and trace elements.[1] In the Chinese Pharmacopoeia (Committee for the Pharmacopoeia of PR China, 2015), chlorogenic acid and luteoloside are two indicators for evaluating the quality of LJ.

From the transcriptome of LJ, three possible routes of chlorogenic acid biosynthesis are proposed.[3,4] The first and main chlorogenic acid pathway is presented in Figure 1.[3,5,6] Through catalytic reactions of phenylalanine ammonia-lyase (PAL, EC 4.3.1.24), cinnamate 4-hydroxylase (C4H, EC 1.14.13.11), and 4-hydroxycinnamoyl-CoA ligase/4-coumarate-CoA ligase (4CL, EC 6.2.1.12), phenylalanine produces the universal precursor p-Coumaroyl-CoA, and then, through hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT, EC 2.3.1.99), chlorogenic acid is synthesized. Another branching reaction of p-Coumaroyl-CoA leads to the biosynthesis of luteoloside through the catalytic reactions of chalcone synthase (CHS, EC 2.3.1.74), chalcone isomerase (CHI, EC 5.5.1.6), flavone synthase (FNS, EC 1.14.11.22), cytochrome P450, and glycosyltransferase (flavone 7-O-beta-glucosyltransferase, EC 2.4.1.81). The genes *PAL1*, *PAL2*, *PAL3*, *C4H1*, *C4H2*, *4CL1*, *4CL2*, *HQT1*, *HQT2*, *CHS1*, *CHS2*, *CHI1*, *CHI2*, *FNS1*, and *FNS2* have been identified in LJ.[3,6,7]

LJ is native to East Asia but is invasive on almost every continent except Antarctica.[8] This wide habitat range of LJ demonstrates that it can survive and reproduce in various environmental conditions. In China, Pingyi County in Shandong Province (SD) is a famous geo-authentic production area of LJ, and the plant production is locally called "dongyinhua." We have introduced LJ from SD to Leye County, Guangxi Zhuang Autonomous Region (GX). Leye County is a poverty-stricken county in China mostly because serious karst rocky desertification problems have limited its economic development. For great viability and important medical value, LJ was identified as a good contributor to ecosystem restoration in GX. We found that LJ still has normal growth and development after introduction, and the flowers of LJ can be harvested four times a year as in SD. However, whether the quality of LJ in GX is the same as that in SD is poorly understood. Here, we chose the flower buds of LJ to analyze the bioactive compounds and their corresponding gene expression changes after 3-year introduction, and endogenous plant hormones, such as indole-3-acetic acid (IAA), gibberellins (GA_3), isopentenyladenine (iPA), zeatin riboside (ZR), abscisic acid (ABA), and jasmonic acid (JA), were detected to discuss the underlying regulation mechanism.

MATERIALS AND METHODS

Plant material and sample collection

In September 2014, approximately 25 cm long cuttings with three nodes were taken from vigorous branches of 2-year-old *Lonicera japonica* Thunb. cv. Tomentosa, and then, seedlings from the cutting propagation were planted in Shaoshanhou Village, Liuyu Town, Pingyi County, Shandong Province (35°21'N, 117°37'E) and Sihe Village, Gantian Town, Leye County, Guangxi Zhuang Autonomous Region (24°36'N, 106°30'E). On May 8–15, 2017 (the first LJ harvest time, which makes up the bulk of the annual harvest), LJ with relatively uniform growth was chosen to collect flower buds which were bloomed into whole white alabastrum. Each sample was randomly collected from six plants and divided into three groups for different purposes. One group was freeze-dried flower buds used for liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to analyze bioactive compound contents. Another group was quick-frozen fresh flower buds used for RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction (PCR) to detect gene expression levels. The third group was quick-frozen fresh flower buds for indirect enzyme-linked immunosorbent assay (ELISA) to determine endogenous phytohormone contents. Each group had three repetitions, and all samples were authenticated by Prof. Qinghua Wu.

Liquid chromatography coupled with tandem mass spectrometry analyses

Freeze-dried flower buds of LJ were used for the preparation of five analytes: chlorogenic acid, luteoloside, luteolin, isoquercitrin, and caffeic acid. Samples were accurately weighed and sonicated in 50% methanol for chlorogenic acid determination and in 70% methanol for other analyte determinations at a ratio of 1:20 (w/v) for 30 min using an SB-800 DTD sonicator (Ningbo Xinzhi Biotechnology Co., Ltd, Ningbo, China; Power: 100 W; Frequency: 40 kHz). The extract was centrifuged at 12,000 g for 15 min, and the resulting supernatant was filtered through a 0.22 μm filter for subsequent LC-MS/MS analysis as described by Nebot *et al.*[9]

Extracts were injected into an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm i.d., 1.8 μm), ACQUITY UPLC™ I-Class system (Waters, Milford, MA, USA). A binary mobile phase composed of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B) at a constant flow rate of 0.6 mL/min was applied with an injection volume of 1.0 μL. The column oven temperature was at 40°C. The linear gradient conditions were optimized as follows: 0 min, 5% B; 2 min, 25% B; 3.5 min, 40% B; and 5 min, 60% B.

The effluent from the HPLC column was directed into the electrospray ionization (ESI) source (Applied Biosystems, Toronto, Canada) of the AB SCIEX API 6500 triple quadrupole MS. The ESI was operated in negative-ion mode, which was carried out by optimization of the product ion obtained from the fragment of the isolated precursor ion for each analyte. The ion spray potential was −4500 V, and the source temperature was set at 550°C. Once the product ions were chosen, the multiple reaction monitoring conditions for each standard were further optimized to achieve maximum sensitivity. GS1 and GS2 flows are also 55 L/min, and the curtain gas flow is 30 L/min. The retention times (t_R), quantitative ion pairs, declustering potentials, collision energy, and cell exit potential are listed in Table 1.

Table 1: Retention times, quantitative ion pairs, declustering potentials, collision energy, and cell exit potential

Analyte	t_R (min)	Quantitative ion pairs m/z	DP/V	CE/V	CXP/V
Chlorogenic acid	1.92	352.9/191.0	−45	−22	−18
Luteoloside	1.53	447.1/284.9	−215	−37	−22
Luteolin	2.26	285.0/133.0	−144	−40	−15
Isoquercitrin	1.48	463.1/300.0	−191	−36	−22
Caffeic acid	2.56	179.0/134.8	−99	−21	−16

t_R: Retention times; DP: Declustering potentials; CE: Collision energy; CXP: Cell exit potential; V: Voltage

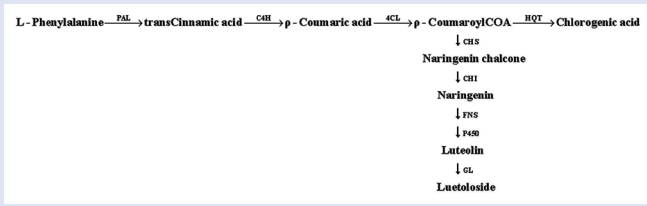

Figure 1: The main biosynthesis pathway of chlorogenic acid and luteoloside. PAL: Phenylalanine ammonia-lyase; C4H: Cinnamate 4-hydroxylase; 4CL: 4-Hydroxycinnamoyl-CoA ligase/4-coumarate-CoA ligase; HQT: Hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; CHS: Chalcone synthase; CHI: Chalcone isomerase; FNS: Flavone synthase; P450: Cytochrome P450; and GL: Glycosyltransferase (flavone 7-O-beta-glucosyltransferase)

RNA extraction and quantitative real-time reverse transcription

Total RNA was extracted from fresh flower buds using TRIzol Reagent (Invitrogen, USA) and was pretreated with RNase-Free DNase (Promega, USA) to eliminate genomic DNA contamination. RNA integrity was analyzed on a 1.5% agarose gel. RNA quantity was determined using a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., China). Complementary DNA (cDNA) was synthesized using an AMV First-Strand cDNA Synthesis Kit (Roche, Switzerland). Real-time qPCR was performed using SYBR Green Fast qPCR Master Mix (BBI, Canada) with a StepOne Real-Time PCR system (ABI, USA) and carried out in triplicate. Gene-specific primers were designed by Primer Premier 5.0 software and synthesized by Integrated DNA technologies (Takara, China). The primer sequences of approximately 15 genes related to the biosynthesis of chlorogenic acid and luteoloside are listed in Table 2. The lengths of amplicons range between 100 bp and 250 bp. *18S* was chosen as an endogenous control gene. Standard errors (SE) were calculated from three PCR replicates. The specificity of amplification was assessed by melting curve analysis, and gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.[10]

Determination of endogenous hormones

Six endogenous plant hormones, IAA, GA_3, iPA, ZR, ABA, and JA, were determined by ELISA as described by Yang *et al.*[11] and Wu *et al.*[12]

Samples were homogenized in liquid nitrogen, extracted in cold 80% (v/v) methanol containing 1 mmol/L butylated hydroxytoluene and incubated overnight at 4°C. After centrifugation at 10,000 ×*g* (4°C) for 20 min, the supernatant was passed through a C18 Sep-Pak cartridge (Waters, Milford, MA), and then, the extracts were collected and dried in N_2. Subsequently, the extracts were dissolved in phosphate buffer saline (PBS, 0.01 mol/L, pH 7.4) for analysis by ELISA.

ELISA was performed on a 96-well microtitration plate (Nunc, Roskilde, Denmark). Each well in the plate was coated with $NaHCO_3$ coating buffer (50 mmol/L, pH 9.6) containing synthetic IAA, GA_3, iPA, ZR, ABA, and JA ovalbumin conjugates. Ovalbumin solution (10 mg/mL) was added to block nonspecific binding. The coated plates were incubated for 30 min at 37°C. After washing four times with PBS, plant extracts or IAA, GA_3, iPA, ZR, ABA, and JA standards and antibodies were added and incubated for an additional 45 min at 37°. The antibodies against these hormones were obtained as described by Weiler *et al.*[13] The plates were washed four times according to the above procedure. Then, horseradish peroxidase-labeled goat anti-rabbit immunoglobulin was added to each well and incubated for 1 h at 37°C. The plates were washed as above. Finally, the buffered enzyme substrate (ortho-phenylenediamine) was added, and the enzyme reaction was carried out in the dark at 37°C for 15 min and then terminated using 3 mol/L H_2SO_4. The absorbance was recorded at 490 nm. Calculations of the enzyme immunoassay data were performed as described by Weiler *et al.*[13] In this study, the percentage recovery of each hormone was calculated by adding known amounts of standard hormone to a split extract. The percentage recoveries were all above 90%, and all sample extract dilution curves paralleled the standard curves, indicating the absence of nonspecific inhibitors in the extracts.

Statistical analysis

The results were the means ± SE of three independent experiments. $P < 0.05$ and 0.01 were considered statistically significant and extremely significant, respectively. Error bars and statistical analyses were performed using SPSS software 19.0 (SPSS Inc., Chicago, IL, USA) for Windows.

RESULTS

Active component changes after plant introduction

SD is the genuine producing area of LJ; after 3-year *ex situ* conservation in GX, we found that LJ was able to grow and develop normally as in SD. We chose the first harvested flower buds to detect pharmacological active compound changes after plant introduction. Figure 2 shows that in GX, chlorogenic acid was significantly decreased while luteoloside and luteolin were significantly increased compared to SD. There were no significant differences in isoquercitrin and caffeic acid contents between GX and SD. Because the contents of chlorogenic acid and luteoloside are two biomarkers of LJ drug quality, these results demonstrated significant drug quality changes after LJ introduction.

Gene expression changes after plant introduction

We analyzed the gene expression profiles related to the key enzymes in the chlorogenic acid and luteoloside biosynthesis pathways. These related genes belong to multiple gene families.

PAL, C4H, and 4CL are important enzymes for synthesizing the universal precursor p-Coumaroyl-CoA. HQT leads to direct synthesis from p-Coumaroyl-CoA to chlorogenic acid. Figure 3 shows that the transcript ratios of *PAL1*, *PAL3*, *C4H1*, *C4H2*, *4CL1*, and *4CL2* increased while *PAL2* decreased in GX compared to SD. At the same time, the

Table 2: Primers of real-time quantitative polymerase chain reaction

Gene	GenBank ID	Forward sequence (5′→3′)	Reverse sequence (5′→3′)
PAL1	JX068601	GACTTGAGGCATTTGGAGGA	AGAACCTTGAGGGGTGGAGT
PAL2	JX068602	TGCCGAAAGAAGTCGAAAGT	TGTCAAATACTCGCCCTTCA
PAL3	JX068603	AAATGCGTCGATCTTCCAGA	AACCGGATTCTCACTCTCCA
4CL1	JX068604	GCCAAACTTGGACAGGGTTA	ATAACGGCGTTTCTCACCAC
4CL2	JX068605	CATTCCCCTCAACTCTCCAA	GAGGAGATAAGGGCGGTTTC
C4H1	JX068610	TATACTTGCATTGCCGATTCTC	CTAAACTGCCCTCCTTTCTCC
C4H2	JX068611	GACGGGACATAATGGCTAAAA	ATTGGACAGTTGGATGGTTGA
CHS1	JX068606	GGTATTTCGGACTGGAACTCG	GGTGGTCTTGAACCCATCTGTCC
CHS2	JX068607	ATCCGGACAAGTCTGTCGAG	GCACGTCCTTCAACAAATGA
CHI1	JX068608	TCCCTGCAACTTCTTCCACT	TCCCACCCAAGTACCACTTC
CHI2	JX068609	AACGGGGCAACAATACTCAG	ATGAGCCAAGAGGTGATTGG
FNS1	JX068612	AGGCTAGTGAGGGGGTGAAC	CACCTCACGTACCAATGTCCT
FNS2	JX068613	AGGGAAGCACAGGCTAGTCA	GGTGTTAGCCGAGATGTGGT
HQT1	ACZ52698	TGAGATCCTAGCTGCCCACT	TGGCTGTGAACACCACATTT
HQT2	JF261014	CAATCAAGTCCCAAGGCTGT	GGCAGCTAGGACCTCGTATG
18S	KF160903	TTCTTAGTTGGTGGAGCGATTT	CCTGTTATTGCCTCAAACTTCC

transcript ratio of *HQT1* and *HQT2* decreased in GX. However, these differences were not significantly different between GX and SD.

CHS, CHI, and FNS are essential enzymes for synthesizing luteoloside. Figure 4 shows that the transcript ratios of *CHS1*, *CHI1*, *CHI2*, *FNS1*, and *FNS2* increased while *CHS2* decreased in GX compared to SD. However, most of these gene expression levels were not significantly different between GX and SD except *FNS2*.

Endogenous hormone changes after plant introduction

Plant hormones are involved in many physiological and biochemical functions, such as flower bud differentiation and formation, plant defense, and secondary metabolic regulation. We analyzed content changes of six endogenous plant hormones, IAA, GA_3, iPA, ZR, ABA, and JA, of LJ flower buds after plant introduction. Figure 5 shows that the contents of IAA, ZR, and ABA were extremely significantly lower while the contents of GA_3 and JA were extremely and significantly higher in GX compared to SD. The iPA content was not significantly different between GX and SD.

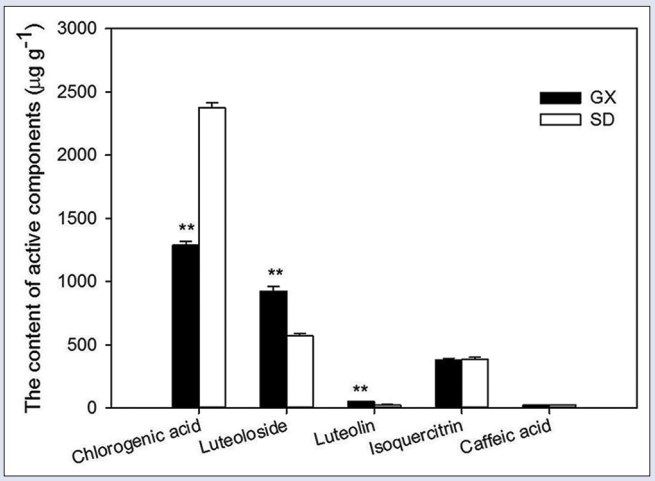

Figure 2: Chlorogenic acid, luteoloside, luteolin, isoquercitrin, and caffeic acid contents of *Lonicera japonica* flower buds. GX: Leye County in Guangxi Zhuang Autonomous Region; SD: Pingyi County in Shandong Province. ** indicates extremely significant differences at $P < 0.01$ level

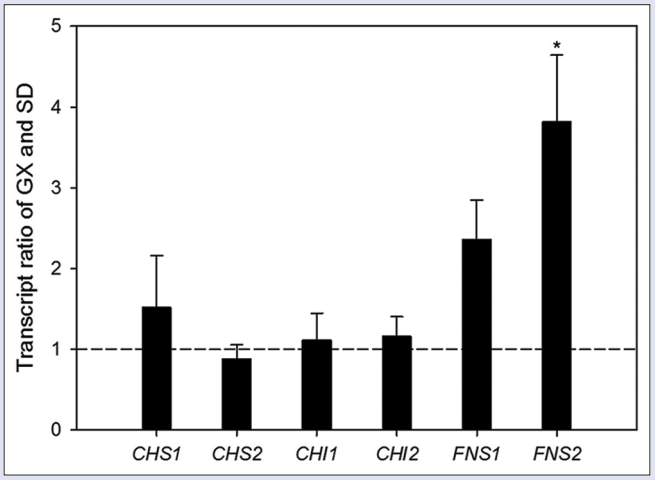

Figure 4: The transcript ratios of key genes in the luteoloside biosynthesis pathway of *Lonicera japonica* flower buds. GX: Leye County in Guangxi Zhuang Autonomous Region; SD: Pingyi County in Shandong Province. The expression level of genes in SD was arbitrarily set to 1. * indicates a significant difference at $P < 0.05$ level

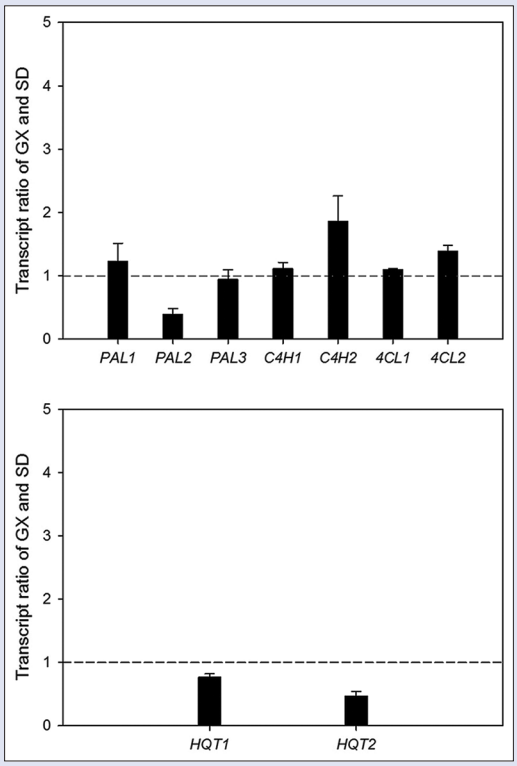

Figure 3: The transcript ratio of key genes in the chlorogenic acid biosynthesis pathway of *Lonicera japonica* flower buds. GX: Leye County in the Guangxi Zhuang Autonomous Region; SD: Pingyi County in Shandong Province. The expression level of genes in SD was arbitrarily set to 1

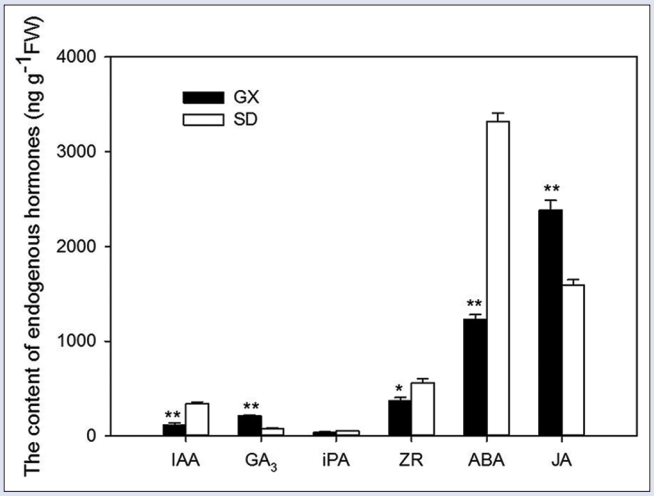

Figure 5: Endogenous hormone contents of *Lonicera japonica* flower buds. IAA: Indole-3-acetic acid; GA_3: Gibberellins; iPA: Isopentenyladenine; ZR: Zeatin riboside; ABA: Abscisic acid; and JA: Jasmonic acid. GX: Leye County in Guangxi Zhuang Autonomous Region; SD: Pingyi County in Shandong Province. * indicates a significant difference at $P < 0.05$ level, and ** indicates an extremely significant difference at $P < 0.01$ level

DISCUSSION

Medicinal quality changed after *Lonicera japonica* introduction

Lonicera japonica Thunb. cv. Tomentosa is the superior variety in Shandong province. We introduced LJ from SD, eastern China, to GX, southern China. As previous studies have stated, the growth and spread of LJ were trivially limited by drought and cold temperature (<−10°C).[8] We observed that LJ adapted well to the soil depletion karst areas of GX. LJ grew easily and densely covered the ground surface. The inflorescence was similar to SD and could also bloom 4 times a year. The first harvest LJ had a much higher yield and quality than the other harvest times. The normal growth and development of LJ outside the authentic area inspired us to study the pharmacological quality changes after plant introduction.

For LJ quality, we also tested compounds such as salicylic acid, kaempferol, isorhamnetin, chrysoeriol, and apigenin, but their contents in LJ were very low, ranging from 0.01 to 8.77 μg/g. These components changes were negligible in our study because they could not be comparable to the five bioactive compounds presented here. Our results showed that chlorogenic acid significantly decreased while luteoloside and luteolin significantly increased after plant introduction. Other active components, isoquercitrin and caffeic acid, were not significantly changed [Figure 2]. In summary, these active ingredients dramatically changed, indicating that LJ introduction altered its medicinal quality.

The five chemical compounds in our study are all ubiquitously distributed natural products in plants. They are low toxicity polyphenols and have high pharmacological potency for their reactive oxygen species (ROS) scavenging and anti-inflammatory abilities.[14,15] Chlorogenic acid and caffeic acid are two hydroxycinnamic acid compounds. Chlorogenic acid is the ester of caffeic acid with quinic acid and has less antioxidative activity but more anti-inflammatory activity than caffeic acid.[16] Luteoloside, luteolin, and isoquercitrin are flavonoids. Luteoloside is a critical medical component in LJ. Luteolin can transform into luteoloside by the catalysis of GT. Isoquercitrin (quercetin-3-O-glucoside) is the glycosylated form of quercitrin (quercetin-3-O-rhamnoside) and exhibits higher ROS scavenging activity than quercitrin.[17] From this point of view, all these bioactive components contribute to the quality of LJ. However, only the contents of chlorogenic acid and luteoloside are used as quality standards by the Chinese Pharmacopoeia. Unlike other studies mainly focused on chlorogenic acid content and believed that LJ quality decreased in nonauthentic producing areas;[18] in our cases, accompanied by the decrease in chlorogenic acid, the notably large flavonoids accumulations demonstrated the benefit of LJ introduction.

In summary, our findings suggest that the growth and quality of LJ in GX evidenced that LJ was suitable for introduction to Leye County. This introduction will help alleviate poverty and encourage ecosystem restoration. Other researchers have also recommended LJ plantations in rock crevices, and LJ displayed superior characteristics for the rehabilitation of karst rock ecology.[19,20]

Catalytic enzyme gene expressions brought about the quality changes in *Lonicera japonica*

Almost all genes for chlorogenic acid and luteoloside are members of diverse gene families. PAL is the first speed-limiting enzyme in the phenylpropanoid pathway and catalyzes L-phenylalanine to trans-cinnamic acid; C4H catalyzes the conversion of cinnamate into 4-coumarate; and 4CL catalyzes coumarate to p-Coumaroyl-CoA.[5] Our results showed that although not significantly different, most isoforms of *PAL*, *C4H*, and *4CL* gene expression levels were upregulated [Figure 3], which means that the contents of p-Coumaroyl-CoA, the starting point of phenylpropanoid compounds, were most likely increased as well.

HQT catalyzes the esterification of quinic acid with caffeoyl-CoA to generate chlorogenic acid. Because HQT leads to the last and direct biosynthesis of chlorogenic acid and is proven to be a rate-limiting enzyme, numerous studies have shown that HQT is positively correlated with chlorogenic acid content.[21-23] Our results revealed that the downregulated gene expression level of *HQT* [Figure 3] was in accordance with the decrease in chlorogenic acid content [Figure 2]. We also concluded that HQT rather than PAL was much more closely related to the chlorogenic acid content, as the PAL catalytic products also functioned in other branches of the phenylpropanoid biosynthesis pathway. In our study, the higher expression levels of *PAL1* and *PAL3* did not stimulate the higher chlorogenic acid biosynthesis [Figures 2 and 3]. However, in other studies, the overexpression of *PAL* family genes improved chlorogenic acid content.[5,24]

CHS produces flavonoid precursors, CHI catalyzes chalcone to flavanone, and FNS catalyzes the direct conversion of flavanones into flavones.[25] Our results also showed that accompanied by the increase in p-Coumaroyl-CoA, most isoform gene expression levels of *CHS*, *CHI*, and *FNS* were also upregulated after plant introduction [Figure 4]. Although most of these differences were not significantly different, these gene expression level increases can partially explain the abundant accumulations of luteolin and luteoloside. Our results are similar to those of other studies. It was found that the expression of *PAL1*, *C4H2*, and *4CL2* and the enzyme activities of PAL, C4H, and 4CL were positively correlated with the accumulation of luteoloside in LJ.[6,22] Moreover, the ectopic overexpression of the *CHS1* gene isolated from the *Freesia hybrid* could restore the pigmentation phenotype of *Arabidopsis thaliana tt4* mutant and alter the flower color of petunia, exhibiting flavonoid accumulation in transgenic plants.[26] In tomato[27] and tobacco,[25] *CHI* gene overexpression and suppression resulted in corresponding flavonoid component changes. In *Lonicera macranthoides*, a substitute for LJ, the expression levels of *FNS2* determined flavone accumulation.[28] Our study also demonstrated the significant increases of *FNS2* expressions in LJ corresponded to the significant increase of luteolin and luteoloside.

Taken together, in the present study, the differential gene expression levels after plant introduction were in line with the bioactive compound changes in LJ.

Plant hormones regulate the quality changes in *Lonicera japonica*

When LJ was transplanted from the optimum growing place to GX, it was exposed to relatively severe environmental conditions, such as rocky and calcium-rich soil, water deficit, and heavy rainfall. Under such adverse conditions, LJ still showed great adaptability. In this process, endogenous phytohormones play vital roles in regulating stress responses. In our study, the contents of ABA and JA were higher than those of other hormones [Figure 5], which was in accordance with their prominent roles against stress.[29,30] Furthermore, JA played special roles in promoting plant secondary metabolites. For example, JA elicited terpenoid indole alkaloid in *Catharanthus roseus*, nicotine in *Nicotiana tabacum,* and artemisinin in *Artemisia annua*.[31] It was speculated that JA activated some transcription factors and induced transcriptional reprogramming in particular cellular networks related to plant secondary metabolite biosynthesis.[32] In the present study, the pronounced increase in JA after plant introduction contributed to the increase in medicinally important luteoloside and luteolin [Figures 2 and 5]. In addition, the crosstalk of ABA and JA with other hormones co-regulated plant defense systems.[29-31] iPA and ZR are two commonly used cytokinins (CKs).[33,34] IAA, GA_3, and CKs alone and their interactions with ABA and JA mediated the stress responses.[31,34]

The yield and quality of LJ are also heavily dependent on the decisive process of flowering. Plant hormones control flower bud differentiation, florescence, flower sex differentiation, and flower senescence.[35] IAA, GA_3, CKs, and ABA are all involved in floral development.[35-37] In our study, the contents of IAA, ZR, and ABA decreased while GA_3 increased after plant introduction [Figure 5]. This discrepancy might explain the superior yield and quality of LJ in the geo-authentic region.

However, the regulatory mechanisms of plant hormones are very complex, as phytohormones individually and collectively mediate wide physiological processes. Further study should be executed to elucidate the different endogenous plant hormone change mechanisms after plant introduction.

CONCLUSION

The quality standard of LJ showed opposite changes when LJ was introduced from SD to GX. The contents of luteoloside increased, and different key enzyme gene expression profiles appeared after plant introduction. Endogenous phytohormones, especially JA, regulate the changes in the quality of LJ.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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