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Full Length Article

Regulation Mechanism of Chlorogenic Acid Accumulation during the Floral Organ Development of *Lonicera confusa*

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Abstract

Regulation mechanism of chlorogenic acid synthesis during the floral organ development of *Lonicera confusa* was investigated. The results showed that among the phenylpropanoid metabolites, the variation trends of chlorogenic acid and flavonoids were similar, and caffeic acid content remained stable in each period. While the lignin contents were nagtively correlated with level of chlorogenic acid. The expression of several gene involved in the phenylpropanoid pathway increased first and then decreased during the floral organ development, which were not consistent with the phenylpropanoid metabolites in the first three flowering stages, inferred that the biosynthesis of phenylpropanoid metabolites were regulated in the post-transcriptional level. The phenylalanine ammonia lyase **(**PAL) and hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase (HQT) maybe the rate limiting enzyme in the biosynthesis of chlorogenic acid. The above results provide theoretical basis for the screening, innovation, standardization, and harvest of honeysuckle germplasm. © 2016 Friends Science Publishers

Keywords: Biosynthesis; Chlorogenic acid; *Lonicera confusa*; Molecular mechanism; Phenylpropanoid metabolites

Introduction

Lonicera japonica Thunb. is widely cultivated as a commercially valuable plant in China and other Asian countries. The dried bud of *L. japonica* (*Flos lonicerae japonicae* FLJ) has been used in Chinese medicine for thousands of years. Massive active ingredients have been isolated from *L. japonica,* among them chlorogenic acid and luteoloside have attracted the most attention from researchers (Yuan *et al*., 2012). Chlorogenic acid and luteoloside have been used as biomarkers for evaluating the quality of FLJ.

The biosynthesis of active components in FLJ are closely correlated with floral developmental stages, for example chlorogenic acid concentration in honeysuckle is highest in first bud stage and gradually decreases thereafter, especially in the blooming flowers (Wang *et al*., 2009; Yuan *et al*., 2012). However, the bud stage is relatively short and individual plants are growing at different rates; therefore, the large-scale harvest of honeysuckle was difficult to carry out.

Chlorogenic acid is a phenylpropanoid formed from the shikimic acid during aerobic respiration in plants. Some studies revealed that aside from chlorogenic acid, honeysuckle also contains other phenylpropanoids, such as flavones, isochlorogenic acid A, caffeic acid, lignin, and so on. There are only two genes of hydroxy cinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT) and allene oxide synthase LjAOS were reported to correlated with the biosynthesis of chlorogenic acid during the floral organ development of *L. confusa* (Jiang *et al*., 2009; Bai *et al*., 2010). The relationship of chlorogenic acid synthesis to the synthesis of other phenylpropanoids in honeysuckle, and the regulation of chlorogenic acid accumulation during the floral organ development of *L. confusa* based on the molecular biological techniques have not been clearly studied.

In the current study, the correlation between the changes in phenylpropanoid production and the regulation of gene expression for the synthesis of secondary phenylpropanoid metabolites during honeysuckle floral organ development were analyzed. Consequently, the regulating mechanisms underlying chlorogenic acid synthesis and transformation were revealed, which laying the foundation for the screening and creating of honeysuckle germplasm and also providing a basis for the standardized cultivation and harvesting of honeysuckle plants.

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Materials and Methods

Plant Materials

Samples were collected from the five years old plant of *L. confusa* which planted in the Nursery of Huazhong University of Science and Technology, Wuhan, China. The collected flowers were divided into six groups of juvenile bud stage (BS), third green stage (TGS), white bud stage (WBS), silver flowering stage (SFS) and golden flowering stage (GFS) (Fig. 1).

Metabolic Profiling of Phenylpropanoid Products

Chlorogenic acid and flavonoids extraction were according to the method of Li *et al*. (2005) with minor modifications: Fresh flowers (0.5 g) of *L. japonica* were cut into 5 mm pieces and mixed with 20 mL of 700 mL L^{-1} ethanol (pH 4.0–4.5), and sonicated two times, each for 30 min. Supernatant was collected twice after centrifugation at 7500 × *g* for 15 min. The above two extracts were mixed together and vacuum dried at 45ºC. At last, 20 mL high-performance liquid chromatography (HPLC)-grade methanol were added to dissolve the extracts and filtered through a 0.45 μm membrane.

Chlorogenic acid, Isochlorogenic acid A, caffeic acid were analyzed by HPLC according to the methods of Wang *et al*. (2009). A standard curve was constructed using 10–50 μg mL^{-1} of chlorogenic acid, Isochlorogenic acid A and caffeic acid. The regression equation of chlorogenic acid (Isochlorogenic acid A) and caffeic acid were as follows:

$$y_{\text{chlorogenic acid}} = 39.054x\text{-}84.922 \ (R^2 = 0.9976)$$

$$y_{\text{caffeic acid}} = 0.0311x\text{+}1.7354 \ (R^2 = 0.9917)$$

Total flavonoid contents (TFC) was determined according to the methods of Laghari *et al*. (2011) with minor modification: Diluted supernatant (1 mL) was mixed with 0.3 mL of sodium nitrite (5%, w/v) and 1 mL of 70% ethanol in a 10 mL volumetric flask and standing for 6 min. And then, 0.3 mL of aluminum chloride (10% w/v) was added, standing for 6 min again. After that, 2 mL of sodium hydroxide (4% w/v) and 0.4 mL distilled water were added and mixed by shaking. After 15 min, the absorbance was measured at 510 nm on a UV-1600(PC) UV/Vis Spectrophotometer (APS-115). The measurements are triplicated for each tested sample. The measurement of standard curve was established with rutin standard (0–50 μg mL^{-1}). The regression equation was $Y_{TFC} = 0.029 \text{ x} + 0.004$ ($R^2 = 0.999$).

Lignin Content Analysis

Lignin contents were analyzed according to the method of Su *et al*. (2005). A standard curve was generated with alkali lignin (Sigma-Aldrich). The measurements are triplicated for each tested sample at 280 nm.

Phenylalanine Ammonia lyase (PAL) Activity Analysis

The extraction of the PAL was according to Lister *et al*. (1996) with minor modification. PAL activity in the partially purified enzyme extracts was assayed at 290 nm as reported by McCallum and Walker (1990).

Real-time PCR Validation

Total RNAs of flower organs were isolated with RNAprep Pure Plant Kit (Polysaccharides and Polyphenolics-rich, Cat: DP441) according to manufacturer's protocol. One μg of total RNAs were transcripted into single-stranded cDNA fragment using reverse transcriptase Superscript II (Invitrogen). Quantitative Real-Time PCR (qRT-PCR) was running on ABI PRISM 7700 DNA Sequence Detection System (Applied Biosystems USA) using SYBR premix Ex Taq Kit (Takara, Japan). Gene-specific primers were showed in Table 1. The PCR was performed under the following conditions: denaturing at 94ºC for 2 min, then followed by 40 cycles of 15 s at 94ºC, 15 s at 60ºC and 20 s at 72ºC. The relative gene expression was measured by comparative threshold cycle method (Livak and Schmittgen, 2001).

Statistical Analysis

Each experiment were repeated three times, and data are the mean values of three replicates, and are expressed as mean ± standard deviation (STDEV). The percentage data for various parameters were arc-sine transformed prior to statistical analysis. Statistical analysis was performed using one-way ANOVA using an SAS software package.

Results

Metabolic Profiling of Main Phenylalanine Products

The main phenylalanine products of *L. confusa* during the development of floral organ were analyzed. The results showed that the highest concentrations of chlorogenic acid, caffeic acid, and total flavonoids occur in the BS stage, and gradually decreased in the TGS, WBS and SFS stages, and subsequently increased slightly during the GFS stage. The total flavonoid concentration was highest among all the main phenylalanine products, which is significantly higher than chlorogenic acid and caffeic acid. Whereas, the caffeic acid concentration was lowest (Fig. 2A-B). The chlorogenic acid concentration was higher than Isochlorogenic acid A. The chlorogenic acid concentration was relatively higher during the BS stage and the TGS stage, but dropped sharply and remained at relatively low levels thereafter. In general, the concentration fluctuated within a significantly wide range (Fig. 2A-B). The lignin concentration gradually increased from the BS stage, and peaked during the GFS stage (Fig. 3).

Table 1: Primers used in the Real-time PCR validation

Gene	Forward primer（5' to 3'）	Reverse primer（5' to 3'）
18S	ATGATAACTCGACGGATCGC	CTTGGATGTGGTAGCCGTTT
PAL1	GCTTGGACTATGGCTTCA	TAGGCTCACGGTGTTCTT
PAL2	GATACTTGAAGCCCTCACTAA	CAAATGGTCCGTAAACTCTG
PAL3	ATTCCTCAACCACAACATCA	GATAAGAGTGCTAGAGTATTCG
CHI1	TATGAAGAGGAGGAAGAAGAG	TGTTGGCTAAGTTGGTGAT
CHI2	AGTTCTTCGCCGACATTG	GCTGCTTGTTCTCTATCACT
4CL1	GGCTAATCCGTTCTTCACT	ACATCATCACATCCTCACAA
CHS1	GACATAGTGGTGGTAGAAGTA	CAGTGATCTCAGAGCAGAC
HQT	GGAGTAGGAGGATGATGGA	AAGTGGAGGGAGGAAACA

Note: PAL1 (Phenylalanine ammonia lyase1), PAL2 (Phenylalanine ammonia lyase2), PAL3 (Phenylalanine ammonia lyase3), CHI1 (Chalcone isomerase1), CHI2 (Chalcone isomerase2), 4CL1 (4-Coumarate-coA ligase1), CHS1 (Chalcone synthase1), HQT (Hydroxycinnamoyl-coA: quinate hydroxycinnamoyltransferase)

These results indicated that the main phenylalanine products metabolic flux might have been changed during the floral organ development of honeysuckle. Chlorogenic acid synthesis was correlated with lignin synthesis. However, the mechanisms underlying the metabolic flux changing need further studies at the molecular level.

Enzyme Activity Analysis of Phenylalanine Ammonia Lyase (PAL)

PAL is the first enzyme in phenylpropane metabolism. The results showed that PAL activity is highest during the BS stage, followed by the TGS stage. The enzyme was relatively inactive during the other flowering phases (Fig. 4), which indicates that the drop in the amount of the main phenylalanine products is correlated with reduced PAL activity during floral organ development of honeysuckle.

Analysis of Key Enzyme Expression in the Phenylpropanoid Pathway

Phenylalanine ammonia lyase (PAL), 4-Coumarate-coA ligase (4CL), Chalcone isomerase (CHI), Hydroxycinnamoyl-CoA: quinate hydroxycinnamoyltransferase (HQT) are the key enzyme in phenylpropanoid metabolism pathway, they are located in the branch or the downstream of phenylpropanoid metabolism pathway, and in charge of the synthesis of precursors. PAL catalyzes the production of L-phenylalanine ammonia, was the rate limiting enzyme in the metabolic pathway of benzene. CHS catlyze the reaction of malonyl-coA and coumaroyl-coA to chalcone, and is the first enzyme that leads to the synthesis of flavonoids. CHI used chalcones as substrates to synthesis flavononeS, the total flavonols in the peer of *chi* overexpression transferred tomatoes can increase 78 times (Verhoeyen *et al*., 2002). 4CL control lignin biosynthesis and is the critical rate-limiting enzyme in the reaction of catalyzing the hydroxy cinnamon to G or S lignin monomer. HQT could catalyze coffee and quinine acid coenzyme A to generate chlorogenic acid (Ulbrich and Zenk, 1979). So the expression of PAL, CHS, CHI, 4CL and HQT was detected through real-time quantitative PCR to analyze the synthesis of the main phenylalanine products during honeysuckle development.

Fig. 1: Different flowering stage of *Lonicera confusa*. BS: green bud stage; TGS: third green stage; WBS: white bud stage; SFS: silver flowering stage; GFS: golden flowering stage

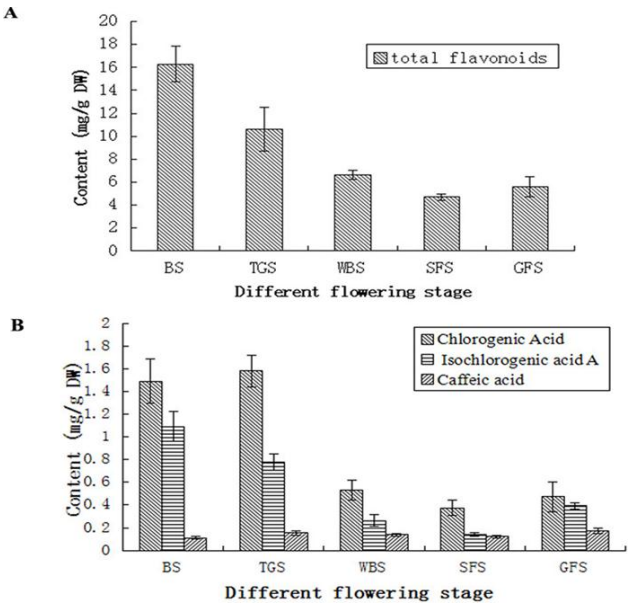

Fig. 2: The biosynthesis of total flavonoids (A) and choloragenic Acid, isochlorogenic acid A and caffeic acid (B) in different flowering stage of *Lonicera confusa*. BS: green bud stage; TGS: third green stage; WBS: white bud stage; SFS: silver flowering stage; GFS: golden flowering stage. Vertical bars referred to standard deviation (SD)

The results showed that the expression of these genes initially increased and then decreased, peaking in the WBS stage (Fig. 5A-B). But the gene expression during the WBS stage, the SFS stage and the GFS stage were consistent with product synthesis. These results are not consistent with previous metabolic analysis (Fig. 2 and 3), inferred that the biosynthesis of phenylpropanoid metabolites were regulated in the post-transcriptional level in the WBS stage. The PAL expression level was highest among all of the enzymes, followed by HQT, CHI, 4CL, and CHS, and the expression levels of PAL2, PAL3, HQT, CHI were 25~175 times higher than the expression of 4CL, CHS, PAL1 in the WBS stage. The differences in the expression level of the homologous gene such as PAL and CHI were also identified. Among the three homologous PAL genes, the expression of PAL3 was 8.4 and 55.9 times higher than those of PAL2 and PAL1 in the WBS stage (Fig. 5A-B). In addition, CHI2 expression was 82.1 times higher than that of CHI1 in the WBS stage (Fig. 5A), these results showed that the founction of homologous genes encoding the same protein are not the same.

Discussion

Chlorogenic acid is an important physiologic activator that is primarily extracted from the floral organs of honeysuckles. Research has indicated that chlorogenic acid concentration is closely correlated with the phases of floral organ development, and chlorogenic acid concentration is relatively higher during the early stages of cell differentiation and budding stage (*i.e*., the BS stage and the TGS stage). The chlorogenic acid concentration in blooming flowers is obviously reduced (Wang *et al*., 2009; Yuan *et al*., 2012). Studies on organisms that synthesize chlorogenic acid during floral development can provide a basis for the screening, development and standardized harvesting of honeysuckle germplasm.

In the present study, we investigated the major phenylalanine substances, as well as the expression levels of key genes involved in the phenylpropanoid pathway during the floral organ development of *L. confusa*. The results indicate that during floral organ development, the synthesis of major phenylpropanoid compounds is similar (except that of lignin), and the biosynthesis of phenylpropanoid metabolites were regulated in the post-transcriptional level. Cause, firstly, the PAL activity was highest in the BS stage and the TGS stage, which are positively correlated with the content of phenylalanine substances. Secondly, the expression of enzymes in the phenylpropanoid pathway initially increased and then decreased during floral organ development, peaking during the WB stage that was inconsistent with the accumulation of chlorogenic acid, flavones and other components. But the gene expression during the WBS stage, the SFS stage, and the GFS stage were consistent with product synthesis, which agrees with the findings of Yuan *et al.* (2012).

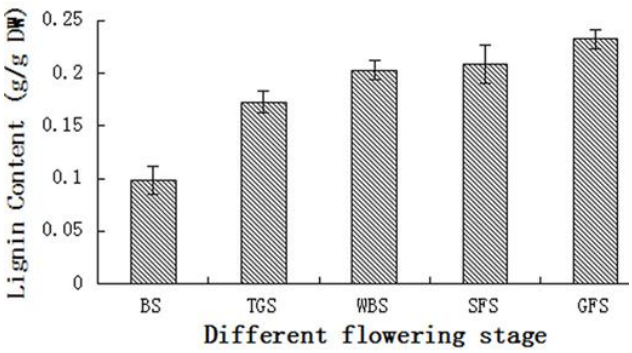

Fig. 3: The lignin content in different flowering stage of *Lonicera confusa*. BS: green bud stage; TGS: third green stage; WBS: white bud stage; SFS: silver flowering stage; GFS: golden flowering stage. Vertical bars referred to standard deviation (SD)

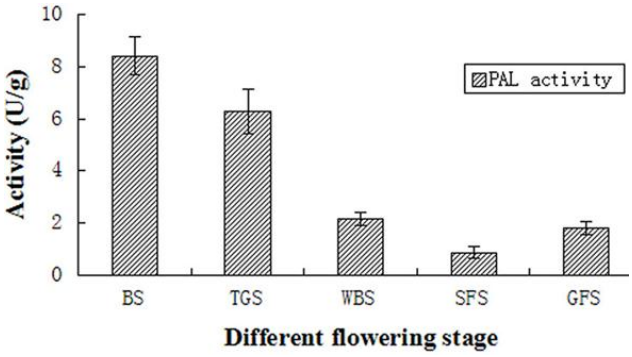

Fig. 4: PAL activity in different flowering stage of *Lonicera confusa.* BS: green bud stage; TGS: third green stage; WBS: white bud stage; SFS: silver flowering stage; GFS: golden flowering stage. Vertical bars referred to standard deviation (SD)

The discrepancy between gene expression and product biosynthesis has been previously reported. Karppinen and Hohtola (2008) detected hyperforin and adhyperforin concentrations in the roots, stems, intralobar parts, leaf epidermis, and flower buds of tartary buckwheat, as well as HpPKS1 and HpPKS2 expression in polyketide synthases. They found that the amount of product were inconsistent with gene expression during some stages. This discrepancy is possibly caused by different gene functions in multigene families, such as most of the genes in the phenylpropanoid pathway. Colliver *et al.* (1997) found that both transcript levels of *CHS* and isoflavonoid and tannin accumulation were improved in transgenic Lotus, which express antisense of French bean *CHS* gene.

The current study also revealed that lignin synthesis is inversely correlated with chlorogenic acid synthesis. Inhibiting the expression of genes related to lignin synthesis maybe increase chlorogenic acid synthesis during the honeysuckle germplasm improvement. This finding has also

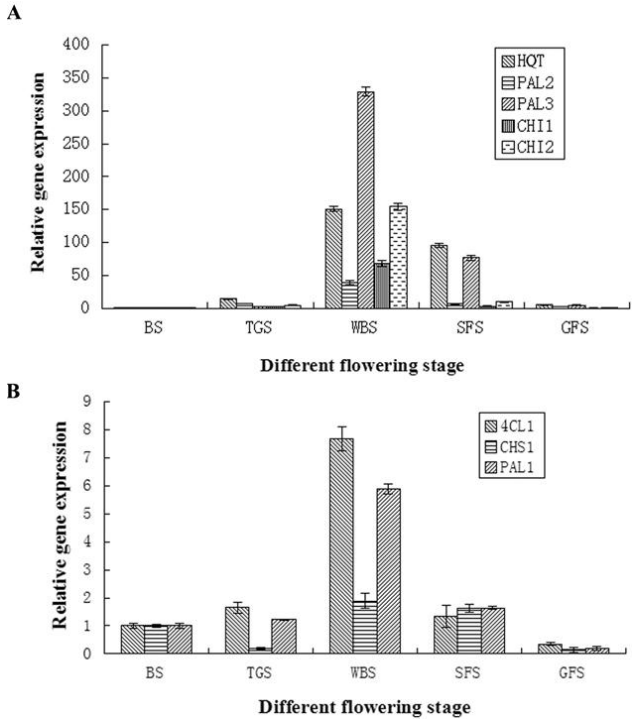

Fig. 5: The expression of phenylalanine metabolic pathway genes in different flowering stage of *Lonicera confusa*. A, HQT (Hydroxycinnamoyl-CoA: quinate hydroxycinnamoyltransferase), PAL2 (Phenylalanine ammonia-lyase2), PAL3 (Phenylalanine ammonia-lyase3), CHI1 (Chalcone isomerase1), CHI2 (Chalcone isomerase2); B, 4CL (4-Coumarate-CoA ligase), CHS1 (Chalcone synthase1), PAL1 (Phenylalanine ammonia-lyase1). BS: Green bud stage; TGS: Third green stage; WBS: White bud stage; SFS: Silver flowering stage; GFS: Golden flowering stage. Vertical bars referred to standard deviation (SD)

been reported in studies on strawberry, arabidopsis and maize (Hoffmann *et al*., 2006; Besseau *et al*., 2007; Fornalé *et al*., 2010; Hoffmann *et al*., 2011; Thévenin *et al*., 2011; Ring *et al*., 2013).

Conclusion

In the current research, we confirmed that at least two elements regulate the chlorogenic acid synthesis during the floral organ development: (1) the expression of PAL and HQT is closely correlated with chlorogenic acid synthesis, and the biosynthesis of phenylpropanoid metabolites were regulated in the post-transcriptional level; (2) lignin synthesis is inversely correlated with chlorogenic acid synthesis. So, inhibiting the expression of genes related to lignin synthesis may increase chlorogenic acid synthesis during the honeysuckle germplasm improvement. Our research provides a solid foundation for improving the honeysuckle germplasm.

Acknowledgments

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