARTICLE

Molecular characterization of flavonoid biosynthetic genes and accumulation of baicalin, baicalein, and wogonin in plant and hairy root of *Scutellaria lateriflora*

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Abstract *Scutellaria lateriflora* is well known for its medical applications because of the presence of flavanoids and alkaloids. The present study aimed to explore the molecular aspects and regulations of flavanoids. Five partial cDNAs encoding genes that are involved in the flavonoid biosynthetic pathway: phenylalanine ammonia lyase (SlPAL), cinnamate 4-hydroxylase (SlC4H), 4-coumaroyl CoA ligase (Sl4CL), chalcone synthase (SlCHS), and chalcone isomerase (SlCHI) were isolated from *S. lateriflora*. Organ expression analysis showed that these genes were expressed in all organs analyzed with the highest levels correlating with the richest accumulation of wogonin in the roots. Baicalin and baicalein differentially accumulated in *S. lateriflora* plants, with the highest concentration of baicalin and baicalein detected in the leaves and stems, respectively. Exogenous methyl jasmonate (MeJA) significantly enhanced the expression of *SlCHS* and *SlCHI*, and accumulation of baicalin (22.54 mg/g), baicalein (1.24 mg/g), and wogonin (5.39 mg/g) in *S. lateriflora* hairy roots. In addition, maximum production of baicalin, baicalein, and wogonin in hairy roots treated with MeJA was approximately 7.44-, 2.38-, and 2.12-fold, respectively. Light condition increased the expression level of *SlCHS*, the first committed step in flavonoid biosynthesis in hairy roots of *S. lateriflora* after 3 and 4 weeks of development compared to the dark condition.

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Dark-grown hairy roots contained a higher content of baicalin and baicalein than light-grown hairy roots, while light-grown hairy roots accumulated more wogonin than dark-grown hairy roots. These results may helpful for the metabolic engineering of flavonoids biosynthesis in *S. lateriflora*.

1. Introduction

More than 6000 flavanoid compounds were detected in higher plants (Ferrer et al., 2008). Flavonoids are reported as one of the essential factors of the environment (Yu and Jez, 2008; Subramanian et al., 2007). Mainly the metabolites belonging to flavonoids were present in food and beverages and are also associated with a reduced risk of some kinds of cancer when consumed in high or increased quantities (Tang et al., 2009; Wilson et al., 2009). Some flavonoids exhibited antioxidant, antiviral, antibacterial, estrogenic, and anti-obesity properties (Wang et al., 2011).

Phenylpropanoid pathway is the backbone for the flavonoid biosynthesis (Fig. 1) (Dixon and Steele, 1999). Many enzymes are involved in the metabolic pathways of the flavanoid biosynthesis; however, chalcone isomerase (CHI) stereo-specifically directs and greatly accelerates the intramolecular cyclization of chalcones to form the flavonoid core molecule called flavanone.

As highly regulated secondary metabolites in plants, flavonoids play an important role to maintain the plants in the environment. Transcription of flavonoid biosynthetic genes and flavonoid content increase after the application of exogenous methyl jasmonate (MeJA), as the induction of secondary metabolite accumulation is an important stress response and jasmonates function as necessary signaling molecules (Repka, 2001; Ruiz-García et al., 2012). The major flavones and expression levels of *PAL*, *C4H*, *4CL*, and *CHS* are up-regulated in *Scutellaria baicalensis* cell suspension cultures treated with MeJA (Xu et al., 2010). Moreover, light is an important environmental factor that affects flavonoid biosynthesis. Previous studies have shown that exposure of grape bunch to light significantly enhance the production of flavonoids, whereas these are reduced by shading (Matus et al., 2009; Cortell and Kennedy, 2006). However, the level of isoflavone increases more in dark-grown plants than in light-grown plants among the three selected soybean genotypes (Kirakosyan et al., 2006). On the other hand, hairy root cultures have been considered as one of the best techniques for the enhanced production of the novel bioactive secondary metabolites (Georgiev et al., 2007; Srivastava and Srivastava, 2007; Zhang et al., 2009; Fattahi et al., 2013; Bourgaud et al., 1999).

American skullcap (*Scutellaria lateriflora* L.) is indigenous to North America and is widely used for insomnia (Joshee et al., 2002; Malikov and Yuldashev, 2002). Commercially, *S. lateriflora* is available in the form of herbal teas, tablets, capsules, and oral liquid preparations (Wills and Stuart, 2004). A large number of compounds, including essential oils (Yaghmai, 1988), diterpenoids (Bruno et al., 1998), amino acids (Awad et al., 2003), and flavonoids (Gafner et al., 2003), have been isolated from *S. lateriflora*. The activity of skullcap may be attributed to several compounds acting on different targets. Results from an animal study suggest that flavonoids are responsible for the anxiolytic activity in the raw plant material extract of *S. lateriflora* (Awad et al., 2003). *S. lateriflora* is rich in various types of flavonoids. Baicalein, baicalin, and wogonin are the major flavonoids in *S. lateriflora* (Brock et al., 2010). Baicalin is one of the most efficient antioxidant and most dominant flavones in the *Scutellaria* species (Boyle et al., 2011). Baicalein protects mitochondria against oxidative damage via the induction of manganese superoxide dismutase (Lee et al., 2011). Wogonin is thought to have very high anticancer activity among the flavonoids (Parajuli et al., 2009).

In this study, 5 partial cDNAs encoding flavonoid biosynthetic genes: phenylalanine ammonia lyase (SlPAL), cinnamate 4-hydroxylase (SlC4H), 4-coumaroyl CoA ligase (Sl4CL), chalcone synthase (SlCHS), and chalcone isomerase (SlCHI) were isolated from *S. lateriflora*. In addition, the expression levels of flavonoid biosynthetic genes and the baicalin, baicalein, and wogonin content were analyzed in the roots, stems, petioles, and leaves of *S. lateriflora*. The transcriptional regulation of flavonoid biosynthetic genes and changes in baicalein, baicalin, and wogonin existences were studied in *S. lateriflora* under MeJA treatment and light/dark conditions.

2. Materials and methods

2.1. Plant materials

Scutellaria lateriflora L. seeds were purchased from the Asian Seed Company (Seoul, Korea), and plants were grown under greenhouse conditions at Chungnam National University (Daejeon, Korea). The plants were cultivated for 180 days to collect the grown parts of roots, stems, petioles, and leaves. The collected parts were thoroughly cleaned and frozen dried immediately for the quantification of the secondary metabolites and for the molecular level studies.

2.2. Establishment of hairy root transformation of *S. lateriflora*

Agrobacterium rhizogenes strain R1000 was used for the development of the hairy root culture. Briefly, hormone-free half MS medium containing cefotaxime (500 mg/L) was used for the transformation experiments. After the formation of the hairy roots, Edwards et al. (1991) method was followed for the extraction of the genomic DNA. Further, 100 mg of fresh hairy roots were mass cultivated under shake flask condition using the growth chamber with a flux rate of 35 $\mu mol\ s^{-1}\ m^{-2}$ and a 16-h photoperiod.

2.3. MeJA treatment and light/dark condition of hairy roots

After 3 weeks, hairy root samples cultured in 30 mL of 1/2 MS medium were treated with MeJA (100 μM, Sigma). The hairy root samples were harvested at 6, 12, 24, 48, 72, and 96 h after MeJA treatment. For dark/light treatment, 100 mg of fresh hairy roots were transferred to new liquid medium under 2

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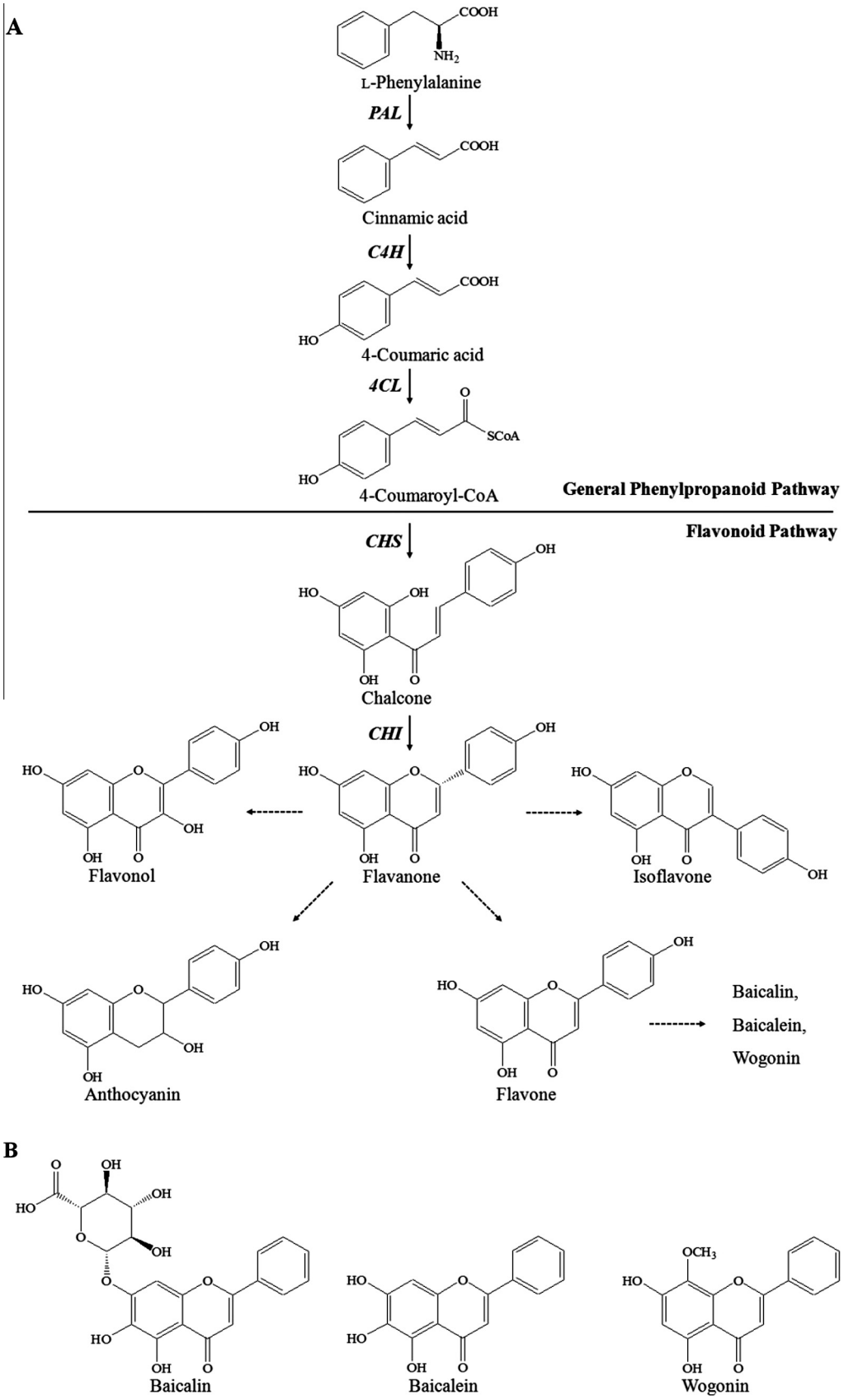

Figure 1 The flavonoid biosynthetic pathway in plants (A) and structure of baicalin, baicalein, and wowonin (B). Enzyme abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumaryl CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase.

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different conditions: light-grown (under cool white fluorescent tubes with a 16-h photoperiod) and dark-grown (under 24-h dark). The hairy root samples were harvested at 1, 2, 3, and 4 week(s) after light/dark treatment. The entire experiment was repeated 3 times, and the mixture of 3 independent replicate hairy roots was used for the further analysis.

2.4. RNA isolation and cDNA synthesis

Plant Total RNA Mini Kit (Geneaid, Taiwan) and ReverTra Ace-R kit (Toyobo, Osaka, Japan), were used for the isolation of total RNA and cDNA preparation. The methodology was followed according to the manual of the providers.

2.5. Cloning of cDNAs encoding flavonoid biosynthetic genes and analysis

To clone PAL, C4H, 4CL, CHS, and CHI, degenerate primers were designed based on the conserved regions of these genes from other higher plants. The sequence results confirmed that the amplified produces were belonged to *S. lateriflora*. BLAST and BioEdit Sequence Alignment Editor, version 5.0.9 were used for the similarity checking and alignments.

2.6. Quantitative real-time PCR analysis

Based on the sequences of SlPAL, SlC4H, Sl4CL, SlCHS, and SlCHI (GenBank accession numbers: KF039679, KF039680, KF039681, KF039682, and KF039683), real-time (RT)-PCR primers were designed using the Primer3 website (http://frodo.wi.mit.edu/primer3/) (Table 1).

2.7. Baicalin, baicalein, and wogonin extraction and analysis

Ten milligrams of the powdered sample was extracted with 70% (v/v) ethanol for 60 min at room temperature. The supernatant was separated by centrifugation and filter sterilized prior to the analysis in the HPLC (C18 column; 250 × 4.6 mm, 5 μm; RStech; Daejeon, Korea). The mobile phase was a gradient prepared from mixtures of methanol and 0.5% acetic acid (conditions: methanol 60% for 5 min, methanol 60–70% for 10 min, methanol 70–78% for 25 min). The flow rate was maintained at 0.6 mL/min. Injection volume of 20 μL and a wavelength of 275 nm were used for the detection. The standard compounds were determined using a standard curve. All samples were analyzed in triplicate.

2.8. Statistical analysis

Gene expression data and content for baicalin, baicalein, and wogonin were analyzed using SAS program. Duncan's multiple range tests was used for further confirmation.

3. Results and discussion

3.1. Cloning and sequence analyses of flavonoid biosynthetic genes from *S. lateriflora*

To clone PAL from *S. lateriflora* and the BLAST search tools confirmed 167 deduced amino acid sequences of SlPAL with its orthologs shows that SlPAL shares high similarity and identity with other PALs. Similarly, degenerate primers of C4H, 4CL, CHS, and CHI used to isolate SlC4H, Sl4CL, SlCHS, and SlCHI consisted of 208 amino acids, 113 amino acids, 263 amino acids, and 169 amino acids, respectively. Sequence analyses of SlC4H, Sl4CL, SlCHS, and SlCHI revealed high similarity and identity with other orthologous genes.

3.2. Expression levels of flavonoid biosynthetic genes and baicalin, baicalein, and wogonin content in different organs of *S. lateriflora*

Expression patterns of SlPAL, SlC4H, Sl4CL, SlCHS, and SlCHI were investigated in the roots, stems, petioles, and leaves of *S. lateriflora*. Organ expression analysis showed that the flavonoid biosynthetic genes were expressed in all organs analyzed of *S. lateriflora* (Fig. 2A). The most abundant transcript level of the first enzyme specific for the flavonoid pathway, SlCHS, was also detected in the roots. The stems exhibited a moderate level of SlCHS expression, whereas the mRNA level of SlCHS was poor in the petioles and leaves.

The HPLC analysis indicated that the accumulation of baicalin was most abundant in the leaves, with a concentration of 33.58 mg/g of dry weight (Fig. 2B). The roots also contained a

Table 1 Primers used for quantitative real-time (RT) PCR.

Primer	Sequence (5′–3′)	Amplicon (base pairs)
SlPAL_RT F	TAGCTAATCCAGTGACCAACCATGT	113
SlPAL_RT R	CTTCAAAATCTCAACCGCTTCACTA	
SlC4H_RT F	TCATGTTCGATAGAAGGTTCGAGAG	181
SlC4H_RT R	CTCTTCGTTTTAACATCCTTGCAGA	
Sl4CL_RT F	GGTTACGGAATGACAGAATCAACTG	152
Sl4CL_RT R	ATGGATCCTGGAGGTAAGTGAGAAC	
SlCHS_RT F	CTCGTCTCAGCTCACCAGACAAT	138
SlCHS_RT R	CTTCAGGCTCTTCTCGATGTTCTTC	
SlCHI_RT F	ATTCACCAAGGTGACGATGATTCTA	113
SlCHI_RT R	ATTCAGCATCCGTGTATTTCCCTAT	
SlActin_RT F	CACAGAGGCACCTCTCAACCCTAAG	102
SlActin_RT R	ACAGCCTGGATGGCAACATCATGG	

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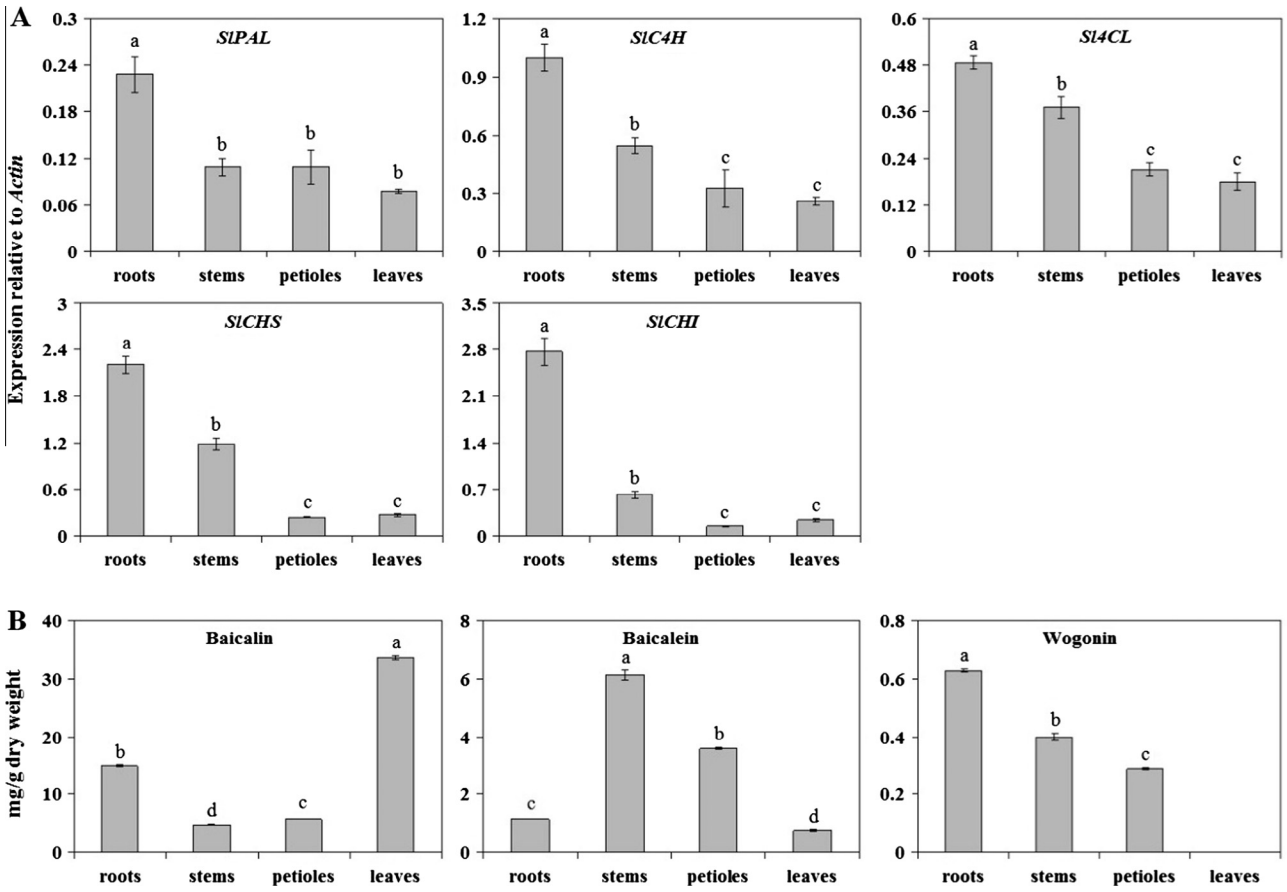

Figure 2 Expression levels of flavonoid biosynthetic genes (A) and baicalin, baicalein, and wogonin content (B) in the roots, stems, petioles, and leaves of *S. lateriflora*. The height of each bar and the error bars show the mean and standard error, respectively from 3 independent measurements. The letters indicate significant differences at the 5% level by Duncan's multiple range test.

relatively high content of baicalin (14.91 mg/g), whereas lower contents of baicalin were observed in the petioles and stems 5.71 mg/g and 4.7 mg/g, respectively. Unlike baicalin, baicalein levels were highest in the stems and petioles 6.14 mg/g and 3.56 mg/g, respectively). The content of baicalein was slightly less in roots (1.13 mg/g) and leaves (0.76 mg/g). Compared to baicalin and baicalein, wogonin was poorly synthesized in *S. lateriflora* and not detected in the leaves. The content of wogonin in the roots was 0.63 mg/g, which was higher than that of stems and petioles 0.4 mg/g and 0.29 mg/g, respectively.

3.3. Transcriptional regulation of flavonoid biosynthetic genes and changes in baicalin, baicalein, and wogonin content were observed in the hairy roots of S. lateriflora under MeJA-induced stress

Fig. 3A displayed the effect of 100 μM MeJA on the expression pattern of *S. lateriflora*. The expression levels of SlPAL, SlC4H, and Sl4CL varied in response to MeJA over 96 h. Specifically, transcript levels of SlPAL were essentially similar until 12 h; thereafter, they decreased and remained constant from 24 h to 96 h after MeJA treatment. Expression levels of SlC4H and Sl4CL did not show large changes under MeJA treatment and reached the highest levels after 24 h and 72 h, respectively. The expression of SlCHS and SlCHI increased after 6 h and reached maximum expression levels at 24 h, followed by a decline until 96 h.

Although baicalin, baicalein, and wogonin were substantially stimulated by MeJA, they exhibited different accumulation patterns throughout the 96-h treatment (Fig. 3). Baicalin content increased throughout the MeJA treatment, with the main induction noted at 72 h and 96 h. The level of baicalein transiently increased 24 h after treatment at which point, it decreased to previously observed lower levels. Wogonin accumulation increased dramatically after 6 h, declined until 48 h, then increased again until 96 h. The maximum production levels of baicalin, baicalein, and wogonin under MeJA treatment were 22.54 mg/g 72 h, 1.24 mg/g 24 h, and 5.39 mg/g 96 h, respectively, i.e., approximately 7.44-, 2.38-, and 2.12-fold higher than that of control hairy roots 3.03 mg/g, 0.52 mg/g, and 2.54 mg/g, respectively.

3.4. Expression levels of flavonoid biosynthetic genes and baicalin, baicalein, and wogonin content in the hairy roots of S. lateriflora under light/dark conditions

As shown in Fig. 4A, expression levels of flavonoid biosynthetic genes in the hairy roots of *S. lateriflora* were varied under light/dark condition during the 4 weeks of development. Expression levels of SlPAL decreased from week 1 to 2 then were maintained until week 4 under both light and dark

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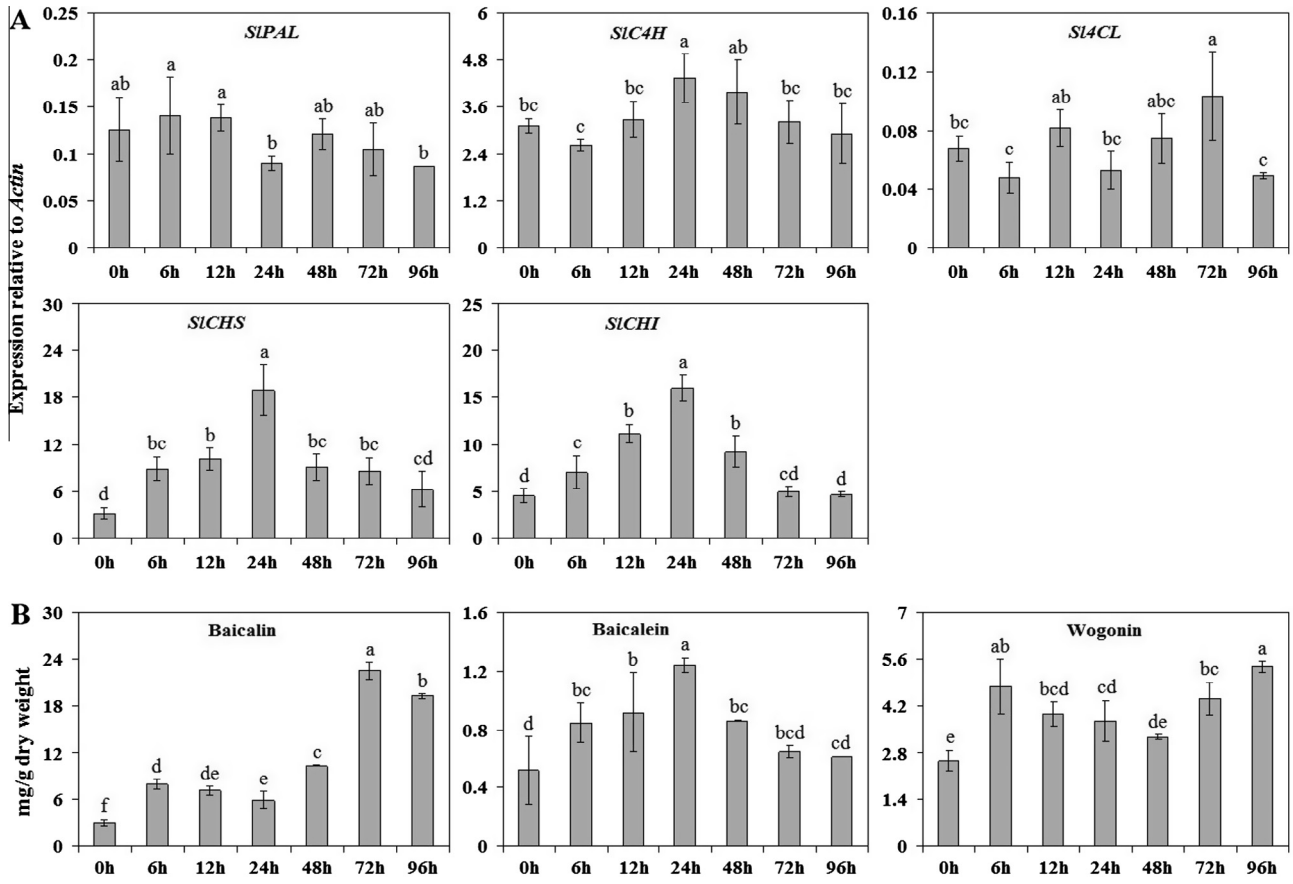

Figure 3 Expression levels of flavonoid biosynthetic genes (A) and baicalin, baicalein, and wogonin content (B) in the hairy roots of *S. lateriflora* treated with 100 μM MeJA. Units on the horizontal axis indicate the number of hour(s) after MeJA treatment. The height of each bar and the error bars show the mean and standard error, respectively, from 3 independent measurements. The letters indicate significant differences at the 5% level by Duncan's multiple range test.

conditions. SlC4H, Sl4CL, SlCHS, and SlCHI revealed different expression patterns of SlPAL in the *S. lateriflora* hairy roots during the 4 weeks of development. In general, transcript levels of SlC4H, Sl4CL, SlCHS, and SlCHI were similar in first 3 weeks, followed by an increase after 4 weeks. Notably, levels of SlCHS expression in light-grown hairy roots were higher than those in dark-grown hairy roots after 3 and 4 weeks.

Light differentially influenced the accumulation of baicalin, baicalein, and wogonin during the 4 week development of *S. lateriflora* hairy roots (Fig. 4B). Baicalin content in dark-grown hairy roots was higher than that in light-grown roots from 2 to 4 weeks. One- and 3-week hairy roots under dark condition contained higher levels of baicalein than hairy roots grown under light condition. A significant difference in wogonin accumulation between light and dark condition was found only after 4 weeks with a higher content of wogonin in light-grown hairy roots. The highest production of baicalin and baicalein was observed after 4 weeks 8.10 mg/g and 3 weeks 0.78 mg/g, respectively, in the hairy roots under dark condition, while the largest accumulation of wogonin was observed after 4 weeks in the hairy roots under light condition 3.18 mg/g.

Despite extensive use and previous investigations of flavonoids, there are no reports on flavonoid biosynthetic genes in *S. lateriflora*. This study reports the first isolation and characterization of flavonoid biosynthetic genes from *S. lateriflora*. The partial cDNAs encoding flavonoid biosynthetic genes, including PAL, C4H, 4CL, CHS, and CHI, from *S. lateriflora* share high similarity. The expression patterns of flavonoid biosynthetic genes are essentially similar in different organs of *S. lateriflora*, with the highest transcript level found in the roots and the lowest level found in the petioles and leaves. In Arabidopsis, maize, and petunia, MYB-, MYC-, and WDR-type transcription factors form a complex that binds to structural gene promoters, thereby controlling the expression of flavonoid biosynthetic genes (Matus et al., 2010).

It has been reported that *S. lateriflora* is rich in flavonoids of various types (Gafner et al., 2003; Brock et al., 2010). As expected, a significant amount of baicalin, baicalein, and wogonin was detected in different organs of *S. lateriflora* in this study. Baicalin and baicalein differentially accumulated in *S. lateriflora* with the highest content of baicalin detected in the leaves and roots, while the highest content of baicalein was detected in the stems and petioles. Baicalein is a precursor metabolite of baicalin synthesis (Ohtsuki et al., 2009), and it has been suggested that the high synthesis of baicalein results in the low content of baicalin in *S. lateriflora* stems and petioles. In contrast, the abundant accumulation of baicalin in the leaves and roots is responsible for the small amount of baicalein found in these organs. Wogonin was not detected in the

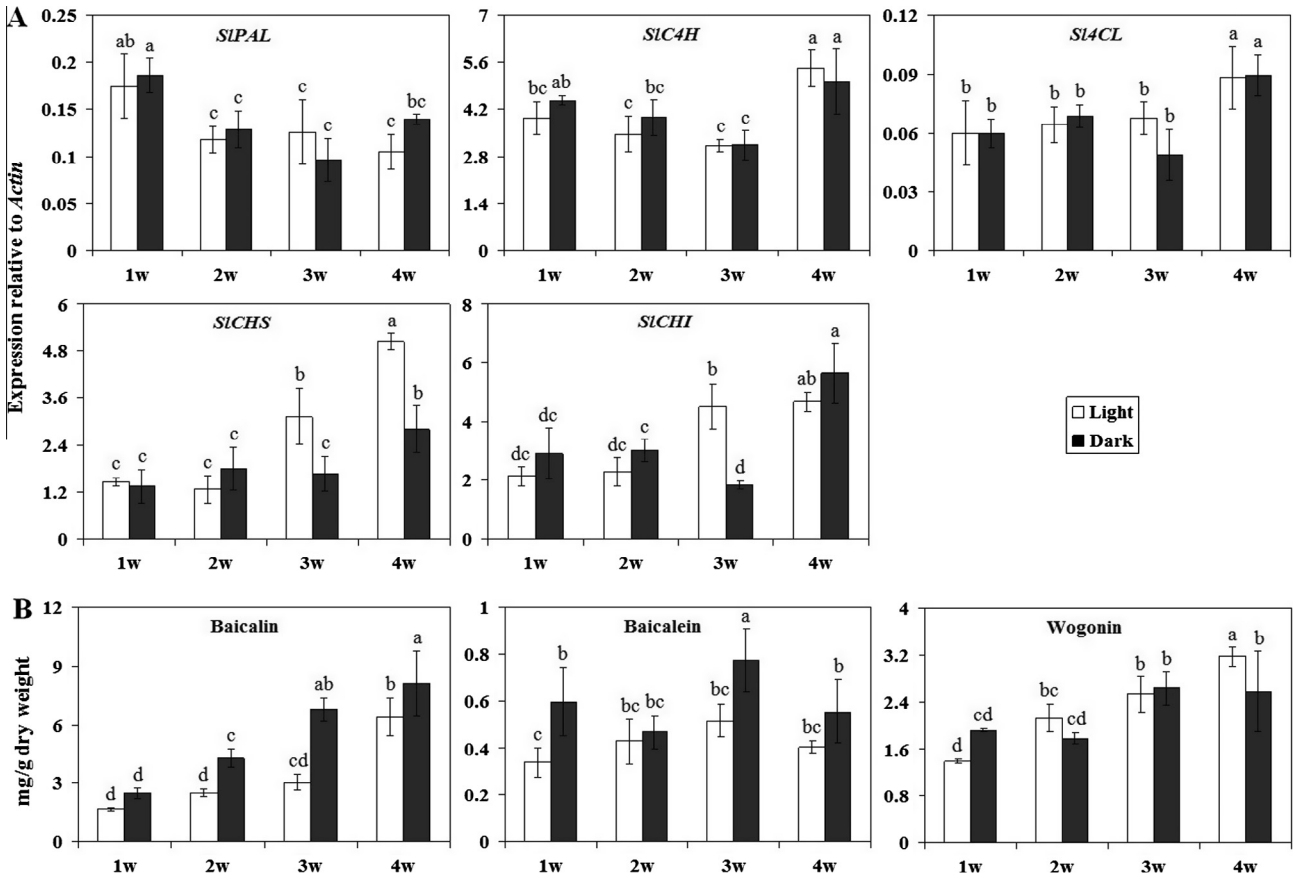

Figure 4 Expression levels of flavonoid biosynthetic genes (A) and baicalin, baicalein, and wogonin content (B) in the hairy roots of *S. lateriflora* under light/dark conditions. Units on the horizontal axis indicate the number of week(s) after light/dark treatment. The height of each bar and the error bars show the mean and standard error, respectively from 3 independent measurements. The letters indicate significant differences at the 5% level by Duncan's multiple range test.

leaves; however, the highest accumulation was detected in the roots, which is the most important part of *S. lateriflora*. Fig. 2 shows that expression patterns of SlPAL, SlC4H, Sl4CL, SlCHS, and SlCHI generally correlate with the accumulation patterns of wogonin, with the highest levels found in the roots, but they do not correlate with the accumulation patterns of baicalin and baicalein. It suggested that these genes have active roles in the flux of the wogonin biosynthetic branch rather than the baicalin and baicalein biosynthetic branche in *S. lateriflora*. Furthermore, flavonoid transportation from one tissue to another may explain for the low accumulation of baicalin and baicalein in the roots where significant expression levels of flavonoid biosynthetic genes were detected (Braidot et al., 2008).

The effect of MeJA has been well reported on secondary metabolites (Palazon et al., 2003; Komaraiah et al., 2003; Hayashi et al., 2003). Exogenous MeJA also significantly enhances the expression of SlCHS and SlCHI, and accumulation of baicalin, baicalein, and wogonin in *S. lateriflora* hairy roots. Moreover, the expression patterns of SlCHS and SlCHI are tightly correlated with the accumulation patterns of baicalein in the hairy roots of *S. lateriflora* throughout the 96-h MeJA treatment, with the highest levels observed at 24 h. This is not in accordance with the independent expression of SlCHS and SlCHI and baicalein accumulation in *S. lateriflora* plants, in which SlCHS and SlCHI expression patterns correlate with the accumulation patterns of wogonin. These data indicate that the biosynthetic mechanism of flavonoids in *S. lateriflora* is dependent on the plant tissues examined. Further studies are required to completely understand the biosynthetic mechanism in *S. lateriflora*. In addition, we discovered that in MeJA-induced hairy roots, the biosynthesized content of baicalin 22.54 mg/g, baicalein 1.24 mg/g, and wogonin 5.39 mg/g was significantly higher at 14.91 mg/g, 1.13 mg/g, and 0.63 mg/g, respectively, than in the natural roots of *S. lateriflora*. Hairy roots may, therefore, be an excellent alternative material for the production of baicalin, baicalein, and wogonin.

Light plays an important role in almost all plant developmental processes and regulates the biosynthesis of plant secondary metabolites (Hemm et al., 2004; Liu et al., 2002). Light has been proved to influence flavonoid biosynthesis by regulating the transcription of CHS (Jenkins et al., 2001; Sakuta, 2000). In *S. lateriflora*, light condition increases the expression level of SlCHS in hairy roots after 3 and 4 weeks of development compared to dark condition. However, dark-grown hairy roots contain higher content of baicalin and baicalein than light-grown hairy roots. It has been suggested that expression of SlCHS may be more involved in the production

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of other flavonoids than baicalin and baicalein in light-grown hairy roots of *S. lateriflora*.

4. Conclusions

The characterization of flavonoid biosynthetic genes along with the content of baicalin, baicalein, and wogonin may provide the background information to clarify the detail molecular analysis of flavonoid biosynthesis in *S. lateriflora*. In addition, our current study demonstrates that exogenously supplied MeJA has a positive effect on both enzyme gene expression and flavonoid accumulation in *S. lateriflora* hairy roots. Our results may be applied to the establishment of new approaches for maximum production of flavonoids as well as biomass productivity and the metabolic engineering of flavonoid biosynthesis in *S. lateriflora* as has been successfully described in other skullcap plants (Park et al., 2011).

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