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Expression analysis of key enzymes involved in the accumulation of iridoid in *Rehmannia glutinosa*

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Abstract

As the traditional Chinese herb, *Rehmannia glutinosa* (*R. glutinosa*) has significant effects on health. The main active ingredient of *R. glutinosa* is iridoid. In this study, root, stem and leaf of *R. glutinosa* at five different growth stages were collected, the content of iridoid in *R. glutinosa* was determinated, and the expression of key enzymes in *R. glutinosa* was analyzed by quantitative real-time PCR (q-PCR). We found that the content of iridoid was increased continuously during the first three growth stages (I–III) of *R. glutinosa.* It reached the maximum value in root and leaf at growth stage III of *R. glutinosa.* However, the content of iridoid was not lower in leaf, compared with root. 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), Geranyl pyrophosphate synthase (GPPS), Geraniol 10-hydroxylase (G10H) and 10-hydroxygeraniol oxidoreductase (10HGO) were the key enzymes involved in the synthesis of iridoid in plant, but their expression pattern or level was different in various tissues and stages of *R. glutinosa.* All appeared tissue specificity and their expression was related to the accumulation of iridoid in *R. glutinosa*. Thus, DXR, GPPS, G10H and 10HGO might take part in the synthesis of iridoid in *R. glutinosa* and could be differently regulated during the growth and development of *R. glutinosa*, which would provide theoretical basis for the research on secondary metabolism of iridoid in *R. glutinosa*.

Keywords: *Rehmannia glutinosa*; iridoid; secondary metabolism; qRT-PCR.
Abbreviations: IPP_isopentenyl pyrophosphate; DMAPP_dimethylallyl pyrophosphate; MVP_mevalonate pathway; MEP_2C-methyl-D-erythritol-4-phosphate pathway; DXR_1-deoxy-D-xylulose-5-phosphate reductoisomerase; GPPS_Geranyl pyrophosphate synthase; G10H_Geraniol 10-hydroxylase; 10HGO_10-hydroxygeraniol oxidoreductase; *R. glutinosa_Rehmannia glutinosa*; qRT-PCR_quantitative real-time PCR; UV_ultraviolet; ANOVA_analysis of variance.

Introduction

Terpenoid is one of the largest family of secondary metabolites in plant, and could be divided into monoterpene (C10), sesquiterpene (C15), diterpene (C20), triterpenes (C30) and polyterpenes (Ashour et al., 2010). Terpenoid is synthesized from precursors of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Newman and Chappell, 2008). There are two synthetic pathway of IPP in plant, such as mevalonate pathway (MVP) and 2C-methyl-D-erythritol-4-phosphate pathway (MEP) (Laule et al., 2003). Iridoid is one kind of monoterpenes, and its synthesis may be completed by MEP pathway in the plant (Li et al., 2010).

In MEP pathway, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) is the branch point of carbon flow, and could convert 1-deoxy-D-xylulose-5-phosphate into methyl erythritol phosphate, which is the important precursors in the synthesis of terpenoid (Luo et al., 2003). Geranyl pyrophosphate synthase (GPPS) is one of key enzymes in MEP pathway. It can catalyze the synthesis of C10 skelecton in monoterpene (Yu et al., 2013). For example, IPP and DMAPP are synthesized by GPPS (Yang et al., 2016). Furthermore, Geraniol 10-hydroxylase (G10H) could catalyze geraniol hydroxylated into 10-hydroxy geraniol in the synthesis of iridoid (Sung et al., 2011), and would control the first committed step in the synthesis of secologanin and TIAs (Wang et al., 2010). The oxidation of 10-hydroxygeraniol to aldehyde 10-oxogeraniol is catalyzed by 10-hydroxygeraniol oxidoreductase (10HGO) (Verma et al., 2012). The dehydrogenases isolated from *C. roseus* may be responsible for this reaction and were able to produce iridoidial as incubated in the presence of iridoid-related synthase (Thamm et al., 2016).

Rehmannia glutinosa (*R. glutinosa*) belongs to one kind of perennial herb, has a lot of functions, such as nourishing yin, supplementing kidney, blood hemostasis, and so on (Kang and kim, 2011). There are a variety of compounds in *R. glutinosa*, such as iridoid, sesquiterpene, phenylethanoid glycoside and triterpene (Zhang et al., 2013, Zhou et al., 2018). Iridoid is the main active ingredient of *R. glutinosa*, and more than 30 kinds of iridoids have been identified from *R. glutinosa*. However, the research on biosynthesis and metabolism regulation of

iridoid in *R. glutinosa* is scarce. In order to explore the synthesis of iridoid in *R. glutinosa*, the accumulation of iridoid was analyzed, and the expression of some key enzymes in the synthesis of iridoid in *R. glutinosa* was studied by quantitative real-time PCR (qRT-PCR). Therefore, this research will help to study metabolic pathway of iridoid in *R. glutinosa*, and can provide proposal for utilization of *R. glutinosa*.

Results

Content of iridoid in R. glutinosa

The content of iridoid in root increased continuously at growth stage Ⅰ-Ⅲ of *R. glutinosa*, and peaked with 7.55% at Ⅲ (Fig. 1, a). After growth stage Ⅲ, the content of iridoid was decreased, but was still higher compared to growth stage Ⅰ. Similar results were found in Fig. 1 (b), where the accumulation of iridoid in leaf was also increased continuously at growth stage Ⅰ-Ⅲ of *R. glutinosa* and reached the maximum value at growth stage Ⅲ (6.52%). It subsequently began to decrease, but was still higher than that at growth stage Ⅰ (2.42%) (Fig. 1, b). Thus, the accumulation of iridoid appeared in a similar trend in root and leaf during the growth and development of *R. glutinosa*, and the content of iridoid in root or leaf was the highest at growth stage Ⅲ.
Compared with root, the content of iridoid in leaf of *R. glutinosa* was higher at growth stage Ⅱ, while it was lower at growth stage Ⅲ - Ⅳ (P<0.01). There was no significant difference between growth stages of Ⅰ and Ⅴ, so the accumulation of iridoid is also important in leaf, especially at growth stage Ⅱ of *R. glutinosa*, indicating that leaf of *R. glutinosa* could be utilized to alleviate shortage of resources.

Expression of DXR in R. glutinosa

After growth stage Ⅰ, the expression of *DXR2* was decreased continuously in root, stem or leaf (Fig. 2, a), and its expression peaked at level 1.0, 1.254 or 4.075, respectively. At the same growth stage of *R. glutinosa*, the expression of *DXR2* in leaf was higher as compared to that in root or stem (P<0.01).
As shown in Fig. 2 (b), the expression of *DXR4* presented an abrupt increase in root, stem and leaf at growth stage Ⅱ, subsequently increased tardily, but was still lower, compared with growth stage Ⅱ. Furthermore, the expression of *DXR4* was significantly different in root, stem and leaf at growth stage Ⅱ, as the following trends: stem> root> leaf (P<0.01), but was higher in leaf at growth stages Ⅳ or Ⅴ (P<0.01). In addition, the expression of *DXR4* in root, stem or leaf was respectively higher than that of *DXR2* during the growth and development of *R. glutinosa* (P<0.01).

Expression of GPPS in R. glutinosa

As shown in Fig. 3, the expression of *GPPS3* was higher in root and stem after growth stage Ⅰ, and peaked at growth stage Ⅱ-Ⅲ (Fig. 3a). However, the expression of *GPPS3* in leaf reached the peak value at growth stage Ⅳ, and was higher than that in root and stem during the growth and development of *R. glutinosa*, which was especially significant at growth stage Ⅰ, Ⅱ, or Ⅳ (P<0.01). Furthermore, at growth stage Ⅱ-Ⅲ of *R. glutinosa*, the expression of *GPPS3* in stem was significantly higher than that in root (P<0.01).
Compared with the expression of *GPPS3*, similar result was found in *GPPS1* (Fig. 3b). The expression of *GPPS1* was especially higher in leaf than that in root and stem at growth stages Ⅰ, Ⅱ and Ⅳ (P<0.01), but was similar in root and stem during the growth and development of *R. glutinosa* (Fig. 3b). In addition, the expression of *GPPS3* and *GPPS1* in *R. glutinosa* had the similar expression pattern, and there was no difference between them, indicating that *GPPS1* and *GPPS3* might be one gene, which could encode geranyl pyrophosphate synthase.

Expression of G10H in R. glutinosa

In this study, the expression tendency of *G10H1* and *G10H2* showed similar phenomenon during the growth and development of *R. glutinosa* (Fig. 4). It reached the maximum value in root, stem and leaf at growth stage Ⅱ, and the minimum value appeared at growth stage Ⅴ.
However, the expression of *G10H1* and *G10H2* in root, stem and leaf was different (Fig. 4), at growth stage Ⅰ. The expression of *G10H1* in leaf and stem was similar, and was significantly higher than that in root (Fig. 4a), yet the significant difference was found among expressions of *G10H2* in various tissues (Fig. 4b), stem> leaf> root (P<0.01). Furthermore, at growth stage Ⅱ, the expression of *G10H1* and *G10H2* both showed the following: leaf> stem> root (P<0.01), and was also higher in leaf at growth stage Ⅲ-Ⅳ of *R. glutinosa* (P<0.01). In addition, the expression of *G10H2* was higher compared to that of *G10H1* (Fig. 4), conjecturing that *G10H1* and *G10H2* might be two different genes encoding geraniol 10-hydroxylase although their expression patterns were consistent.

Expression of 10HGO in R. glutinosa

The expression of *10HGO33* was different in root, stem and leaf of *R. glutinosa* (Fig. 5, a), during the growth and development of *R. glutinosa.* The expression of *10HGO33* in root had a downward trend, and peaked at level 1.067 in growth stage Ⅰ (P<0.01). However, it was up-regulated in stem and reached the maximum value at growth stage Ⅴ. The expression of *10HGO33* in leaf was also up-regulated, and peaked at level 2.0 in growth stage Ⅱ (P<0.01), then decreased and was still higher than that at growth stage Ⅰ.
As shown in Fig. 5b, the expression tendency of *10HGO6* appeared different in root, stem and leaf during the growth and development of *R. glutinosa*. The expression of *10HGO6* in root and stem both reached maximum value at growth stage Ⅱ, yet was the highest in leaf at growth stage Ⅳ. At the same growth stage of *R. glutinosa*, the expression of *10HGO6* in various tissues appeared significantly different except for growth stage Ⅱ-Ⅲ. For example, the expression of *10HGO6* at growth stage Ⅳ-Ⅴ was significantly higher in leaf and root than that in stem, especially in leaf (P<0.01). Further analysis found that the expression of *10HGO33* and *10HGO6* was different in *R. glutinosa* (Fig. 5), indicating that *10HGO33* and *10HGO6* may belong to two different genes encoding 10-hydroxygeraniol oxidoreductase.

Table 1. The primers used in qRT-PCR.

Primer	Sequence of primer (5'-3')
DXR2-F	TCCACTGTAATCTTCTTTCCCATA
DXR2-R	TCTCAGGCGGATACTTTTGACT
DXR4-F	ATTTGGCTTACGCTGCTGG
DXR4-R	TTCTGCTCGGTGCTTATCG
GPPS1-F	TCGGGCAACAGGCTCAT
GPPS1-R	GAACAGTGGGTCGGAATCTTT
GPPS3-F	GATACAAGGAGGGGAATCGG
GPPS3-R	CCACAACTTTAGCCAAGAGGG
G10H1-F	TCCGTCGTCTGGCTTCCG
G10H1-R	TTTCCTACACCTGAGATGCTGATT
G10H2-F	GTAATGCTTGGTTCGCTTTTG
G10H2-R	GGAGTGGTTGAGCCTTCTGC
10HGO6-F	TTCCAGATTCGCCGTAAGC
10HGO6-R	CACAGGAGATGATTGATTTTGC
10HGO33-F	GCCTGGGCACGAGATTG
10HGO33-R	TGAGTATTTGTTTGGGGCAGTA
TIP41-F	TGGCTCAGAGTTGATGGAGTGCT
TIP41-R	CTCTCCAGCAGCTTTCTCGGAGA

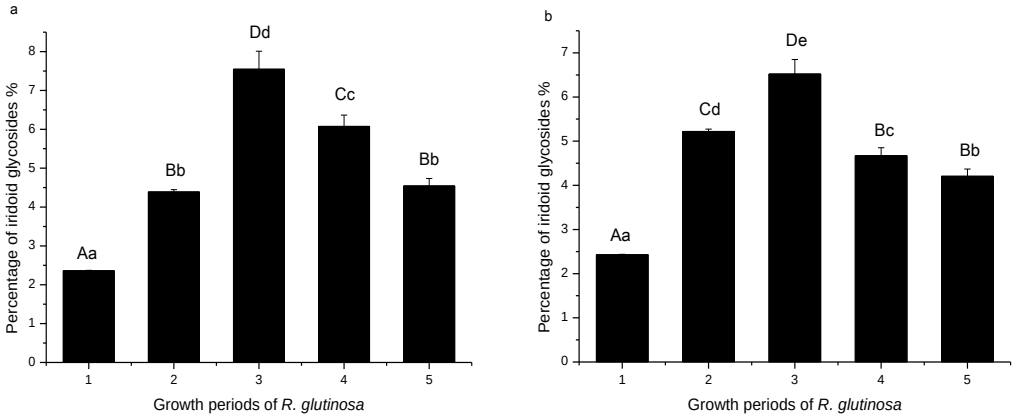

Fig 1. The content of iridoid in *R. glutinosa* (a) The content of iridoid in root of *R. glutinosa*; (b) The content of iridoid in leaf of *R. glutinosa*. 1, 2, 3, 4 or 5, respectively, represented plants at growth stage of Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ in *R. glutinosa*. The lower cases and capital letters represented significant differences (P<0.05) or extremely significant difference (P<0.01), respectively.

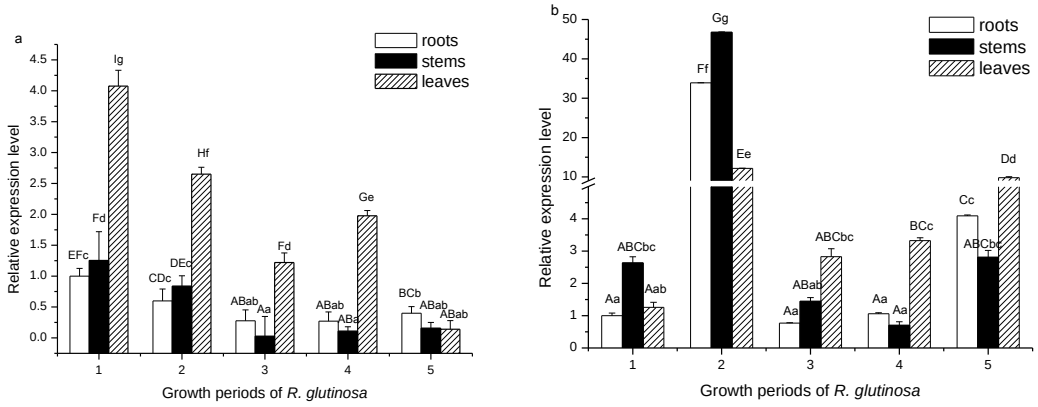

Fig 2. The expression of *DXR* in *R. glutinosa* (a) and (b) represented the expression of *DXR2* or *DXR4*, respectively. The numbers 1, 2, 3, 4 or 5 respectively represented plants at Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ growth stage of *R. glutinosa*. The lower cases and capital letters represented significant differences (P<0.05) or extremely significant difference (P<0.01), respectively.

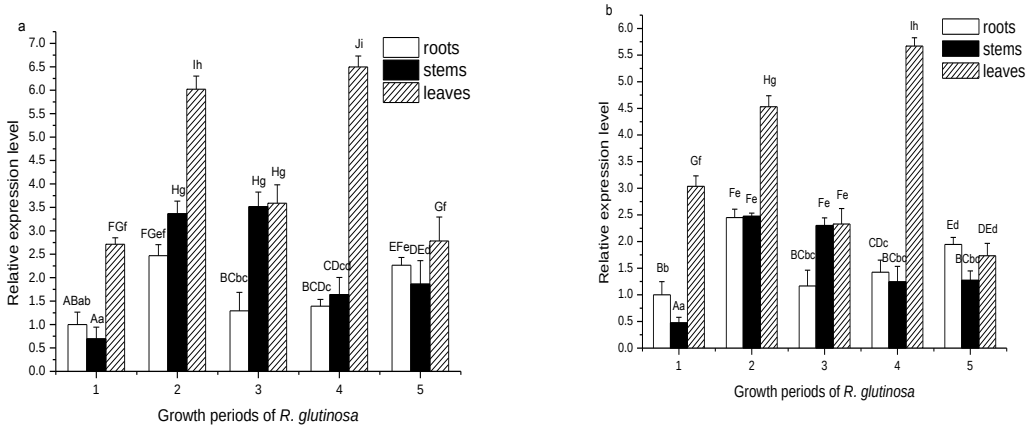

Fig 3. The expression of *GPPS* in *R. glutinosa* (a) and (b) represented the expression of *GPPS3* and *GPPS1* in *R. glutinosa*, respectively. The numbers 1, 2, 3, 4 or 5 respectively represented plants at Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ growth stages. The lower cases and capital letters represented significant differences (P<0.05) or extremely significant difference (P<0.01), respectively.

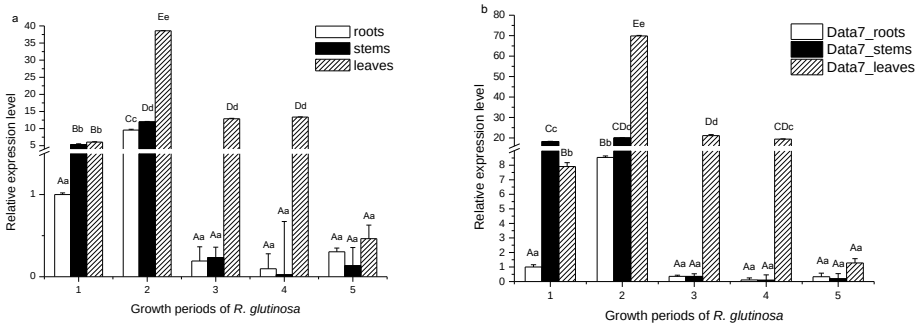

Fig 4. The expression of *G10H* in *R. glutinosa* (a) and (b) represented the expression of *G10H1* and *G10H2* in *R. glutinosa,* respectively. The numbers 1, 2, 3, 4 or 5 represented plants at Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ growth stage of *R. glutinosa,* respectively. The lower cases and capital letters represented significant differences (P<0.05) or extremely significant difference (P<0.01), respectively.

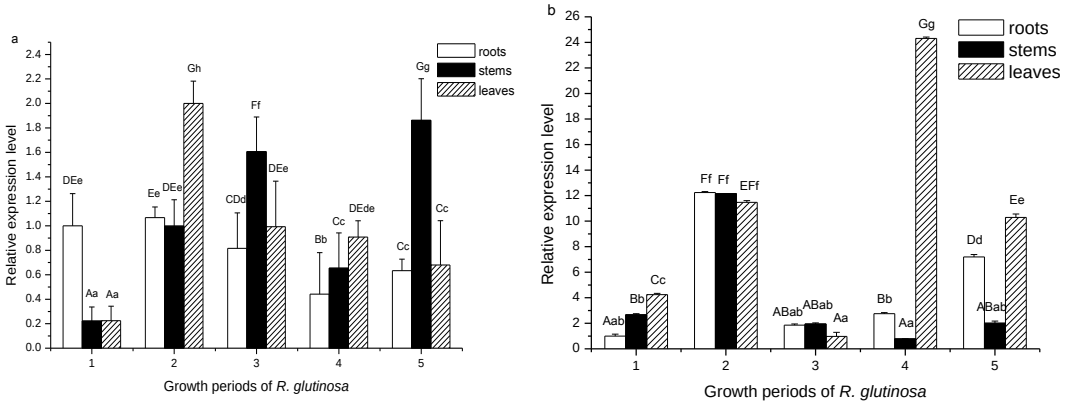

Fig 5. The expression of *10HGO* in *R. glutinosa* (a) and (b) represented the expression of *10HGO33* and *10HGO6*, respectively. The numbers 1, 2, 3, 4 or 5 represented plants in Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ growth stage of *R. glutinosa*, respectively. The lower cases and capital letters represented significant differences (P<0.05) or extremely significant difference (P<0.01), respectively.

Discussion

It is well known that terpenoid is one important kind of secondary metabolites, and distributes widely in plant (Peng et al., 2002). Iridoid is one kind of terpenoid, exhibits antimicrobial properties and is important in defensive response to bacteria and fungus (Wang et al., 2009). However, the distribution of secondary metabolite is various among species, grow stages and tissues of plants (Nu et al., 2012). In this study, the content of iridoid in *R. glutinosa* increased continuously during the first three growth stages, and reached the maximum value in root and leaf at growth stage Ⅲ. The similar results were also found in other researches (Ji et al., 2014). In addition, the content of iridoid in leaf of *R. glutinosa* was not lower compared with that in root. Therefore, the leaf of *R. glutinosa* may become one important source of iridoid.

The synthetic pathway of iridoid is mainly MEP, in which it serves as the precursor for many substances, such as IPP, DMAPP, vitamin B1, and so on (Fitzpatrick et al., 2007). Some studies showed that DXR would play a critical role in directing intermediate flux into IPP and DMAPP synthetic pathway (Xing et al., 2010). The GPPS is responsible for enzymatic formation of GPP towards most of monoterpenoid and diterpenoid in plastid (Nagegowda, 2010; Schmidt et al., 2010; Xi et al., 2016). The G10H is a rate-limiting enzyme for the synthesis of terpenoid (Dagnino et al., 1995; Cui et al., 2015; Kai et al., 2015), and 10HGO could take part in the synthesis of ring-opening iridoid (Ji, 2014). In this study, the expression of two unigenes for DXR, GPPS, G10H and 10HGO were analyzed in *R. glutinosa*. We found that *DXR2* and *DXR4* had different expression. The expression of *10HGO6* and *10HGO33* was also different, although *G10H1* and *G10H2* had the same expression pattern, their expression level were different. However, the expression of *GPPS3* and *GPPS1* in *R. glutinosa* was similar, inferring that *GPPS3* and *GPPS1* might be two parts of GPPS, which was consistent with the previous research (Ji, 2014). Thus, two unigenes for DXR, G10H and 10HGO might be both differently regulated during the growth and development of *R. glutinosa*.

Further analysis showed that expression patterns or levels of DXR, GPPS, G10H, 10HGO were different during the growth and development of *R. glutinosa*, where all appeared tissue specificity, and their expression was relative to the accumulation of iridoid in *R. glutinosa.* This indicates that these enzymes might take part in the synthesis of iridoid in *R. glutinosa*, but could be differently regulated during the growth and development of *R. glutinosa*, which was also found in other plants (Zhu et al., 2016). Given that many steps and enzymes are involved in the synthesis of iridoid in plant (Liu et al., 2004), its metabolism regulation is complex in plant. Therefore, this research provided evidence for synthesis and metabolism regulation of iridoid in *R. glutinosa*. However, the regulatory network of iridoid synthesis is very complicated and need to be further studied in *R. glutinosa*.

Materials and Methods

Plant materials

R. glutinosa Wen 85-5 was used to be experimental material in this study, and its root tubers were kindly provided by Agricultural Research Institute of Wenxian County, Henan, China. Root tubers of *R. glutinosa* were grown in test field, Wenxian County, Jiaozuo City, Henan, China. Type of planting soil was loam, planting density was 40×40 cm, and *R. glutinosa* was managed by conventional field management.

In this study, *R. glutinosa* was analyzed at five different growth stages (Duan et al., 2018), such as growth stage Ⅰ (seedling root is not fleshy), Ⅱ (plant root is fleshy and cylindrical), Ⅲ (plant root is expansive in the middle), Ⅳ (plant root appears late expansion), Ⅴ (plant root is spindle-shaped). Root, stem and leaf of *R. glutinosa* at each growth stage were frozen in liquid nitrogen and stored at -80℃ for RNA preparation, or were baked in Electro-Thermostatic Blast Oven at 50℃ for 48 h and then crushed into powder.

Determination of iridoid

Extraction of iridoid from leaf and root of *R. glutinosa* was performed as following: 1.0 g power of leaf or root was added into 100 mL conical flask with 10 mL 70% alcohol. Then, it was mixed and put into ultrasonic cleaner for 45 min. After the mixture was filtered, the filtrate was added into 50 mL volumetric flask with 70% alcohol, then 2.5 mL mixture was drawn into another 50 mL volumetric flask with 70% alcohol and used to be reaction liquid. The content of iridoid was measured at 463 nm with ultraviolet (UV) spectrophotometer according to methods by Zeng (Zeng et al., 2010).

In addition, 5.5 mg catalpol reference substance was dissolved with 70% alcohol to make the concentration of catalpol solution of 0.22 mg/L, then 1.5, 2.0, 2.5, 3.0, 3.5 or 4.0 mL solution was drawn into 10 mL volumetric flask and was constant with 70% alcohol, respectively. The measurement method of catalpol reference substance was same to that of reaction liquid, and the standard curve was in the following, $y=0.0754*x+0.065$, $R^2=0.998$ (y: concentration, x: absorbance).

Extraction of total RNA

Total RNA was extracted from root, stem and leaf of *R. glutinosa* with RNAiso Plus (TaKaRa, Japan) according to the instructions. Furthermore, in this study, DNase/RNase-free treatment and phenol-chloroform extraction were used to remove DNase, and RNA was dissolved in RNase-free dH_2O. The integrity of total RNA was verified by 1.0% agarose gel, the yield of total RNA was determined at 260 nm with UV spectrophotometer, and the purity of total RNA was checked by determining A260/A280 ratio.

qRT-PCR

Expression of key enzymes taking part in the synthesis of iridoid such as DXR, GPPS, G10H and 10HGO was studied. According to transcriptome sequencing results of *R. glutinosa*, two unigenes for each key enzyme were chosen as *DXR2*, *DXR4*, *GPPS1*, *GPPS3*, *10HGO6*, *10HGO33*, *G10H1* and *G10H2*, respectively (Table 1). In this study, the internal reference gene was *TIP41*, all primers were synthesized by Yingjie Ji Trade Co., Ltd. (Shanghai, China) and their sequences were listed in Table 1.

The qRT-PCR was conducted by LightCycler 96 Real-time PCR reaction, and cDNA used in qRT-PCR was synthesized by PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time: TaKaRa, Japan). The total volume of qRT-PCR reaction system was 20 μl, composed of 10 μl 2×SYBR Premix Ex Taq II (TliRNaseH Plus) (TaKaRa, Japan), 0.5 μl each primer (10 μM), 2

μl of diluted cDNA mix and 7 μl dH_2O. The reaction procedure of qRT-PCR was 95℃ for 30s, followed by 40 cycles of 95℃ for 5s, 60℃ for 30s, then for melting carve stage. In addition, the relative expression level of gene was normalized and analyzed by the comparative Ct ($2^{-\Delta\Delta ct}$) method (Livak and Schmittgen, 2001).

Statistical analysis of data

Statistical analysis of data in this study was performed as the following: content of iridoid and expression of genes were tested by significance level, ANOVA (analysis of variance) and multiple comparisons of Duncan's multiple range. Content of iridoid and expression of genes were calculated and analyzed by Excel, and all histograms were drawn by Origin.

Conclusion

In this study, the content of iridoid was increased continuously during the growth and development of *R. glutinosa.* It reached the maximum value at growth stage Ⅲ, and the content of iridoid in leaf was not lower as compared with that in root. Furthermore, expression pattern or level of *DXR*, *GPPS*, *G10H*, *10HGO* was different in various tissues and stages of *R. glutinosa*, and their expression was related to the accumulation of iridoid in *R. glutinosa*. Thus, this research would help to study metabolic pathway of iridoid in *R. glutinosa*, and can provide proposal for utilization of *R. glutinosa*.

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