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# An overexpression of chalcone reductase of *Pueraria montana* var. *lobata* alters biosynthesis of anthocyanin and 5′-deoxyflavonoids in transgenic tobacco☆

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**Abstract**

We isolated the chalcone reductase (*pl-chr*) gene of *Pueraria montana* var. *lobata* by using a PCR strategy from cDNA pools of storage roots. A high level of expression of RNA was found in both stems and roots. The genomic Southern blot result suggests that *pl-chr* exists as a member of a small gene family. By introducing a *pl-chr* gene under the control of the 35S CaMV promoter into the pink-flowering Xanthi line of *Nicotiana tabacum*, the flower color was changed from pink to white-to-pink. The contents of anthocyanin in the flowers of the transgenic lines were dramatically decreased by 40%, but the total UV absorption compounds remained unchanged. The production of liquiritigenin in *pl-chr* overexpressed transgenic tobacco lines was confirmed by HPLC and MS analysis. The introduction of *pl-chr* gene provides a method to redirect the flavonoid pathway into 5′-deoxyflavonoid production in non-legume crops, in order to manipulate the phenylpropanoid pathway for isoflavonoid production.


*Keywords:* Chalcone reductase; Isoflavonoid; *Pueraria montana* var. *lobata*; Anthocyanin; 5′-Deoxyflavonoids; Transgenic tobacco

Isoflavonoids have a very limited distribution to leguminous plants, but have very important functions such as inducers of Rhizobium nodulation genes and antimicrobial phytoalexins [1,2]. Many isoflavonoids also exhibit medicinal properties and are common constituents in human diets [1,3].

The storage root of *Pueraria montana* var. *lobata* (Willd.) Ohwi contains diverse isoflavonoids [4]. In Korea, the extracts of them have been traditionally used as medicinal supplements for fever, pain, myalgia, alcohol poisoning, and abortifacients. Flavonoids are synthesized via the phenylpropanoid pathway. Chalcone reductase (CHR) is an enzyme that co-acts with chalcone synthase (CHS) to produce 4,2′,4′-trihydroxychalcone (isoliquiritigenin), which is a precursor of 5-deoxy-(iso)flavonoids, a branch in the first step of the flavonoid pathway. This chalcone is the precursor of the 5-deoxy series of flavonoids and isoflavonoids [5], which include nodulation–induction factors as well as pterocarpen phytoalexins of the Leguminosae [6]. A cDNA encoding chalcone reductase has been cloned from soybean [7], alfalfa [8], *Glycyrrhiza echinata* [9], and *Sesbania rostrata* [10]. In recent years, many genes encoding the enzymes involved in isoflavonoid biosynthesis have been cloned [11–14]. There have also been many reports on metabolic engineering for isoflavone production in non-legume dicot and monocot plants [15–18].

We are currently studying the production of isoflavones in non-legume crops by introducing isoflavonoid pathway genes. From the viewpoint of efficient metabolic engineering, it is very important to determine a method to control the overall metabolic flux within the targeted pathway and endogenous competing pathways. In this study, we report on the isolation and

☆ *Pueraria montana* var. *lobata* chalcone reductase sequence has been submitted to GenBank and the Accession No. awarded is AF462632.

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characterization of cDNAs of the isoflavonoid pathway, *P. montana* var. *lobata* chalcone reductase (*pl-chr*) gene. We show that the overexpression of *pl-chr* gene in transgenic tobacco flower affects the biosynthesis of anthocyanins and 5′-deoxyflavonoids, and provides a useful method to produce 5′-deoxyflavonoids in non-legume crops that do not naturally possess this 5′-deoxyflavonoid pathway.

## Materials and methods

*Cloning of chalcone reductase cDNA*. We used a polymerase chain reaction (PCR) strategy to clone *P. montana* var. *lobata* chalcone reductase cDNA. The storage roots of *P. montana* var. *lobata* grown in a field were used for extraction of total RNA. Total RNA was isolated from the storage root according to the phenol/SDS methods [19], and first-strand cDNA was synthesized with oligo(dT)-anchor primer using the 5′/3′-rapid amplification of a cDNA ends (RACE) Kit (Roche Molecular Biochemicals). Based on the amino acid sequences conserved among three Leguminosae chalcone reductase genes (gene products of soybean (X55730), *Glycyrrhiza glabra* (D86559), and *Medicago sativa* (U13924) clustered in the phylogenetic tree, a degenerate primer was designed. Using a degenerate primer CHR-F1 and PCR anchor primer, we amplified a DNA fragment with an expected size of about 950 bp from the *P. montana* var. *lobata* cDNA. The degenerate primer CHR-F1 was based on the peptide sequences GYRH FDT with the nucleotide sequence 5′-GGTTA(T/C)AGACA (T/C)T T(T/C)GA(T/C)ACTG-3′. The 950-bp band was cloned into a pCR-BluntII-TOPO vector (Invitrogen) and the nucleotide sequence was determined. The deduced amino acid sequence from this DNA fragment showed a significant identity with the chalcone reductase from soybean, and thus, the putative corresponding gene was named chalcone reductase of *P. montana* var. *lobata*. A 5′-terminal portion of cDNA was isolated by the 5′-rapid amplification of cDNA ends (RACE) method using a newly synthesized first-strand cDNA with a specific antisense primer, CHR-R1 (5′-GAGCAGATTCTGAAGCT TCTTGACAGA-3′). Another specific antisense primer, CHR-R2 (5′-AAGGCAAGAGATCCTCAACCTCAA-3′), was used for the 5′-RACE using the 5′/3′-RACE System (Roche Molecular Biochemicals). The full-length cDNA (designated *pl-chr*, GenBank Accession No. AF462632) was achieved by a final PCR using primers from the 5′- and 3′-untranslated regions and the cDNA pools as a template.

*Construction of chalcone reductase plant expression vector*. A *P. montana* var. *lobata* CHR coding region DNA fragment starting with the start codon and continuing through the stop codon was produced by PCR amplification using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) with primers: 5′-TCTAGAAACTAGTTACAATGG-3′ and 5′-TTATATTTCATCATCCCAGAG-3′, and the cDNA clone as a template. The resulting 963-bp fragments were subcloned in a Zero-blunt PCR Cloning Kit (Invitrogen). After cutting out the *Eco*RI fragment from this cloning vector, *pl-chr* gene fragment was ligated into the *Eco*RI site of the pCAMBIA1390-35S binary vector between the CaMV 35S promoter and the *Nos* 3′ region that contains a hygromycin resistance marker gene for plant selection. We confirmed the sense-oriented pCAMBIA1390-35S-pl-chr vector (pCAM35-pl-chr) by sequencing.

*Transformation of tobacco*. pCAM35-pl-chr was transformed into *Agrobacterium tumefaciens* strain LBA4404 and introduced into *Nicotiana tabacum* cv. Xanthi by leaf disc transformation following standard procedures [20]. Plants regenerated from transformed calli on 5 mg/L of hygromycin containing plates were transferred to MS media and subcultured for 2 weeks before analysis. Transgenic lines of high expression level were selected by Northern blot analysis of *pl-chr* gene, transferred to soil, and grown for flowering and chemical analysis.

*Southern blot analysis of pl-chr in the P. montana var. lobata*. To examine the copy number of the *P. montana* var. *lobata* chalcone reductase gene, Southern blot analyses were performed on genomic DNA extracted from leaves of *P. montana* var. *lobata*. Genomic DNA was isolated according to the protocol [21]. After digestion with the appropriate restriction enzymes, the DNA was subjected to electrophoresis through a 0.7% agarose gel and transferred to a nylon membrane. The membranes were hybridized with probes labeled with digoxigenin (DIG) using a PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals). Hybridization lasted for 16 h at 42 °C in a hybridization buffer, a DIG Easy Hyb. The membranes were washed twice in 2× SSC, 0.1% (w/v) SDS at room temperature for 5 min each and then washed twice in 0.1× SSC, 0.1% SDS at 68 °C for 15 min each. Target DNAs were detected by using a DIG luminescent detection kit as described by the manufacturer's instructions (Roche Molecular Biochemicals).

*Northern blot analysis of pl-chr mRNA*. The expression of *pl-chr* genes was investigated by Northern blot experiments in *P. montana* var. *lobata* plant. Total RNAs were isolated from the samples according to the phenol/SDS methods [19]. To recognize the expression of the *pl-chr* genes, 25 μg of total RNA was separated by electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde and then transferred to a nylon membrane. The membranes were hybridized with probes labeled with digoxigenin (DIG) using a PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals). Hybridization lasted for 16 h at 50 °C in a hybridization buffer, a DIG Easy Hyb. The membranes were washed twice in 2× SSC, 0.1% (w/v) SDS at room temperature for 5 min each, and then washed twice in 0.1× SSC, 0.1% SDS at 65 °C for 15 min each. Target RNAs were detected by using chemiluminescence as described by the manufacturer's instructions (Roche Molecular Biochemicals).

*Analysis of anthocyanin and total UV-absorptive compounds*. Anthocyanin analysis was performed as described in the following protocol [22]. Plant tissues were ground in liquid nitrogen and extracted with 80% (v/v) methanol as described above. The methanol extracts were mixed with an equal volume of 0.5% (v/v) HCl and then extracted with 2 ml of chloroform. The aqueous/methanol phase was assayed by spectrophotometer at $A_{530}$, with $A_{657}$ subtracted, and the resulting absorbance value was normalized to the fresh weight for each sample. Total UV-absorptive compounds were assayed according to the protocol [23], as follows. Plant tissues were ground in liquid nitrogen and extracted with 80% (v/v) methanol/1% (v/v) HCl. The extracts were cleared by centrifugation at 14,000*g* for 10 min, followed by filtration through nylon centrifuge tube filters (Corning, Corning, NY). The flavonoid compounds were assayed by spectrophotometer at $A_{330}$ and absorbance values were normalized to the fresh weight of each sample.

*Identification of liquiritigenin in transgenic tobacco*. Samples were prepared from selected transformants and a control wild-type plant. The flower part of the plant was ground in liquid nitrogen, extracted with 80% ethanol at room temperature for 2 h, and passed through a Puradisc 25 AS filter (Whatman). Extraction solutions were concentrated by vacuum-evaporation and 1 N HCl was added to the extract, followed by incubation at 95 °C for 2 h to hydrolyze any conjugated forms of isoflavonoids. After hydrolysis, the samples were extracted once with ethyl acetate, dried under nitrogen, and re-suspended in 80% methanol. The resultant filtrates were analyzed by photodiode array HPLC after TLC analysis was carried out on a KC 18F reverse phase plate (Whatman). Samples were assayed on an HPLC system (Waters 510 Pump 2× 1000 PSI, Waters 991 Photodiode Array Detector). Chromatography was performed on a Phenomenex Ultracarb 5 μm ODS (30) HPLC column (250 × 4.60 mm), separated using an isocratic 65% MeOH condition as a mobile phase at a flow rate of 1 ml/min and the effluent monitored at 280 nm. Levels of liquiritigenin were quantified from standard curves by using authentic liquiritigenin standards. ESI-negative mass spectra were acquired using a Finnigan Navigator mass spectrometer (Finnigan, UK).

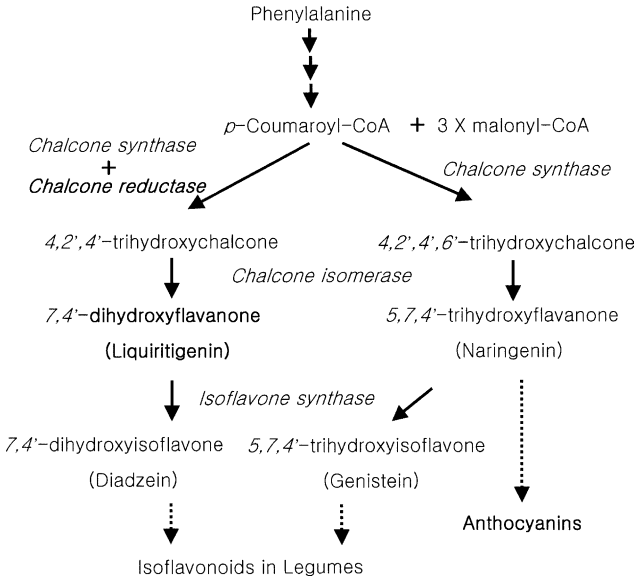


Fig. 1. A partial diagram of the flavonoid biosynthetic pathway. The enzymes in bold are encoded by genes overexpressed as transgene and the metabolites in bold are compounds accumulated or reduced in this study. Dotted arrows represent multiple steps. Enzymes are indicated in italics.

## Results and discussion

### *Cloning and characterization of pl-chr*

The cDNA sequence is 1109 nucleotides long and consists of an open reading frame encoding a peptide of 314 amino acids (Fig. 2), with a calculated molecular mass of 35.4 kDa and an isoelectric point of 6.4. The BLAST analysis [24] on the homology of the deduced amino acid sequence shows high homologies with soybean (X55730), *G. echinata* (D83718), and *M. sativa* (U13925) (93%, 87%, and 83% identities, respectively) (Fig. 2). Moreover, *pl-chr*, like other aldo/keto reductases, contains a number of $K^+$ ion channel beta chain regulatory domains having oxidoreductase activity [25]. Among other aldo/keto reductases except Leguminosae chalcone reductase genes, *pl-chr* from *P. montana* var. *lobata* was the most similar to *Papaver somniferum* NADPH-dependent codeinone reductase (AF108435), with an overall amino acid identity of 54%.

To examine the copy number of the *P. montana* var. *lobata* chalcone reductase gene, Southern blot analyses were performed on genomic DNA extracted from leaves

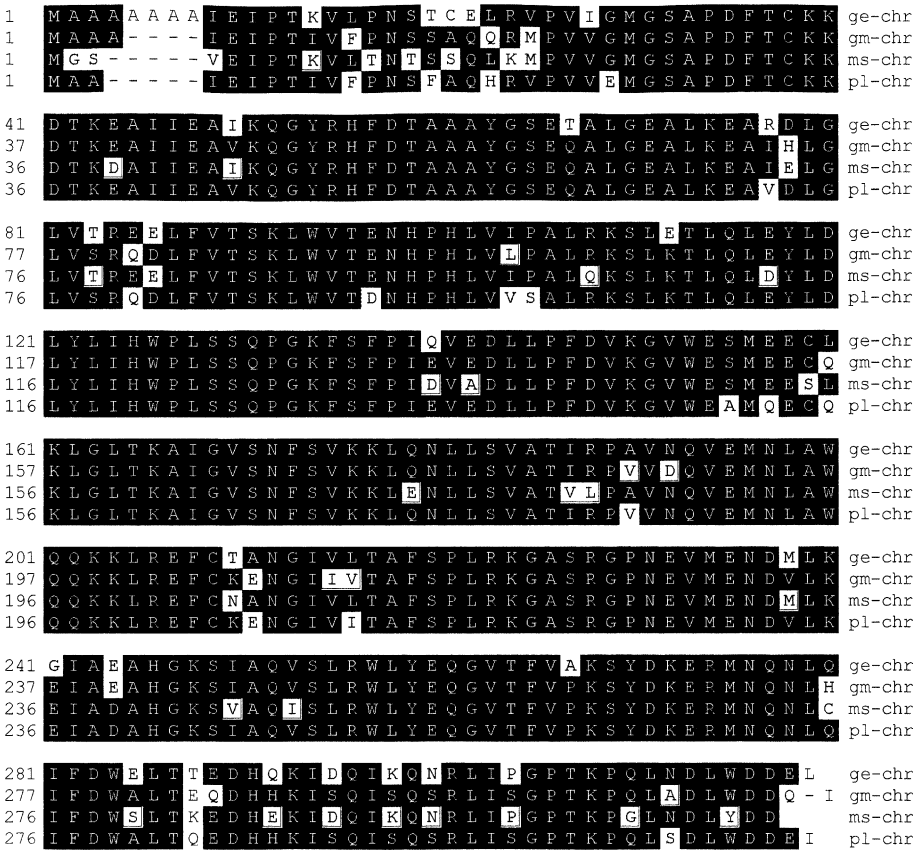


Fig. 2. Alignment of predicted amino acid sequences of chalcone reductase of *P. montana* var. *lobata* (*pl-chr*). The amino acid sequences conserved in more than two proteins are shaded in the figure. *Abbreviations:* ge, *G. echinata* (D83718); gm, *Glycine max* (X55730); ms, *M. sativa* (U13924); and pl, *P. montana* var. *lobata* (AF462632).

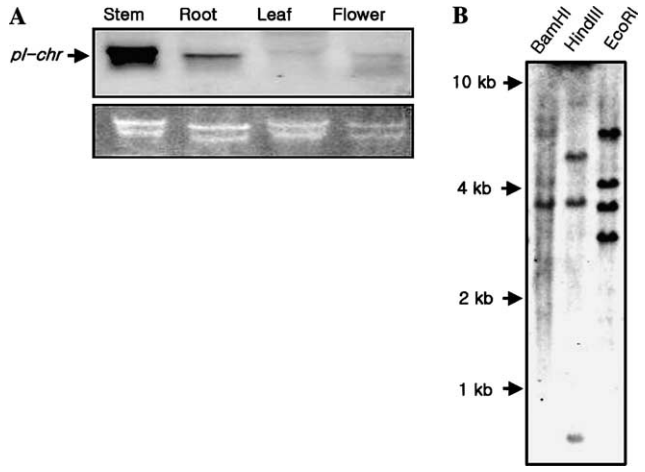


Fig. 3. The expression profile of *pl-chr* genes in different tissues of *P. montana* var. *lobata* plant was investigated by Northern blot experiments (A) and copy number prediction of *pl-chr* by genomic Southern blot analysis (B). To recognize the expression of the *pl-chr* genes, 25 mg of total RNA was separated by electrophoresis and then transferred to a nylon membrane. Ethidium bromide-stained gel indicates equal loading of the RNA samples (A). Genomic DNA was digested with restriction enzymes, subjected to electrophoresis through a 0.7% agarose gel, and transferred to a nylon membrane (B). The membranes were hybridized with probes labeled with digoxigenin (DIG) using a PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals).

of *P. montana* var. *lobata*. There are two internal *Hin*dIII sites in the *pl-chr* cDNA sequence. We also made DIG probes using a PCR DIG probe synthesis kit with two specific primers located between two *Hin*dIII sites. There is one 80-bp intron between the two specific primers (data not shown). As shown in Fig. 3B, digestion of genomic DNA with *Hin*dIII generates hybridizing fragments of 0.4, 3.7, and 5.0 kb, respectively. Since there are no *Eco*RI and *Bam*HI sites in *pl-chr*, we can expect that there are at least three copies of *pl-chr* genes (Fig. 3B). Therefore, the genomic Southern blot result suggests that *pl-chr* exists as a member of a small gene family. The results of the Northern blot analysis (Fig. 3A) showed that staining with ethidium bromide was similar in each sample, suggesting that approximately an equal amount of total RNA from leaves, roots, stem, and flowers was loaded. High levels of expression of RNA were observed in stem and roots, while expression in leaves and flowers was very weak.

*Expression of pl-chr in transgenic tobacco changes metabolic flux in anthocyanin biosynthesis*

Transgenic tobacco plants harboring the T-DNA region including the CaMV 35S::CHR expression cassette were readily regenerated using *Agrobacterium*-mediated leaf disc transformation. Fifteen independent transgenic lines were recovered. Highly expressed transgenic plants were selected by Northern blot analysis from the lines that were PCR positive for the CHR construct (Fig. 4A). The transgenic H1 line expressing *pl-chr* gene at the highest level was further grown in a greenhouse for the analysis of liquiritigenin. Surprisingly, the flower color of the transgenic H1 line was changed dramatically from pink to white-to-pink (Fig. 4B). The total content of anthocyanin in the floral tissue of transgenic line H1 was decreased by 40%, but the total content of flavonoids was not changed (Fig. 4C). It is interesting that metabolic flux in competing pathways, anthocyanin biosynthesis, is easily affected by the

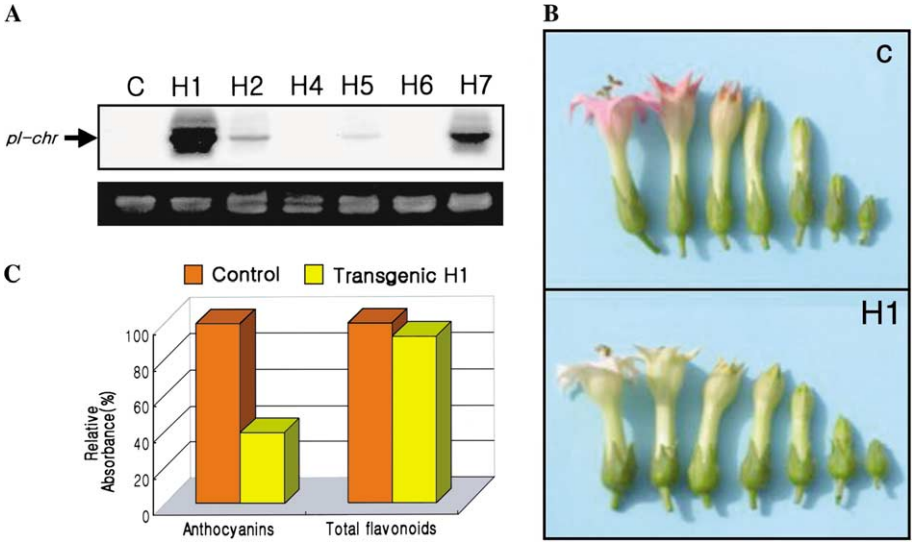


Fig. 4. Screening of transgenic tobacco expressed with high level of *pl-chr* gene by Northern blot analysis and the changing pattern of anthocyanin and total UV-absorptive compounds. (A) Northern blot of RNA from CHR transgenic tobacco plants. Lane C, Wild-type tobacco; Lanes H1, 2, 4, 5, 6, and 7, transgenic numbers 1, 2, 4, 5, 6, and 7. (B) Different stages of flowers of transgenic H1 plant harbouring the CHR gene (H1) and wild-type tobacco plant (C). (C) Contents of total anthocyanin and UV-absorptive compounds in the flowers of transgenic H1 and wild-type tobacco plant.

introduction of foreign genes of 5′-deoxyflavonoid pathways. In flavonoid biosynthetic pathways, chalcone isomerase catalyzes the cyclization of chalcone and 6′-deoxychalcone, both of which are synthesized by the upstream enzyme chalcone synthase into naringenin and isoliquiritigenin, respectively. The introduced chalcone reductase enzyme co-acts with chalcone synthase enzyme and uses the same substrates, *p*-coumaroyl-CoA and malonyl-CoA. Focusing on the availability of the substrate, there might be a competition between 5′-deoxy and 5′-hydroxy pathways (Fig. 1). Therefore, as shown in Fig. 4B, in the transgenic line H1, the substrate availability for chalcone synthase to produce anthocyanin biosynthesis was reduced, as the introduced chalcone reductase enzyme activity to accumulate 5′-deoxyflavonoids increased at a higher level. These results suggest that the method of controlling the overall metabolic flux within the targeted pathway and endogenous competing pathways is a very important aspect to consider in metabolic engineering.

*Accumulation of liquiritigenin in transgenic tobacco*

The two major groups of isoflavonoids, with 5-hydroxy and 5-deoxy A-rings, respectively, apparently use the same chalcone synthase activity, but the synthase is associated with a reductase activity to produce the 5-deoxy compounds [26]. Two types of chalcone isomerases have been found. The isomerase from legumes such as *Phaseolus vulgaris* that contain both 5-hydroxy and 5-deoxy products catalyzes the isomerization of both 6′-hydroxy- and 6′-deoxychalcones, while that from plants such as *Petunia hybrida* that synthesize only 5-hydroxyflavonoids functions only with 6′-hydroxychalcones [27].

In the tobacco system, since there are no chalcone reductase and isoflavone synthase enzymes, we were not able to detect any isoliquiritigenin and liquiritigenin. However, if we introduce chalcone reductase enzymes of legumes such as *P. montana* var. *lobata*, then we could detect isoliquiritigenin and liquiritigenin. In addition, if the chalcone isomerase isoliquiritigenin to tobacco could not isomerize to liquiritigenin as the chalcone isomerase of *P. hybrida* does, liquiritigenin would not be detected at all.

From the HPLC profiles of leaf extracts between the wild-type and transgenic H1 line, we did not find any different peaks. It has been reported that tobacco plants have a natural tissue-specific activation of the phenylpropanoid pathway, leading to production of anthocyanins in the flower petals [17]. From floral tissues of a transgenic H1 line, extracts were prepared, hydrolyzed, and partially purified by preparative TLC. After extraction, the sample was analyzed by HPLC. A large peak in the HPLC profile corresponding to liquiritigenin was found in the transgenic H1 line (Fig. 5A). There are no other detectable different peaks between the wild-type and transgenic H1 line. The UV–VIS spectrum of that peak exactly matched that of an authentic standard liquiritigenin (Fig. 5B). The ESI negative mass spectrum of that compound gave a $M^-$ peak at $m/z$ 255.2 (Fig. 5C). From the HPLC and UV and mass spectra data, the

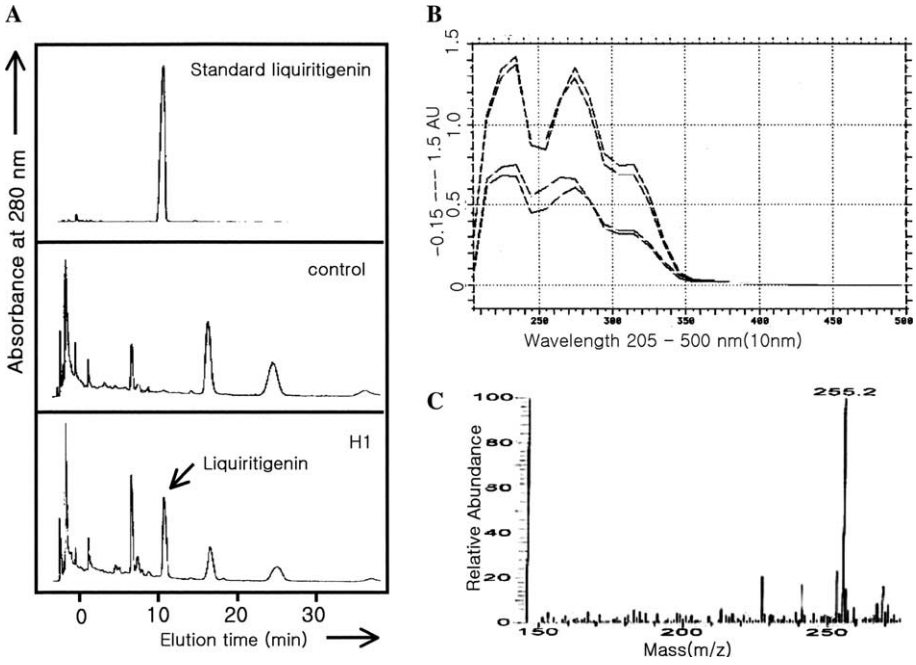


Fig. 5. Chemical identification of liquiritigenin from flowers of transgenic H1 tobacco plants. (A) HPLC profiles of extracts from flowers of transgenic H1 and wild-type tobacco plant. (B) Comparison of UV–VIS spectrum data of compounds isolated from flowers of transgenic H1 plants and standard liquiritigenin. (C) ESI-MS spectral data of compounds isolated from flowers of transgenic H1 plants.

compound was identified as 7,4′-dihydroxyflavanone, liquiritigenin. The transgenic H1 line produced about 20 ng of liquiritigenin per gram fresh weight of sample after acid hydrolysis of conjugates. These results imply that the chalcone isomerase of tobacco, like that of legumes, is able to isomerize isoliquiritigenin to liquiritigenin. Therefore, isoliquiritigenin, synthesized by the tobacco chalcone synthase and the introduced chalcone reductase of *P. montana* var. *lobata*, was successfully isomerized to liquiritigenin by the endogenous chalcone isomerase. However, since there is no isoflavone synthase in tobacco plants, the synthesized liquiritigenin may be accumulated in a glycosylated form. Further engineering of the isoflavone synthase of legumes into the transgenic H1 line may be possible for high production of isoflavonoids, diadzein, in non-legume plants. Developing transgenic crops for production of valuable plant secondary metabolites is a very promising field of study. However, for efficient metabolic engineering, we need a greater understanding of regulatory mechanisms of the biosynthetic pathways and have to consider about the overall metabolic flux within the targeted pathway and endogenous competing pathways.

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