

Heterologous biosynthesis of isobavachalcone in tobacco based on in planta screening of prenyltransferases

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Abstract

Isobavachalcone (IBC) is a prenylated chalcone mainly distributed in some Fabaceae and Moraceae species. IBC exhibits a wide range of pharmacological properties, including anti-bacterial, anti-viral, anti-inflammatory, and anti-cancer activities. In this study, we attempted to construct the heterologous biosynthesis pathway of IBC in tobacco (*Nicotiana tabacum*). Four previously reported prenyltransferases, including GuLDT from *Glycyrrhiza uralensis* , HIPT1 from *Humulus lupulus* , and SfILDT and SfFPT from *Sophora flavescens* , were subjected to an in planta screening to verify their activities for the biosynthesis of IBC, by using tobacco transient expression with exogenous isoliquiritigenin as the substrate. Only SfFPT and HIPT1 could convert isoliquiritigenin to IBC, and the activity of SfFPT was higher than that of HIPT1. By co-expression of GmCHS8 and GmCHR5 from *Glycine max*, endogenous isoliquiritigenin was generated in tobacco leaves (21.0 mg/g dry weight). After transformation with a multigene vector carrying GmCHS8 , GmCHR5 , and SfFPT , *de novo* biosynthesis of IBC was achieved in tobacco calluses (0.46 mg/g dry weight). The yield of IBC in calluses was nearly equal to that in SfFPT transient expression experiments, in which substrate supplement was sufficient, indicating that low IBC yield was caused by the low activity of SfFPT. Our research provided a prospect to produce valuable prenylflavonoids using plant-based metabolic engineering.

Key Message

In planta screening showed that SfFPT and HIPT1 could convert isoliquiritigenin to isobavachalcone, and *de novo* biosynthesis of isobavachalcone was achieved in tobacco calluses by introducing *GmCHS8*, *GmCHR5*, and *SfFPT*.

Introduction

Prenylflavonoids are a subclass of naturally occurring flavonoids that contain at least one lipophilic prenylated side-chain in the flavonoid skeleton. They are mostly found in a small number of families, including Fabaceae, Moraceae, Cannabaceae, Clusiaceae, Apiaceae, and Euphorbiaceae (Awouafack et al. 2020; Yang et al. 2015). Prenylflavonoids act as phytoalexins and defend plants against infections through their antibacterial activities (Ahuja et al. 2012). They are also bioactive substances that have a variety of biological effects, such as estrogenic (An et al. 2016; Stulikova et al. 2018), antioxidant (Botta et al. 2005), anti-cancer ((Saito et al. 2018; Sastre-Serra et al. 2019), and antiviral (Grienke et al. 2016). The addition of various prenyl groups to the aromatic ring of flavonoids not only gives rise to the structural diversity, but also significantly increases their biological activity and bioavailability in comparison to their non-prenyl flavonoid parent compounds. Such enhancement may be caused by the presence of a lipophilic prenyl side-chain (Kretzschmar et al. 2010; Zhao et al. 2003). It is thought that the prenyl side-chain improves the membrane permeability of prenylated flavonoids, which subsequently provides enhanced biological activities (Botta et al. 2005; Levisson et al. 2019; Mukai 2018).

Prenylflavonoids often exist at trace levels and are distributed in limited species (Gomes et al. 2022; Yang et al. 2015). Isobavachalcone (IBC), a prenylated chalcone at the 3' position, is mainly isolated from several species belonging to the Fabaceae and Moraceae including the traditional Chinese medicinal materials *Psoralea corylifolia* as one of the major active components (Chen et al. 2012; Gao et al. 2016; Xing et al. 2022). According to reports, this prenylated chalcone exhibits a wide range of physiological and pharmacological properties, including antioxidant, anti-inflammatory, anti-rheumatoid arthritis, anticancer, attenuate hepatocyte injury, and neuroprotective properties (Dzoyem et al. 2017; Li et al. 2019; Song et al. 2021; Wang et al. 2022; Zhou et al. 2019). In *P. corylifolia*, IBC is only synthesized in its seeds at low concentration (Tsai et al. 1996). Due to regioselectivity, stereoselectivity, and laborious chemical reaction steps, the chemical synthesis of prenylflavonoids is subjected to various restrictions, which have hampered its production at scale and commercial application (Gester et al. 2001; Grealis et al. 2013; Nemoto et al. 2012; Tischer and Metz 2007).

Based on the structure, isoliquiritigenin is thought to be the biosynthetic precursor of IBC (Li et al. 2018). The biosynthesis of isoliquiritigenin is derived from the phenylpropanoid pathway (Fig. 1). Phenylalanine is converted to naringenin chalcone (2',4,4',6'-tetrahydroxychalcone) by the successive catalysis of phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate: coenzyme A ligase (4CL), and chalcone synthase (CHS) (Yi et al. 2010). Naringenin chalcone is then isomerized to naringin either spontaneously or via chalcone isomerase (CHI) (Ralston et al. 2005). In the Fabaceae family, CHS-catalyzed condensation reaction is coupled with chalcone reductase (CHR)-catalyzed reduction reaction to generate isoliquiritigenin (2',4,4'-trihydroxychalcone) (Mameda et al. 2018). Although no specific prenyltransferase catalyzing the biosynthesis of IBC has been characterized from *P. corylifolia*, several chalcone- and flavanone-specific prenyltransferases have been identified from some legume and nonlegume species. CtIDT from *Cudrania tricuspidata*, MaIDT from *Morus alba* (Wang et al. 2014), GuLDT from *Glycyrrhiza uralensis* (Li et al. 2018), and SfILDt from *Sophora flavescens* (Sasaki et al. 2011) can recognize isoliquiritigenin as a substrate; CtIDT, MaIDT, and GuLDT catalysis the prenylation of isoliquiritigenin at C-3' position to generate IBC, while SfILDt prenylates isoliquiritigenin at C-5' position. On the other hand, HIPT1 from *Humulus lupulus* recognizes naringenin chalcone as a substrate to form desmethylxanthohumol (Tsurumaru et al. 2012). In addition, SfFPT (Chen et al. 2013) and SfN8DT (Sasaki et al. 2008) from *S. flavescens* exhibit substrate specificity towards liquiritigenin, which is the isomerization product of isoliquiritigenin.

In this study, we supposed that it would be possible to construct the biosynthesis pathway of IBC in a plant chassis using the previously characterized prenyltransferases. An *in planta* screening of prenyltransferases using tobacco (*Nicotiana tabacum*) transient expression system revealed that SfFPT and HIPT1 could convert exogenous isoliquiritigenin to IBC. By co-expression of *GmCHS8* and *GmCHR5* (Mameda et al. 2018), isoliquiritigenin was synthesized in tobacco as the endogenous substrate. Finally, the *de novo* biosynthesis of IBC was achieved in tobacco (0.46 µg/g DW) by introducing a multigene expression vector containing *GmCHS8*, *GmCHR5*, and *SfFPT* genes. Our study provided a potential source of this valuable natural compound.

Materials And Methods

Plant materials and chemicals

After sterilization with 70% ethanol, tobacco (*Nicotiana tabacum*) seeds were sown on soil made up of a 3:1:1 combination of peat moss, perlite, and vermiculite (v/v/v). A fluorescent light cycle of 16/8 hours and a temperature of 26°C were used to cultivate seedlings. Seeds of soybean (*Glycine max*), *Sophora flavescens*, *Glycyrrhiza uralensis*, and *Humulus lupulus* were cultivated under the same condition. The standard compounds isobavachalcone (IBC) (CAS: 20784-50-3) and isoliquiritigenin (CAS: 961-29-5) were purchased from ShanghaiyuanyeBio-Technology Co., Ltd. (Shanghai, China).

Nucleic acid extraction and cDNA synthesis

Six-week-old plants of soybean, *S. flavescens*, *G. uralensis*, and *H. lupulus* were treated with 1% yeast extract for 24 h. Roots and leaves were collected in 2 mL tubes after being frozen in liquid nitrogen, and stored at -80°C until use. A 5 mm glass bead was placed within each tube. An automated grinder (Jingxin, Shanghai, China) was used to homogenize the liquid nitrogen cooling samples. Total RNA was isolated from samples less than 80 mg by using an EasyPure Plant RNA Kit (TransGen Biotech, Beijing, China) following the standard protocol. cDNA was synthesized from total RNA using a RiverTra Ace qPCR RT Master Mix and the gDNA Remover Kit (Toyobo, Tokyo, Japan). cDNA was diluted to 1/10 with nucleic acid-free water.

Gene cloning and plasmid construction

cDNA isolated from the soybean, *S. flavescens*, *G. uralensis*, and *H. lupulus* was used as templates for the cloning of genes. The coding sequence of *GmCHS8* (AY237728), *GmCHR5* (LC309095), *SfFPT* (KC513505), *SfILDT* (AB604223), *GuILDT* (KR139751), and *HIPT1* (AB543053) were amplified using a KOD plus DNA polymerase (Toyobo, Tokyo, Japan). *attB1* and *attB2* sequence was attached to 5' sites of forward and reverse primers, respectively. A recognition site for the *BsaI* restriction enzyme on *GmCHS8* was changed by point mutation using PCR. Primers for mutation were designed based on the degenerate codons. The PCR amplified products were subsequently ligated using a pEASY-Basic Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China). The sequence of primers was listed in Table S1. Amplified products were analyzed on a 1.2% agarose gel with 1×TAE and run at 100 V. The gel was stained with GelGreen (Biotium, CA, USA), and the bands were cut out under blue light. The bands were purified with an AxyPrep DNA Gel Extraction Kit (Axygen, CA, USA). Each gene was introduced into a pDONR222 vector by the Gateway BP reaction (Invitrogen, CA, USA), and then introduced into a pGWB402W vector (Nakagawa et al. 2007) by the Gateway LR reaction (Invitrogen, CA, USA), generating six single gene expression vectors, i.e., pGWB402W-GmCHS8, pGWB402W-GmCHR5, pGWB402W-SfFPT, pGWB402W-SfILDT, pGWB402W-GuILDT, and pGWB402W-HIPT1. The multigene expression vector was constructed based on the Golden Gate assembly strategy (Bell and Molloy 2022; Engler et al. 2008) using a T4 DNA Ligase (New England Biolabs, CA, USA) and the restriction enzyme *BsaI*-HF v2 (New England Biolabs, CA, USA). The region spanning the promoter-CDs-terminator of each gene in pGWB402W vector

was amplified. By using the Golden Gate assembly, these amplicons were connected with a *nptII* gene conferring kanamycin resistance plants, an *Sm^R* gene conferring spectinomycin resistance to bacteria, and *Ori* and *OriV* replicons to construct the multigene expression vector. This vector was named pGK-IBC. Golden Gate Assembly Tool (<https://goldengate.neb.com/>) was used to design the overhanging sequences. The coding sequence of *AtMYB28* (NM_125535) from *Arabidopsis thaliana* was cloned using primers flanked with *KpnI* restriction enzyme sites. The amplicon was subcloned into the pGWB402Ω vector, and *AtMYB28* was removed by enzymatic cleavage to generate an empty vector.

Transient expression

The single gene expression vector pGWB402W-SfFPT, pGWB402W-SfILDT, pGWB402W-GuILDT, and pGWB402W-HIPT1, as well as multigene expression vector pGK-IBC were transformed into *Agrobacterium tumefaciens* strain GV3101. After validation of transformation by colony PCR, the bacteria were grown in a liquid LB medium for 24 hours at 28°C. The suspension was discarded after the bacteria were separated by centrifugation for 10 min at 4000 g at 4°C. The pellets were gently resuspended to an OD600 of 1.5 in sterilized water containing 0.2 mM acetosyringone, 10 mM MgCl₂, and 10 mM MES (pH = 5.8). The suspension was shaken at 120 rpm for two hours in dark at room temperature. Tobacco leaves that were six weeks old and fully expanded were employed for agroinfiltration. Using a 1 ml needleless syringe, *Agrobacterium* suspension was injected into the whole leaf area through stomas from the abaxial side of the leaves. Plants were cultivated after agroinfiltration for 24 hours in the dark, and then transferred to the normal condition. For isoliquiritigenin supplement, agroinfiltrated tobacco leaves were injected with 100 mM isoliquiritigenin five days after agroinfiltration. After cultivation under normal conditions for 24 h, injected leaves were sampled. Tobacco leaves infected with *Agrobacterium* carrying an empty vector served as a negative control.

Reverse transcription-PCR

GmCHS8, *GmCHR5*, and *SfFPT* genes were amplified by using KOD plus DNA polymerase (Toyobo, Tokyo, Japan). Diluted cDNA from tobacco leaves agroinfiltrated with pGK-IBC was used as templates, and that with the empty vector was used as negative control. Primers for cloning were used for RT-PCR. *ACTIN1* gene from tobacco (*NtACTIN1*, AB158612) was used as an endogenous reference. PCR amplification was carried out using the following thermal cycle conditions: initial denaturation at 94°C for 2min; 30 cycles of denaturation at 98°C for 10s, annealing at 60°C for 30s, and extension at 68°C for 1min. Amplicons were analyzed on a 1.2% agarose gel with 1×TAE and run at 100 V, the gel was stained with GelRed (Biotium, CA, USA).

Flavonoids extraction and analysis

Freeze-dried tobacco leaves were ground into powder. Approximately 500 mg dry powder was extracted with 80% methanol (v/v) by ultrasonic for 30min. After methanol was evaporated under nitrogen gas, the residue was re-dissolved in 2 ml 80% methanol and analyzed using ultra-performance liquid

chromatography-quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF). For each analysis, five biological replicates were applied.

A 3 ml injection of each sample was made onto ACQUITY UPLC C18 1.7 mm (2.1 × 50 mm) Column (Waters, USA) and analyzed by liquid chromatography-electrospray ionization tandem mass spectrometry (LC/ESI/MS) using an Agilent 1290 UPLC at 254 nm coupled with an Agilent Q-TOF 6560 mass spectrometer system. Data acquisition was performed in positive ion mode with a scan range of 20-750 Da. ESI source parameters were set as following: capillary voltage, 3500 V; drying gas temperature, 225°C; drying gas flow rate, 5 L/min; sheath gas temperature, 400°C; sheath gas flow rate, 12 L/min; nebulizer pressure, 20 psi; nozzle voltage, 500 V; data acquisition frequency, 1 spectrum/s. MS/MS spectra were used to obtain fragments and identify compounds. The solvents were acetonitrile (solvent A) and water containing 0.1% formic acid (solvent B) at the flow rate of 0.3 ml/min. The gradient of the solvent was: 0–5min, 3%–10% solvent A; 5–20 min, 10%–70% solvent A; 20–27 min, 70%–90% solvent A; 27–30min, 97% solvent A.

Results

In planta screening of prenyltransferase for the biosynthesis of isobavachalcone

Four prenyltransferases exhibiting the substrate specificity towards isoliquiritigenin and liquiritigenin were subjected to an *in planta* screening for the biosynthesis of isobavachalcone (IBC) by transient expression, including *GuILD*T, *HIPT*1, *SfILD*T, and *SfFPT*. Tobacco was selected as the chassis of transient expression, because there is no endogenous isoflavonoid or flavonoid/chalcone-specific prenyltransferase in tobacco. Four single expression vectors carrying each prenyltransferase gene were constructed, in which the CDs was flanked with an enhanced cauliflower mosaic virus (CaMV) 35S promoter and a *NOS* terminator. These vectors were independently delivered into *Agrobacterium* GV3101, and transformed into tobacco leaves by agroinfiltration. Aqueous solution of isoliquiritigenin (100 mM) was supplied by injection five days after agroinfiltration to serve as an exogenous substrate. IBC was then detected by UPLC-Q-TOF.

The sample of IBC standard compound showed a peak with the retention time of 13.0 min (Fig. 2a). Although the previous *in vitro* assay demonstrated that *GuILD*T catalyzed the formation of IBC from isoliquiritigenin (Li et al. 2018), our *in planta* experiment showed that *GuILD*T failed to produce IBC by transient expression in tobacco leaves when isoliquiritigenin was supplied as the substrate (Fig. 2b; Table 1). The same result was observed when *SfILD*T was agroinfiltrated (Fig. 2c; Table 1). By contrast, when *SfFPT* or *HIPT*1 was applied to transient expression, a new peak with the same retention time as IBC standard was observed (Fig. 2d, e; Table1). This peak was verified by UPLC-Q-TOF analysis in the electrospray ionization (ESI)-positive ion mode. The main fragments of a molecular ion peak with a mass-to-charge ratio (m/z) of 325.1420 $[M+H]^+$ are m/z 269.0812 $[M+H]^+$, m/z 205.0842 $[M+H]^+$, and m/z 149.0265 $[M+H]^+$, which was consistence with the IBC standard (Fig. 2g–i). By contrast, when an empty vector was transiently expressed, or a prenyltransferase gene was expressed without isoliquiritigenin

supplement, this peak was not detected (Fig. 2f; Table 1). These results suggested that SfFPT and HIPT1 were able to convert exogenous isoliquiritigenin to IBC in the transient expression system. Quantitative analysis revealed that the concentration of IBC in *SfFPT*-infiltrated samples was significantly higher than that in *HIPT1*-infiltrated samples, indicating stronger catalytic activity of SfFPT (Table 1).

Re-construction of isoliquiritigenin biosynthesis pathway in tobacco

Compared with the heterologous biosynthesis of natural compounds in microorganisms, it is unfeasible to supply exogenous substrates when plants are applied for the large-scale production of natural compounds under field conditions. To achieve the heterologous biosynthesis of IBC in tobacco, endogenous biosynthesis of isoliquiritigenin is necessary. The presence of isoliquiritigenin is resulted from the combined enzymatic activities of CHS and CHR. CHR is mainly distributed in legume plants. Therefore, *GmCHR5* was selected for the generation of isoliquiritigenin in tobacco, and *GmCHS8* was also utilized to enhance the metabolic fluxes towards chalcone pathway.

Two single gene expression vectors carrying *GmCHS8* and *GmCHR5* were constructed and co-expressed by transient expression in tobacco leaves as mentioned above. The samples were subjected to UPLC-Q-TOF analysis. The presence of chromatographic peaks with an identical retention time as the isoliquiritigenin standard was observed (Fig. 3a, B). QTOF-MS/MS analysis revealed that the sample showed MS ion of m/z 257.0801 $[M+H]^+$ and fragments of m/z 137.0233 $[M+H]^+$, which was identical as the isoliquiritigenin standard (Fig. 3d–f). This result indicated that the heterologous biosynthesis of isoliquiritigenin in tobacco was achieved by introducing *GmCHS8* and *GmCHR5*. Quantitative analysis showed that the samples generated isoliquiritigenin with concentrations around 21.0 mg/g.

Construction of multigene expression vectors

To establish the genetic transformation of tobacco producing IBC, a multigene expression vector carrying three selected genes, i.e., *GmCHS8*, *GmCHR5*, and *SfFPT*, was constructed. One recognition site of *BsaI* restriction enzyme on *GmCHS8* was changed through point mutation without changing the amino acid sequence. Then the promoter-CDs-terminator cassette of each gene was amplified from its single gene expression vector. The sequence of recognition and cleavage sites of *BsaI* restriction enzyme was added in the primers. All cassettes were assembled into an pPZP vector backbone (Hajdukiewicz et al. 1994) based on Golden Gate assembly protocol (Engler et al. 2008). In the resulting multigene expression vector (named as pGK-IBC), three genes were tandemly arranged, and their expression was independently control by their own promoter and terminator (Fig. 4a). The pGK-IBC vector was transformed into *Agrobacterium* GV3101. After agroinfiltration into tobacco leaves, the expression of three genes was validated by RT-PCR (Fig. 4b).

De novo biosynthesis of IBC in transgenic tobacco

Finally, we engineered IBC-producing tobacco plants. Genetic transformation of tobacco was carried out by infection with *Agrobacterium* carrying the pGK-IBC or empty vector. After induction, the transgenic

calluses (Fig. S1) were subjected to UPLC-Q-TOF analysis. The appearance of IBC was detected by peaks with the identical retention time of IBC standard (Fig. 5a–c), confirmed by mass spectra with the MS ion of m/z 325.1432 $[M+H]^+$, fragments of m/z 269.0810 $[M+H]^+$, m/z 205.0878 $[M+H]^+$, and m/z 149.0233 $[M+H]^+$ (Fig. S2). The concentration of IBC was 0.46 mg/g (DW). The presence of isoliquiritigenin in transgenic calluses was also detected (Fig. 5d–f) with the concentration of 12.3 mg/g (DW). It is noticeable that the contents of IBC generated in transgenic calluses was relative to that in the *SfFPT* transient expression experiment, in which sufficient isoliquiritigenin was supplied as substrate. These results indicated that increased substrate supplement was not able to increase the accumulation of IBC, and the rate-limiting step of heterologous IBC pathway was caused by the low enzyme activity of *SfFPT*.

Discussion

In this study, we perform an *in planta* screening of previously reported prenyltransferases for the biosynthesis of IBC, and construct the *de novo* heterologous biosynthesis of IBC in tobacco by introducing *GmCHS8*, *GmCHR5*, and *SfFPT*. An interesting issue raised from our results is the different functions of these prenyltransferases from previous studies. At present, about 17 flavonoid pathway-specific PTs have been reported, and the *in vitro* functions of most of them have been well characterized by yeast recombinant proteins. By contrast, their *in vivo* functions were poorly mentioned, including four PTs we selected in this study. When *GulLDT* was expressed in yeast, their microsome extraction containing the recombinant *GulLDT* enzymes was able to convert isoliquiritigenin to IBC (Li et al. 2018). However, when *GulLDT* was expressed with isoliquiritigenin in tobacco in our study, no IBC accumulation was observed (Fig. 2b; Table 1). Such *in vivo* and *in vitro* difference seems to be not occasional in prenyltransferase studies, since the functions of *SfN8DT* (Sasaki et al. 2008) and *LaPT2* (Liu et al. 2021) were reported to be different between transgenic *Arabidopsis thaliana* and recombinant proteins. This phenomenon is probably caused by different microenvironments *in vitro* and *in planta*, including substrate concentration and accessibility, subcellular localization of enzymes, co-factors, competitive inhibition of enzyme activity, etc. Therefore, when a prenyltransferase is applied for plant-based metabolic engineering, its *in planta* function should be carefully confirmed. Fortunately, two prenyltransferases showed the activity to convert isoliquiritigenin to IBC in tobacco in our screening (Fig. 2d, e; Table 1). The substrate specificity of *SfFPT* (Chen et al. 2013) and *HIPT1* (Tsurumaru et al. 2012) towards liquiritigenin and naringenin chalcone, respectively, was demonstrated by using recombinant proteins, whereas isoliquiritigenin was not checked in these studies. Based on the structural similarity of isoliquiritigenin to liquiritigenin and naringenin chalcone, it would be reasonable that *SfFPT* and *HIPT1* can catalyze the prenylation of isoliquiritigenin in tobacco.

Although the *de novo* biosynthesis of IBC was achieved in our transgenic tobacco calluses, its concentration was not satisfying. Generally, low production rate in metabolic engineering is attributed to the insufficient substrate supplement. However, sufficient supplement of isoliquiritigenin in our transient expression experiment (Table 1) could not increase the accumulation of IBC when compared with the *de novo* biosynthesis of IBC, suggesting that the poor yield of IBC was caused by the low activity of *SfFPT*

enzyme. SfFPT is isolated from *S. flavescens*, in which IBC is not the predominant prenylflavonoids and the level of IBC is extremely low. Therefore, the major activity of SfFPT in *S. flavescens* is probably not subjected to isoliquiritigenin. In this respect, further identification of isoliquiritigenin-specific PT in some IBC-producing plants, such as *P. corylifolia* and *Erythrina variegata*, is expected to improve the IBC production rate in metabolic engineering.

Another fact is that prenyltransferases have putative transmembrane domains and are localized in plastids (Chang et al. 2021). However, the flavonoid biosynthetic enzyme complexes, as well as the flavonoids as substrates, are localized in ER. The subcellular compartment of prenylflavonoid enzymes inhibits the substrate accessibility of parent flavonoids with prenyltransferases, and thus limits the efficiency of prenylation of flavonoids. An alternative strategy is to re-construct the IBC biosynthesis pathway in the chloroplast. The crowded microenvironment in the chloroplast consisting of membrane and stroma may shorten the distance between prenyltransferases and other flavonoid biosynthetic enzymes, thereby enhance the efficiency of IBC biosynthesis. Abundant phenylalanine is also provided as a substrate of the flavonoid pathway without transport to the cytosol. More important, lots of enzymes involved in the biosynthesis and secondary modification of flavonoids exist in the cytosol, which may consume the target products via competitive or non-specific reactions. As an organelle for primary and energy metabolism, the chloroplast possesses relatively “pure” environment for secondary metabolism. Re-construction of flavonoid pathway in the chloroplast can avoid undesirable reaction. Recently, the biosynthesis pathway of dhurrin and precursors of taxane has been constructed in the tobacco chloroplast (Gnanasekaran et al. 2016; Li et al. 2019). It will be interesting to construct the biosynthesis pathway of prenylflavonoid in the chloroplast.

Although IBC exhibits excellent pharmacological effects such as antibacterial and antiviral activities, it has not been commercially used as yet due to the difficult availability based on natural plant extraction. The current price of IBC is around 50,000 USD/kg in China market. As the first report of prenylflavonoid metabolic engineering in plant chassis, our study provided a strategy to produce high valuable natural compounds. However, several critical challenges are raised. The metabolic background in the plant cells is more complicated than that in the microorganisms. As shown in this study, genes with identified functions via *in vitro* experiments might play different roles in plant cells. Numerous enzymes in the plant cytosol may also direct the metabolic flux to branch pathways, or catalyze the target compound to undesired derivatives. In addition, the gene manipulation in plants is more difficult than that in microorganism. For stable genetic transformation of plants, selected genes have to be transformed simultaneously by constructing multigene expression vectors. Despite all these challenges, once an ideal yield of the target compound is achieved in the plant-based metabolic engineering, a much lower cost of production is expected due to its electricity-free and non-pollution properties, even compared with the microorganism-based fermentation.

Abbreviations

PT, prenyltransferase; IBC, isobavachalcone; RT-PCR, reverse transcription polymerase chain reaction; EIC, extracted ion chromatogram; UPLC-Q-TOF, ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry.

Declarations

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Author Contribution Statement LG, HC, and YL conceived and designed the experiments; LG, WZ, YW, and YY performed the experiments; LG, CW, and JG analyzed the data; LG, ZY, and YL prepared the manuscript; JD, AB, and MYH provided advice on the experiments and revised the manuscript; YL, HC, and MYH provided the fundings. All authors read and approved the final manuscript.

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Data availability All data supporting the findings of this study are available within the paper. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest All authors have no conflict of interest to declare.

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Tables

Table 1
In planta conversion of isoliquiritigenin to isobavachalcone by prenyltransferases

Transgene	Substrate	Concentration of IBC
<i>GuILD</i>	isoliquiritigenin	N.D.
	none	N.D.
<i>SfILD</i>	isoliquiritigenin	N.D.
	none	N.D.
<i>SfFPT</i>	isoliquiritigenin	0.54 ± 0.08
	none	N.D.
<i>HIPT1</i>	isoliquiritigenin	0.27 ± 0.09
	none	N.D.
Empty vector	isoliquiritigenin	N.D.
	none	N.D.

Values shown are the concentration of isobavachalcone (IBC) (µg/g dry weight). Values are the mean of five replicates ± standard error. N.D., not detected.

Figures

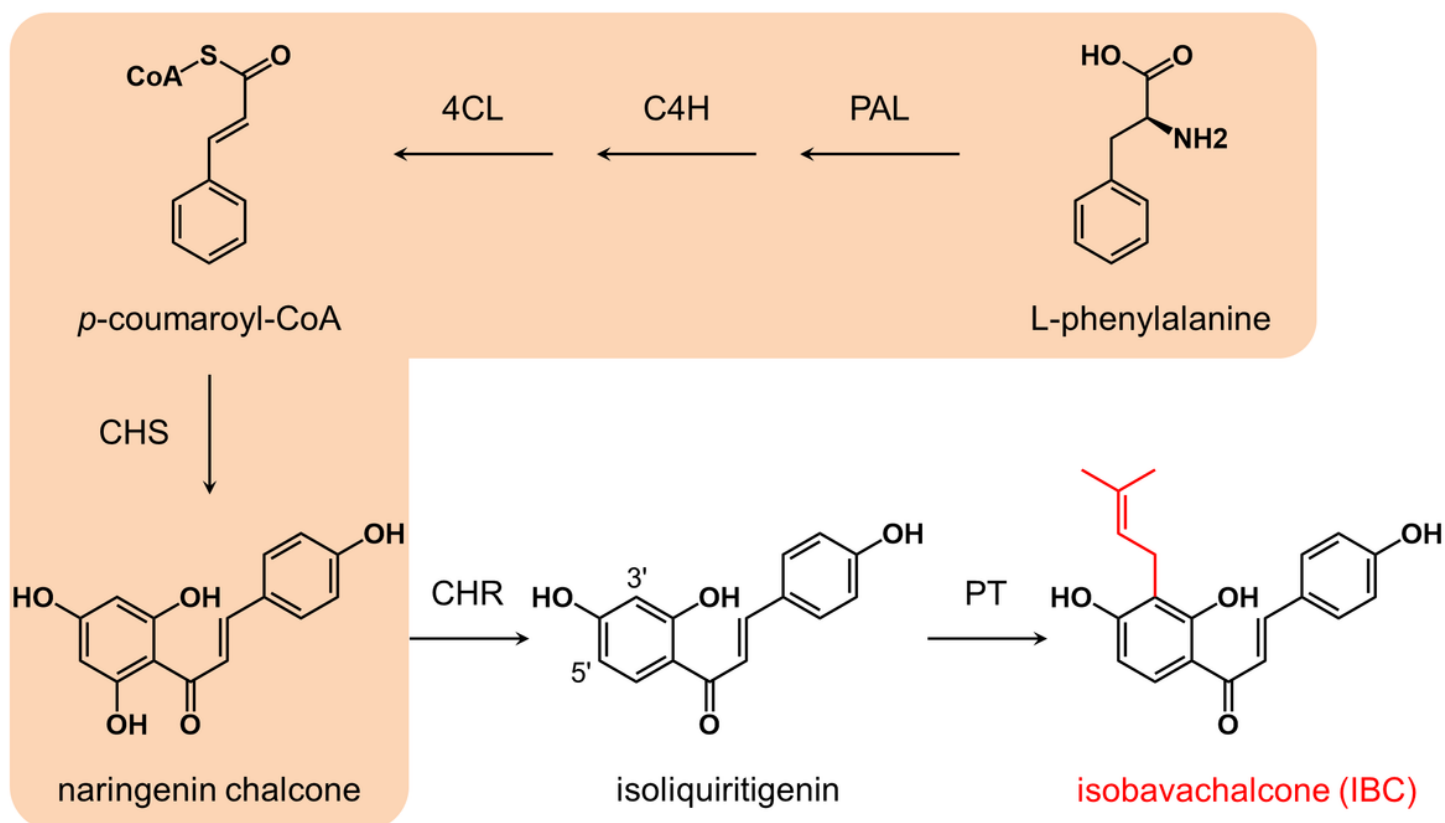


Figure 1

Biosynthesis pathway of isobavachalcone. Enzymes and compounds in the orange shadow commonly exist in higher plants, while CHR is mainly distributed in leguminous plants. Isobavachalcone is supposed to be synthesized via 3' prenylation of isoliquiritigenin by a PT. PAL, phenylalanine ammonia-lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4-coumarate: coenzyme A ligase; CHS, chalcone synthase; CHR, chalcone reductase; PT, prenyltransferase.

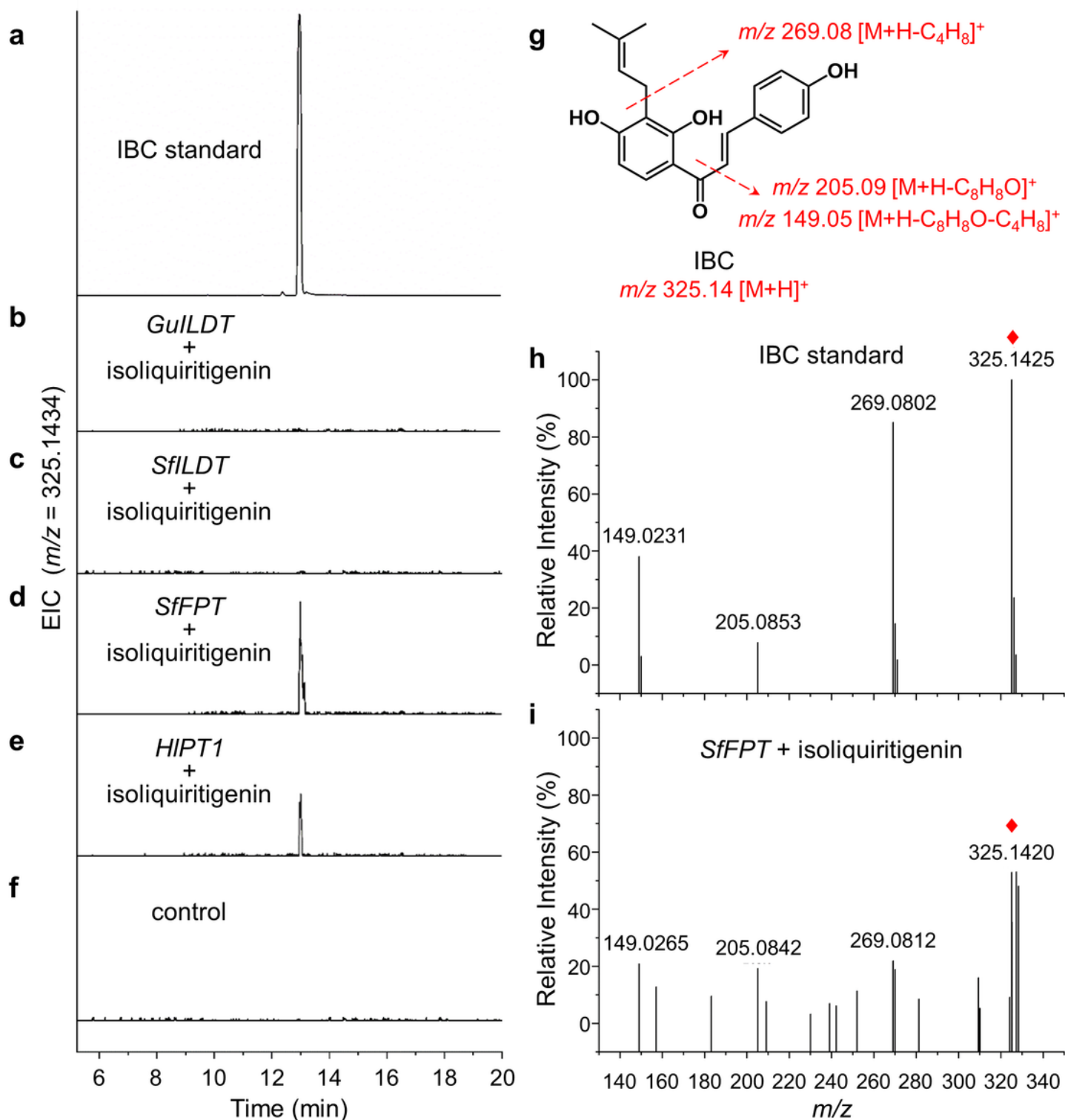


Figure 2

Screening of prenyltransferases for the conversion of isoliquiritigenin to isobavachalcone by transient expression. Four prenyltransferases were transiently expressed in tobacco leaves supplied with isoliquiritigenin as the substrate, to validate their *in planta* activities for the biosynthesis of isobavachalcone (IBC). Representative extracted ion chromatogram (EIC) of (a) IBC standard, (b) *GuILD*T, (c) *SfILD*T, (d) *SfFPT*, (e) *HIPT1* expressed samples, and (f) negative control was shown. (g) The structure of IBC and its fragmentation pattern was proposed by dotted arrows. Positive ion modes of MS/MS spectra of (h) IBC standard and (i) *SfFPT* expressed tobacco leaves was shown. Red rhombs indicated the precursor ions of IBC.

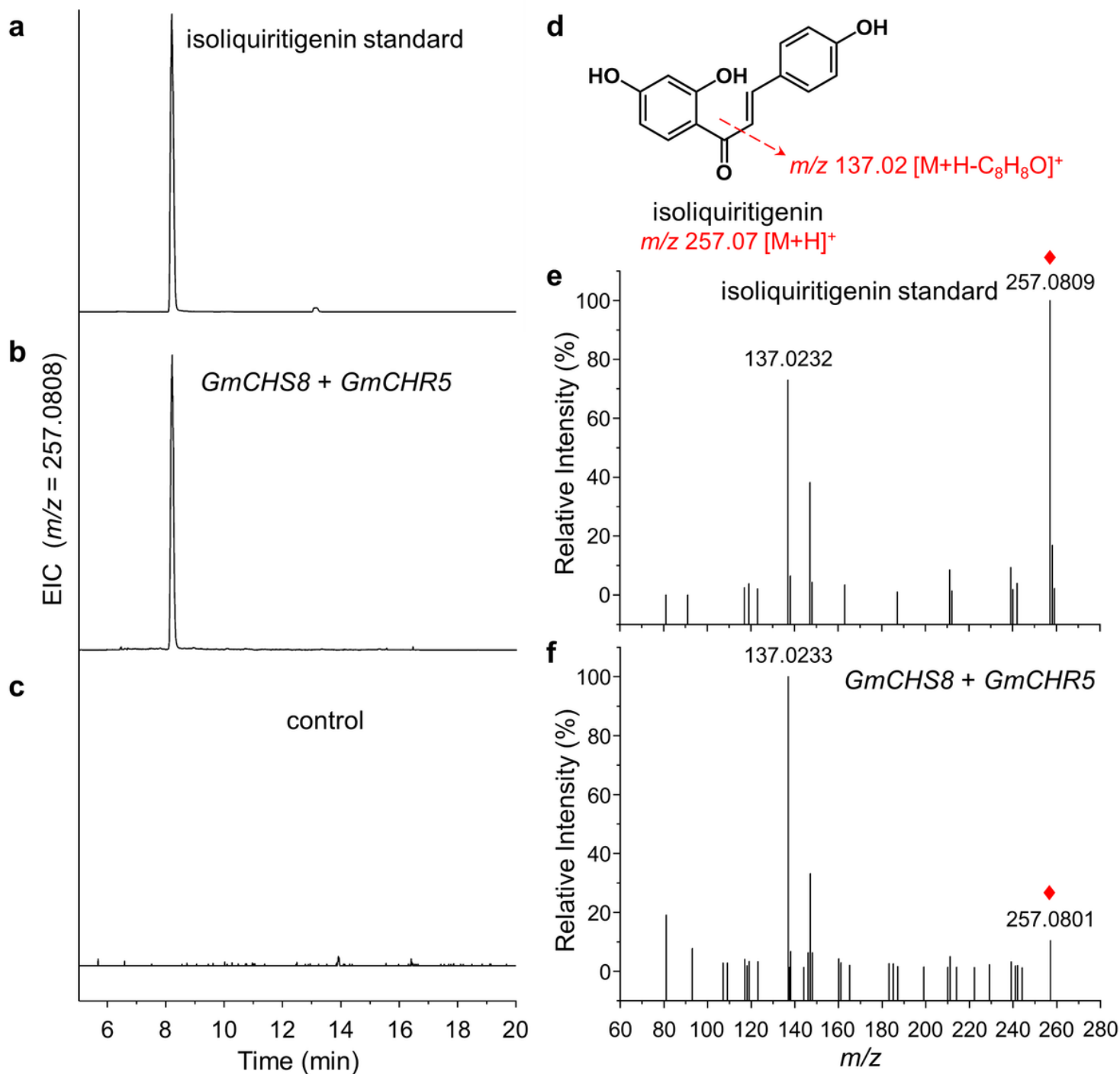


Figure 3

Generation of isoliquiritigenin in tobacco by transient expression. *GmCHS8* and *GmCHR5* was transiently co-expressed in tobacco leaves. Representative extracted ion chromatogram (EIC) of (a) isoliquiritigenin standard, (b) *GmCHS8* and *GmCHR5* co-expressed samples, and (c) negative control was shown. (d) The structure of isoliquiritigenin and its fragmentation pattern was proposed by dotted arrows. Positive ion modes of MS/MS spectra of (e) isoliquiritigenin standard and (f) *GmCHS8* and *GmCHR5* co-expressed samples was shown. Red rhombs indicated the precursor ions of isoliquiritigenin.

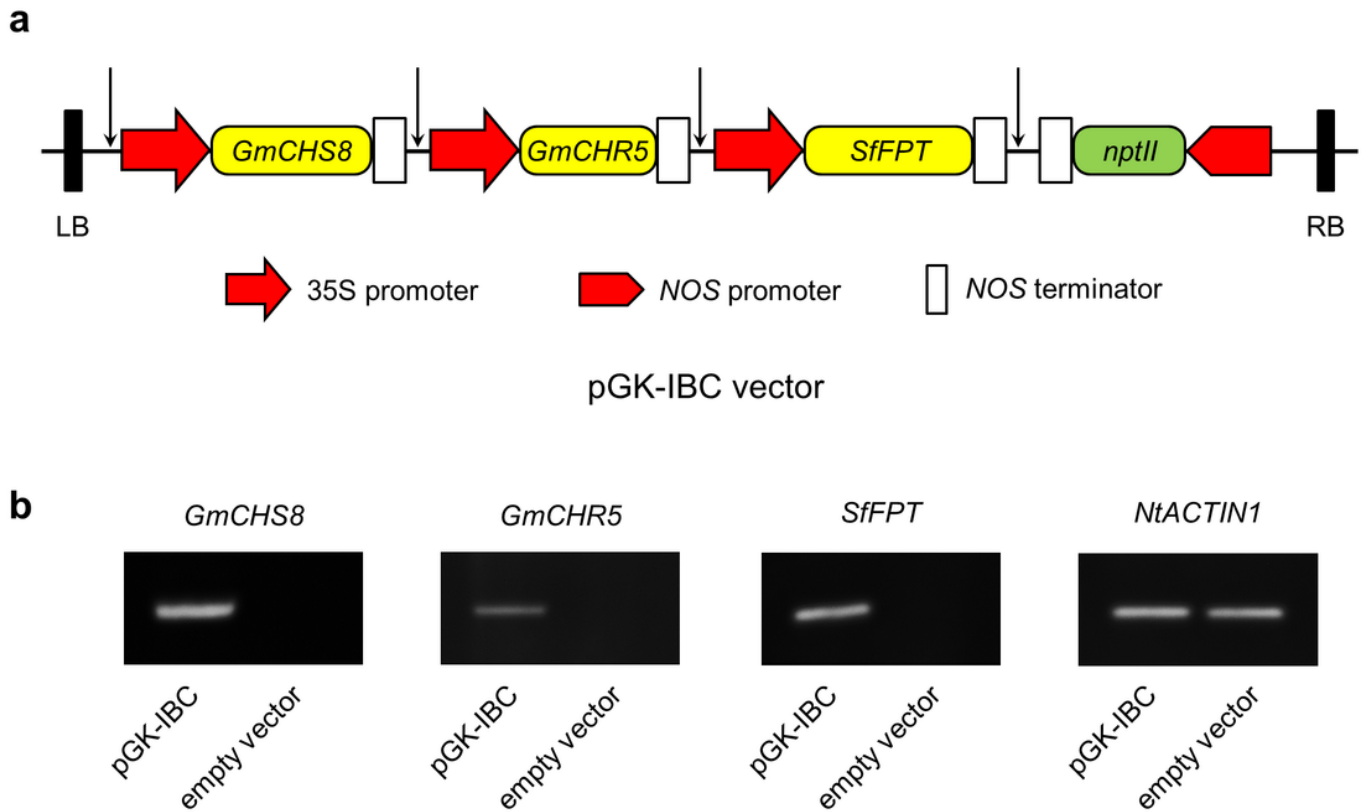


Figure 4

Schematic structure of the multigene expression vector. (a) The region spanning the LB and RB was indicated. Arrows indicated the conjunction sites after Golden Gate assembly. (b) The pGK-IBC and empty vectors were transformed into tobacco, and the expression of *GmCHS8*, *GmCHR5*, and *SfFPT* was confirmed by RT-PCR in transformed calluses. LB, left border; RB, right border; *nptII*, neomycin phosphotransferase.

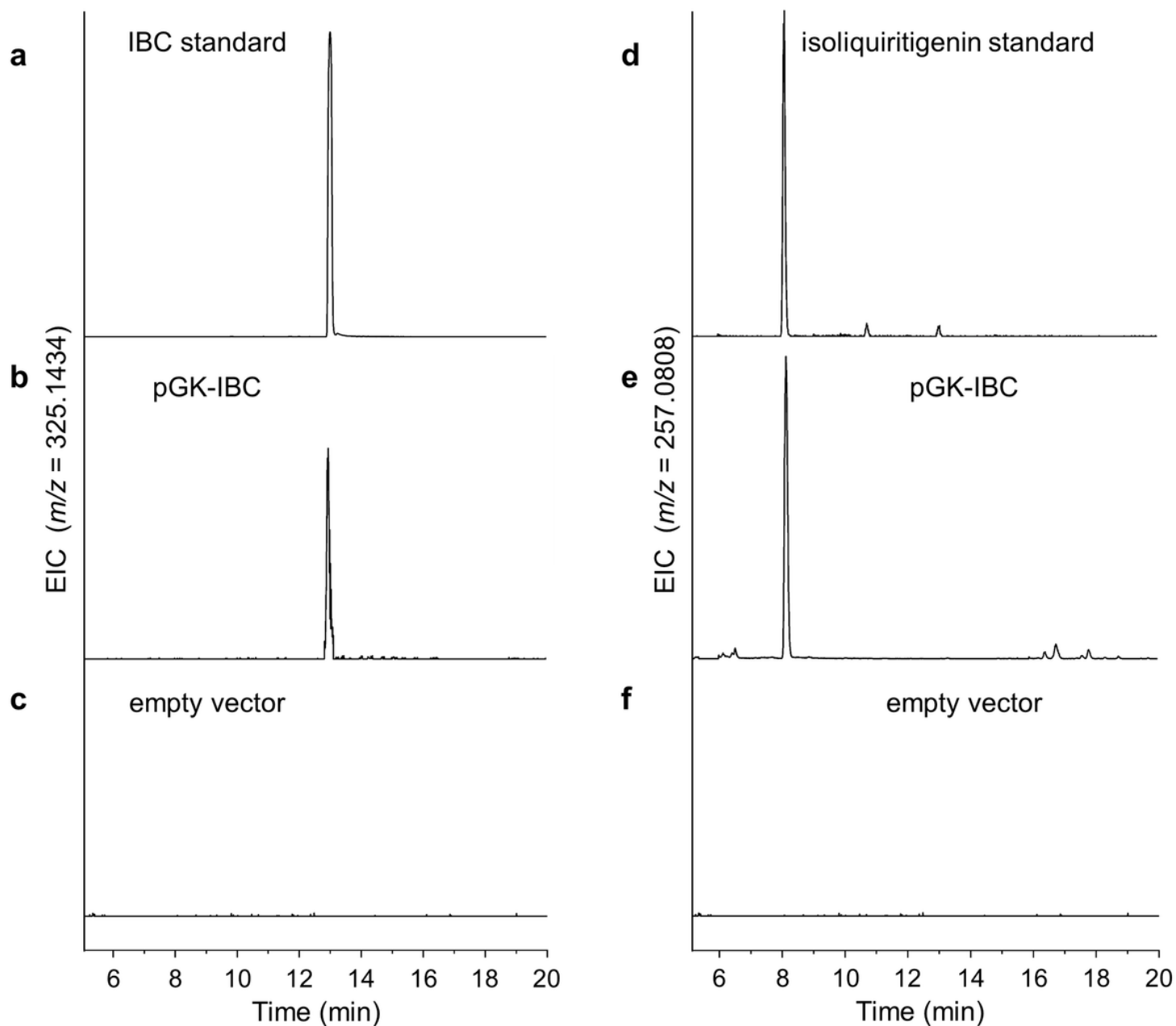


Figure 5

***De novo* biosynthesis of isobavachalcone in transgenic tobacco calluses.** Representative extracted ion chromatogram (EIC) for m/z 325.1434 of (a) isobavachalcone (IBC) standard, (b) pGK-IBC vector transgenic tobacco calluses, and (c) empty vector transgenic tobacco calluses, as well as EIC for m/z 257.0808 of (d) isoliquiritigenin standard, (e) pGK-IBC vector transgenic tobacco calluses, and (f) empty vector transgenic tobacco calluses was shown.

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