Fe induced a dynamic and biased allocation of material flux within terpenoid metabolism is controlled by *CbNudix* in *Conyza blinii*

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Abstract

Conyza Blinii (*C. blinii*) is a unique traditional Chinese medicine growing in Sichuan, China, which has soils with an abundant iron content. This Fe enrichment environment contributed to a variety of terpenoids of *C. blinii*, blinin and saponin which can play an important role in the process of resisting abiotic stress. The relationship between Fe and terpenoid metabolism was studied to explore Fe tolerance mechanism of *C. blinii*. In this study, *C. blinii* was treated with ferrous iron solutions, and the effect of ferrous iron on the synthesis of blinin and saponins was further studied by spectrophotometry and liquid chromatography. Also, gene expression was detected by qRT-PCR. Under ferrous treatment, the blinin content of *C. blinii* increased, while the total saponin content decreased. When the ferrous concentration reached 200 μM , the difference in metabolite production was the largest. Furthermore, it was found that blinin and saponin have synchronous and opposite accumulation trends, accompanied by time dependence. The genes expression results of key enzyme in the MVA and MEP pathways showed the same trends. In this process, the expression of *CbNudixs* played a key role to switch the material flux between MVA and MEP, via catalyzed the dephosphorylation of isoprenoid diphosphate. This study explored the effect of ferrous iron on the saponin and blinin synthesis of *C. blinii* and found the unique role of *CbNudixs* in regulating material flux between diterpene and triterpene synthesis, which greatly deepened our understanding of the mechanism of Fe tolerance of *C. blinii*.

1. Introduction

Iron, one of the most abundant elements in the earth's crust(Ruiz et al. 1961), is an important component of plant growth factors(McCandliss and Herrmann 1978; Crichton and Ward 1992). The active valence transition between bivalent iron and trivalent iron provides convenient conditions for electron transfer, which involved in the synthesis of chlorophyll, oxidation-reduction reactions, electron transfer and respiration(Imsande 2010; Gong et al. 2015) and plays a very important role in plant life activities(Bashir et al. 2011). In long-term waterlogged paddy fields or acidic soil, plants may suffer from iron toxicity when its extremes met. The phenotypic symptoms of iron toxicity in different plants vary, but they all show obstruction of growth and development. High concentrations of iron can significantly inhibit the growth of the aboveground parts and root systems of rice, and even cut down the yield(Audebert and Fofana 2009; Samaranayake et al. 2012; Sanglard et al. 2019). The study of pea seedlings treated with excessive ferrous ions also showed that the high concentration of ferrous ions significantly inhibited the growth of pea seedlings and caused significant oxidative damage(Kannan 1981; Becker et al. 1998). With the increasing frequency of human activities, ferrous pollution in soil is becoming increasingly serious. The reduction in crop quality and safety caused by high concentrations of iron is increasingly concerning(Becker and Asch 2005; Samaranayake et al. 2012). However, the mechanism of the effect of iron on plants is unclear.

Terpenoids(Keeling and Bohlmann 2012), one class of plant secondary metabolites occurring through natural selection, constitute a large variety of natural products widely existing in plants and are generally distinguished into two groups: compounds with 10 carbons as the basic unit, such as geranyl

pyrophosphate, and compounds with 15 carbons as the basic unit, such as farnesyl pyrophosphate; these two subclasses of terpenoids are synthesized by the methyl erythritol phosphate pathway (MEP) and mevalonate pathway (MVA), respectively (McGarvey and Croteau 1995; Aharoni et al. 2003). These natural compounds are biosynthesized from different tissues and organs, such as leaves, roots, glandular trichomes and others (Takabayashi et al. 1994; Bartram et al. 2006; Kleine and Müller 2014), and can act as bodyguards of plants to effectively protect plants from herbivores, phytopathogenic fungi and bacteria (Cheng et al. 2007; Tholl 2015). Fortunately, terpenoids also have immeasurable value for human health, such as antimalarial and antineoplastic characteristics (Iranshahi et al. 2008; Tu 2016; Ma et al. 2017). Recently, based on the products induced by specific regulation, a study found that the pool of isopentenyl pyrophosphate (IPP)/isopentenyl phosphate (IP) and dimethylallyl pyrophosphate (DMAPP)/dimethylallyl phosphate (DMAP) regulated by the Nudix hydrolase family affects the balance of material flux linking the cytoplasm and plastid between the MVA and MEP pathways.

Nudix hydrolase is an enzyme superfamily that needs metal ions, exists in more than 250 species, including viruses, bacteria, archaea and eukaryotes, and catalyzes the hydrolysis of nucleoside diphosphate (McLennan 2006; Kraszewska 2008). Nudix enzymes play protective, regulatory and signaling roles in metabolism via hydrolyzing these compounds, include intact and oxidatively damaged nucleoside triphosphate, dinucleotide polyphosphate, nucleotide sugar, capped RNA and dinucleotide coenzyme (McLennan 2006). Subsequently, another status of Nudix hydrolase was exposed. Not long after the discovery of metabolic alternative to the classical mevalonate pathway (Dellas et al. 2013), AtNudix1 and AtNudix3 were proved to promote the allocation of intermediate product between MVA and MEP pathway in cytoplasm, by modulating the proportion of IPP/IP (Henry et al. 2018).

Since *C. blinii* normally grows in soils with a high iron content, it can be used as model specimen to study the mechanism of iron tolerance in plants. In this study, the blinin and saponin contents and the expression model of key genes of *C. blinii* were determined in two temporal experiments. Thus, our study explored the regulatory mechanism between the MVA and MEP metabolic pathways in *C. blinii* as a response to iron at the transcriptional and metabolic levels, which greatly deepened our understanding of the mechanism of iron tolerance in other plants.

2. Materials And Methods

2.1 Plant materials and ferrous iron treatment

The seeds of *C. blinii* were obtained from Hui Chen, Sichuan Agricultural University, China. Seeds germinated after disinfection in 2% sodium hypochlorite solution at $25\pm 2^{\circ}\text{C}$. After germination, the seedlings were transferred to 1/2 Hoagland's solution for further cultivation in a greenhouse, where the temperature was $26\pm 2^{\circ}\text{C}$ and the photoperiod was 16 h light/8 h dark. When the plants grew to two months old, they were treated with a ferrous iron solution.

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{EDTA} \cdot 2\text{Na}_2 \cdot \text{H}_2\text{O}$ were used to prepare ferrous iron solutions. For ferrous ion treatment, each pot of water-cultured *C. blinii* seedlings was cultured in 400 mL 1/2 Hoagland nutrient solution, and the corresponding amount of ferrous iron solution was added into each pot of nutrient solution so that the final concentrations were 50 μM , 100 μM , 200 μM and 400 μM . To avoid the effect of iron deficiency stress in the experiment, the normal 1/2 Hoagland nutrient solution was used as a blank treatment (0 μM) instead of the 1/2 Hoagland nutrient solution with ferrous ions deducted as a blank. The nutrient solution was replaced, and new ferrous iron solution was added every two days.

2.2 Preparation of standard curves of blinin and saponins

A total of 0.003 g (mass fraction $\geq 98\%$, CAS: 125675-09-4) of a blinin standard was accurately measured and dissolved in 30 mL of methanol. Then, standard solutions with concentrations of 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4 and 0.8 $\mu\text{g/mL}$ were prepared. The standard curve was made by HPLC analysis of the standard solutions. The HPLC conditions were as follows: the mobile phase was 40:45:15 methanol/water/acetonitrile; the flow rate was 1 mL/min; the detection wavelength was 210 nm; the injection volume was 10 μL ; and the column temperature was 25°C. The column was a Zorbax eclipse AAA column (4.6 mm $\times$ 150 mm, 3.5 μm). Finally, the standard curve was generated with the blinin concentration as the abscissa and absorption peak area as the ordinate. The stability, reproducibility, precision and recovery of the method were tested.

A total of 20 mg of an oleanolic acid standard was accurately weighed, a 50 mL 0.50 mg/mL oleanolic acid reference solution was prepared with methanol as the solvent. Then, 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mL of the oleanolic acid control solution was accurately pipetted into tubes with caps, and then 0.3 mL of a vanillin (5%) - glacial acetic acid solution and 0.8 mL of perchloric acid were added to each tube. The tubes were heated in a 65°C water bath for 25 min, removed, placed in cold water to stop the reaction, combined with 5 mL of glacial acetic acid, and then shaken. The absorption value of the solution was measured at 544 nm wavelength, and then the standard curve was generated with the mass (mg) as the abscissa and the absorbance (A) as the ordinate.

2.3 Extraction of blinin and HPLC analysis

The extraction of blinin from the whole leaves was carried out in methanol (Sun et al. 2018). First, leaves of *C. blinii* were collected and dried at a constant temperature of 60°C to a constant weight and then ground into powder. The extraction was carried out at a 1:40 dried powder:methanol ratio, the extraction temperature was 37°C, and the extraction was carried out overnight. An organic filter membrane was used to filter the extract, and the filtrate was put into a 2 mL liquid bottle and sealed at 4°C for storage. HPLC was used to detect and quantify the blinin content at a detection wavelength of 210 nm, column temperature of 25 °C, mobile phase of 40:45:15 methanol/water/acetonitrile, and flow rate of 1 mL/min. The retention time of blinin was approximately 6.9 min.

2.4 Extraction of saponins and content analysis

The leaves and stems of *C. blinii* before and after treatment with different concentrations of ferrous iron solution were washed and dried to a constant weight at 55 °C. After drying, the leaves and stems were ground into powder, weighed to 0.5 g, added to 10 mL of 60% ethanol, and extracted by ultrasonication at 60°C for 30 min. The sample was centrifuged at 12000 rpm for 5 min, and the supernatant was processed according to the abovementioned method. The absorbance value a was measured at 543 nm, and then analysis was conducted in triplicate. Compared with the standard curve, the content of total saponins in the sample solution treated with different concentrations of iron salt was determined.

2.5 RNA extraction and qRT-PCR analysis

All the leaves of samples after UV-B treatment were collected and immediately put into liquid nitrogen and stored at -80°C. Before RNA extraction, 0.1% RNase solid-phase scavenging reagent was sprayed onto the samples, or they were soaked in the reagent on an ultraclean bench; moreover, the grinding rod, disposable gloves and other items used for extraction were also extensively cleaned. RNA was extracted from the samples using the EASYspin plant RNA rapid extraction kit (Adlai Biological CO., LTD.).

cDNA was synthesized using reverse transcription kits. The PrimeScript™ RT reagent kit with gDNA eraser (perfect real time) kit was used for reverse transcription. The procedure was performed with a PCR reagent kit and divided into two steps according to the addition sequence. The first step was a reaction at 42°C for 5 min (2 µL of gDNA eraser, 4 µL of 5×gDNA eraser buffer, 10 µL of ddH₂O, and 4 µL of RNA); the second step was a reaction at 37°C for 15 min then at 85°C for 5 s. qRT-PCR was used to determine gene expression. The primers and total reaction system volume used in all the reactions are reported in our previous study (Sun et al. 2018). The reaction system volume was 15 µL: 7.5 µL of SYBR® Premix Ex Taq™ II (Tli RNaseH PI S), 0.5 µL of upstream and downstream primers, 1 µL of cDNA template, and 6 µL of ddH₂O. Triplicate assays were performed for each gene in each sample. The two-step real-time PCR method was adopted, and the real-time PCR program of each gene was roughly the same, with the exception of some differences in the annealing elongation temperature, all of which were predenatured at 95°C for 3 min. At the end of the reaction, all the samples were cooled to 65°C. After heating from 0.5°C to 95°C every 5 s, the samples were held for 5 s, and the fluorescence signals were collected for the fusion curve analysis. Finally, the $2^{-\Delta\Delta C}$ method was used to calculate the gene expression abundance and analysis (Livak and Schmittgen 2001).

2.6 Statistical analysis

CbNudixs were screened from the transcriptome database of *C. blinii* (PRJNA563166) reported in our previous study (Zheng et al. 2020). The amino acid sequence structure and conserved sequences comparison of Nudixs was performed TBtools (Chen et al. 2018a). And using GraphPad Prism 7 statistical software (GraphPad Prism Inc., USA), we carried out an analysis of variance for all data, and differences among treatments were analyzed by one-way analysis of variance (ANOVA) combined with Duncan's multiple range test. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1 Effects of different concentrations of a ferrous iron solution on the saponin content and expression of key genes in the saponin synthesis pathway of C. blinii

To study the effect of ferrous iron on the saponin synthesis of *C. blinii*, we treated *C. blinii* with different concentrations (50 μ M, 100 μ M, 200 μ M and 400 μ M) of a ferrous iron solution for 7 d. Then, saponins were extracted, and content analysis (Fig. 1A) showed that with increasing concentrations of the ferrous iron solution, the content of saponins decreased. This decrease plateaued when the ferrous iron solution concentration reached 200 μ M, with a total decline of 23%.

In terms of the expression of key enzyme genes in saponin synthesis, the result (Fig. 1B) was similar to the change in content presented above. The expression of *CbHMGR* and *CbFPPS* showed a trend of increasing first and then decreasing. Both of these genes reached their maximum values on four days after treatment, with an increase of 30%. On day seven, the expression of both genes began to decline, with a decrease of more than 70%. However, the expression of *CbSQS* and *CbSQE* showed a trend of decreasing the whole time, with the largest decrease of more than 80% on day seven.

3.2 Effects of different concentrations of a ferrous iron solution on the blinin content and expression of key genes in the blinin synthesis pathway of C. blinii

To study the effect of ferrous iron on the blinin synthesis of *C. blinii*, we treated *C. blinii* with the same abovementioned concentrations of a ferrous iron solution for 7 d. Then, blinin was extracted, and content analysis (Fig. 2A) showed that with increasing ferrous iron solution concentration, the content of blinin showed an increasing trend. This increasing trend also reached a stable level when the ferrous iron solution concentration reached 200 μ M, with an increase of 166%.

In terms of the expression of key enzyme genes in blinin synthesis, the results (Fig. 2B) showed that the expression of *CbDXS* and *CbDXR* first increased and then decreased. Both of these genes reached their maximum values four days after treatment, with an increase of 200%. On day seven, the expression of both of these genes began to decline. Similarly, the expression of *CbGGPPS* and *CbCPS* showed a trend of increasing until day four, with the largest increase of more than 800% on day four, and then decreasing thereafter.

3.3 Effect of short-term ferrous iron exposure on the saponin content and expression of key genes in the saponin synthesis pathway of C. blinii

Short-term ferrous iron exposure was carried out on *C. blinii*, and the change in saponin content was determined. The results (Fig. 3A) showed that the content of saponins gradually increased within 5 h.

At the same time, we also found that the expression of key enzyme genes in the saponin synthesis pathway (Fig. 3B) showed different trends within 3 h after treatment. The expression of *CbHMGR* and *CbFPPS* showed a trend of first decreasing and then increasing, with the largest reduction reaching more

than 90% after 1 h of treatment, while the expression of *CbSQS* and *CbSQE* showed a trend of increasing, with the highest increase reaching approximately 150%.

3.4 Effect of short-term ferrous iron exposure on the blinin content and expression of key genes in blinin synthesis pathway of *C. blinii*

The change in blinin content was also detected in the abovementioned short-term iron exposure experiment. The results (Fig. 4A) showed that the content of blinin decreased within the first 3 hours of ferrous iron exposure and then increased gradually.

At the same time, we also found that the expression of key enzyme genes in the blinin synthesis pathway (Fig. 4B) showed different trends within the first 3 hours of exposure. The expression of *CbDXS* and *CbDXR* showed a trend of first decreasing and then increasing, with the largest reduction reaching more than 90% after 1 h of treatment, while the expression of *CbGGPPS* and *CbCPS* showed a trend of rising, with the highest increase reaching approximately 300%.

3.5 Effect of ferrous iron solution on the expression of *CbNudixs*

The expression of *CbNudixs* in *C. blinii* treated with different processing times of ferrous iron solution was also detected. All *CbNudixs* to be tested, including *CbNudix29* (Cluster-16989.29545), *CbNudix30086* (Cluster-30086.0) and *CbNudix6371* (Cluster-16989.6371; orf1, orf2 and orf3), were all screened from the transcriptome database of *C. blinii* (SRR10053375, SRR10053374 and SRR10053373) compared with the Nudix family from *Artemisia annua*, *Arabidopsis thaliana*, *Helianthus annuus* and *Lactuca sativa*. *CbNudix29* and *CbNudix30086* have the same motifs as *AtNudix1* (NP_177044.1). *CbNudix6371* seems to be made up of 3 fragments according to the motif distribution of *AtNudt3* (NP_001320544.1) (Fig. 5A and 5B).

After 7 d of treatment with a 200 μ M ferrous iron solution, the expression of *CbNudix29* in all three *CbNudix* genes increased, with a maximum increase of 5 times, while the other two genes showed stable expression or even a slightly declining trend (Fig. 5C). After 3 hours of 200 μ M ferrous iron solution exposure, the expression of all three *CbNudix* genes showed the same downward trend, with the highest decrease being 10-fold (Fig. 5D).

3.6 Correlation analysis between *CbNudixs* expression, saponin synthesis and blinin synthesis

Correlation analysis of the saponin content, blinin content and expression of all three *CbNudix* genes under long-term and short-term ferrous iron exposure was carried out. As shown in Fig. 6, the color of the block is approximately red, indicating a more positive correlation between the two experiments. The bluer the color of the plot is, the more negative the correlation between the two exposure experiments, and the number on the block represents the specific value of the correlation between the two. As shown in Fig. 6A, it was found that after short-term iron exposure, there was no correlation between the expression of *CbNudix30086*, *CbNudix6371*, and *CbNudix29* and the blinin content, but the gene expression showed a negative correlation with saponin content. Conversely, the content of saponins and blinin was correlated

with the expression of *CbNudix29*, *CbNudix30086* and *CbNudix6371* under long-term iron exposure, as shown in Fig. 6B. The correlation between the expression of these three genes and the blinin and saponin contents showed a completely opposite phenomenon. There was a strongly negative correlation between the expression of *CbNudix29* and the saponin content but a strongly positive correlation with its expression and the blinin content. In contrast, there was a positive correlation between the expression of *CbNudix30086* and *CbNudix6371* and the saponin content but a positive correlation with their expression and the blinin content. The changes in saponin and blinin contents presented a strong negative correlation with each other under long-term iron exposure, and the Pearson index even reached -0.979.

4. Discussion

Many previous studies found that biotic and abiotic factors both enhance and inhibit the synthesis and accumulation of certain terpenoids in plants, contributing to specific ecological function differentiation (Arimura et al. 2004; Cheng et al. 2007). For example, herbivore-induced, pathogenic microbial infection or ultraviolet irradiation produces a specific profile of terpenoids that function as phytoalexins in plant defense and environmental adaptation.

In this study, we found that the contents of blinin and saponins, the best-known terpene metabolites of *C. blinii*, responded differently and opposite to each other to iron exposure at different concentrations. Surprisingly, the content of *C. blinii* saponins decreased with increasing iron concentration, indicating that the presence of iron ions inhibited the accumulation of *C. blinii* saponins in leaves. In contrast, the blinin content showed a strong oscillation, and its maximum variation was nearly 2.5 times that at the beginning of the experiment between the iron concentrations of 50 μM and 400 μM ; however, during exposure to 400 μM iron, the blinin content significantly decreased to the level of the control group. It was revealed that the stimulation of the appropriate concentration of iron ions contributed greatly to the accumulation of blinin in the leaves of *C. blinii*, but the stimulation of excessive iron ions caused tissue damage in *C. blinii*. In the absence of clear degradation pathways, transport and secretion mechanisms and natural effects of terpene metabolites stimulated by iron ions, this study focuses on the regulation and changes in terpene metabolic pathways.

4.1 Effects of ferrous iron on the synthesis of blinin and saponins in *C. blinii* presented a time-dependent manner

To further explore whether ferrous iron had a close regulatory relationship with the synthesis and accumulation of terpenoids, we carried out long- and short-term gradient exposure experiments. After long- and short-term exposure, we found a very subtle regulatory relationship. In the long-term exposure experiment, the contents of *C. blinii* saponins and blinin showed completely opposite trends, with the content of the former decreasing and the content of the latter increasing (Fig. 1A and Fig. 2A). During the short-term exposure experiment (Fig. 3A and Fig. 4A), the *C. blinii* saponin content did not decrease but increased rapidly, while the blinin content decreased first and then increased. According to the contrast between the long- and short-term exposure results, it can be speculated that a transport or degradation

mechanism in the downstream metabolic or transport pathways of saponin and blinin exists in *C. blinii* leaves, similar to herbivore-induced terpenoid emission (Takabayashi et al. 1994; Chen et al. 2003; Mithöfer et al. 2005), and is regulated by the ferrous iron signaling pathway, and such regulation is time-dependent to some extent.

This time dependence was also reflected in the variation in the expression levels of key enzyme genes in the MVA and MEP metabolic pathways (Fig. 1B, Fig. 2B, Fig. 3B and Fig. 4B). From the change in gene expression and correlation analysis, we found that under short-term iron exposure, the correlation between the key enzyme genes in the MVA and MEP metabolic pathways and the accumulation of final metabolites was consistent with the gene expression pattern. Blinin content accumulation was positively correlated with the expression patterns of key enzymes in MEP metabolic pathways (*CbDXS*, *CbDXR*, *CbGGPPS* and *CbCPS*), while saponin content accumulation was negatively correlated with the expression patterns of *CbHMGR* and *CbFPPS* and negatively correlated with the expression patterns of *CbSQS* and *CbSQE*. When the plants were subjected to long-term iron exposure, the correlation reversed dramatically. The correlation between blinin content accumulation and the expression patterns of key enzymes in the MEP metabolic pathway changed from a positive correlation to a negative correlation, while the correlation between saponin content accumulation and the expression patterns of *CbHMGR* and *CbFPPS* changed from a negative correlation to a positive correlation. The positive and negative correlation conversion between gene expression pattern and terpene accumulation also appeared in the correlation between *CbNudix29* expression pattern and blinin accumulation. Not surprisingly, the accumulation of saponins maintained a negative correlation with the *CbNudix29* expression pattern in both the long- and short-term iron exposure experiments.

In other words, the accumulation of metabolic intermediates IPP/IP is directly related to the synthesis and accumulation of metabolic end products, while the downstream direction of IPP/IP is regulated by the expression of *CbNudix29*. Since, Nudix catalyzed the dephosphorylation of isoprenoid diphosphate to drag down MVA metabolism (Henry et al. 2018).

4.2 A possible age-appropriate IPP/IP pool resulted in changes in the diterpene and triterpene metabolic flows of C. blinii under iron treatment

In the past decade, without considering the time scale, a dynamic material flux allocation in IPP/IP both in the cytosol and plastid was confirmed to remove the strict separation between the MVA and MEP pathways by suppressing the two metabolic pathways individually with chemical inhibitors (Bick and Lange 2003; Laule et al. 2003; Bartram et al. 2006). In addition, research on the effect of IP on terpenoid metabolism in *Arabidopsis thaliana* revealed that AtNudix contributes to managing the dynamic equilibrium (Henry et al. 2015, 2018).

In this context, the gene expression levels of the MVA and MEP pathways increased and decreased in the different exposure experiments, which can be understood as follows: while the scale of the age-appropriate IPP/IP pool is determined by the upstream rate-limiting enzyme, Nudixs are deployed as a dispatcher to control the material flux via the expeditious allocation of IPP/IP. When *C. blinii* is stimulated

by iron for the first time, an age-appropriate IPP/IP pool exists in the body of *C. blinii* and is sufficient for the synthesis of downstream and final products. Therefore, the expression of *CbSQS* and *CbSQE* in the downstream saponin metabolic pathway is upregulated, and the content of saponins increases rapidly, while the metabolic pathway of blinin is inhibited. As a result, an expenditure gap occurs in the age-appropriate IPP/IP pool under the continuous stimulation of iron. Subsequently, the upstream rate-limiting enzyme genes (*CbHMGR*, *CbDXS* and *CbDXR*) in both the MVA and MEP pathways are upregulated, and the age-appropriate IPP/IP pool can be restored quickly to a sustainable resource supply. However, how *C. blinii* controls the material flux direction of the IPP/IP pool after the first exposure to iron salt is unknown.

As *CbNudixs* were severely inhibited during the short-term iron exposure experiment, IPP was favored to produce a higher material proportion than of IP, and combined with *CbSQS* and *CbSQE*, IPP led to the dominance of *C. blinii* saponin synthesis. Under continuous iron stimulation (long-term exposure), adaptability was obtained through saponin transport or degradation. *C. blinii* needed to restore, or even enhance, the metabolic accumulation of blinin. Therefore, when the IPP/IP pool reached the appropriate age again, the expression of *CbNudixs* increased, and combined with the upregulated expression of *CbGGPPS* and *CbCPS*, blinin synthesis enhancement and continuous accumulation were observed (Fig. 7). In general, it is speculated that the migration of IPP and IP follows a certain cellular rule, that is, the transport of IPP and IP on chloroplast membrane is unidirectional, from the inside out and from the outside in, respectively. The transporters on the chloroplast membrane could be the expressway, and IPP must get off the expressway while it would like to turn around, be dephosphorylated by Nudixs, and then enter the chloroplast in the form of IP.

5. Conclusion

In this study, it was found that under the stimulation of ferrous iron, the content of terpenoids in *C. blinii* was different, and the metabolic pathways of MVA and MEP were periodically regulated. On the one hand, ferrous iron enhanced the synthesis and accumulation of the diterpene blinin and inhibited the synthesis and accumulation of saponins. On the other hand, under continuous ferrous iron stimulation for a short period and a long period, *C. blinii* regulated the dynamic balance of terpenoid levels by maintaining an age-appropriate IPP/IP pool and transforming the material flux direction in the metabolic hub controlled by changes in *Nudix* expression. In this study, the influence of ferrous iron exposure on the terpene synthesis of *C. blinii* was discussed, and the possible relationship between an age-appropriate IPP/IP pool and *Nudix* gene expression model was highlighted as the key factor for regulation of terpene metabolism pathways, providing a guidance and theoretical basis for artificial selection and cultivation of medicinal plants with terpenes as the main medicinal ingredients.

Declarations

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Author's contributions

J.-Y.Z and T.-R.Z designed research; J.-Y.Z, T.-R.Z, W.-J.S and M.-J.W performed research; J.-Y.Z and T.-R.Z analyzed data; J.-Y.Z and T.-R.Z wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Effects of different concentrations of a ferrous iron solution on the saponin content and expression of key genes in the saponin synthesis pathway of *C. blinii*. *C. blinii* was treated with ferrous iron for 7 days, and then the saponin content (A) and the expression levels of four key enzymes in the saponin synthesis pathway (B) were detected. The CK group did not receive ferrous solution treatment. The values are the means $\pm$ SDs ($n = 3$).

Figure 2

Effects of different concentrations of a ferrous iron solution on the blinin content and expression of key genes in the blinin synthesis pathway of *C. blinii*. *C. blinii* was treated with ferrous iron for 7 days, and then the blinin content (A) and the expression levels of five key enzymes in the blinin synthesis pathway (B) were detected. The CK group did not receive ferrous solution treatment. The values are the means $\pm$ SDs ($n = 3$).

Figure 3

Effects of short-term ferrous iron solution exposure on the saponin content and expression of key genes in the saponin synthesis pathway of *C. blinii*. *C. blinii* was treated with 200 μ M ferrous iron for 5 h, and then the saponin content (A) and the expression levels of four key enzymes in the saponin synthesis pathway (B) were detected. Considering the time required from gene expression to product synthesis, the

time point selected for determination of saponin content was slightly later than that selected for determination of gene expression. The values are the means $\pm$ SDs (n = 3).

Figure 4

Effects of short-term ferrous iron exposure on the blinin content and expression of key genes in blinin synthesis pathway of *C. blinii*. *C. blinii*. was treated with 200 μ M ferrous iron for 5 h, and then the blinin content (A) and the expression levels of five key enzymes in the blinin synthesis pathway (B) were

detected. Considering the time required from gene expression to product synthesis, the time point selected for determination of blinin content was slightly later than that selected for determination of gene expression. The values are the means $\pm$ SDs (n = 3).

Figure 5

Effects of ferrous iron solution on the expression of CbNudixs. All CbNudixs to be tested, including CbNudix29 (Cluster-16989.29545), CbNudix30086 (Cluster-30086.0) and CbNudix6371 (Cluster-16989.6371; orf1, orf2 and orf3), were all screened from the transcriptome database of *C. blinii*. The left side of Fig. 5A and Fig. 5B is the amino acid sequence structure comparison result of screened CbNudix

of *C. blinii* and Nudix of other species identification. Each color block represents a structural motif. The right side of Fig. 5A and Fig. 5B is the conserved sequences comparison between the CbNudix screened and the Nudix of other species. Fig. 5C shows the effects of long-term ferrous iron solution exposure on the expression of CbNudixs. Fig. 5D shows the effects of short-term ferrous iron solution exposure on the expression of CbNudixs. The values are the means $\pm$ SDs (n = 3).

Figure 6

Correlation analysis between CbNudixs expression, saponin synthesis and blinin synthesis. Fig. 6A shows the correlation analysis for short-term ferrous iron exposure, and Fig. 6B shows the correlation analysis for long-term ferrous iron treatment. The color of the block is approximately red, indicating a more positive correlation between the two exposure times. The bluer the color of the block is, the more negative the correlation between the two exposure times, and the number on the block represents the specific value of the correlation between the two experiments.

Figure 7

Mechanism of metabolic flow between the diterpene blinin and the triterpenes saponins in *C. blinii* under different ferrous treatment periods.