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Overexpression of the *Saussurea medusa* chalcone isomerase gene in *S. involucrata* hairy root cultures enhances their biosynthesis of apigenin

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Abstract

Saussurea involucrata is a medicinal plant well known for its flavonoids, including apigenin, which has been shown to significantly inhibit tumorigenesis. Since naturally occurring apigenin is in very low abundance, we took a transgenic approach to increase apigenin production by engineering the flavonoid pathway. A construct was made to contain the complete cDNA sequence of the *Saussurea medusa* chalcone isomerase (CHI) gene under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Using an *Agrobacterium rhizogenes*-mediated transformation system, the *chi* overexpression cassette was incorporated into the genome of *S. involucrata*, and transgenic hairy root lines were established. CHI converts naringenin chalcone into naringenin that is the precursor of apigenin. We observed that transgenic hairy root lines grew faster and produced higher levels of apigenin and total flavonoids than wild-type hairy roots did. Over a culture period of 5 weeks, the best-performing line (C46) accumulated 32.1 mg L^{-1} apigenin and 647.8 mg L^{-1} total flavonoids, or 12 and 4 times, respectively, higher than wild-type hairy roots did. The enhanced productivity corresponded to elevated CHI activity, confirming the key role that CHI played for total flavonoids and apigenin synthesis and the efficiency of the current metabolic engineering strategy.

Keywords: *Saussurea involucrata*; Compositae; Hairy root; Genetic transformation; Chalcone isomerase; Flavonoids; Apigenin

1. Introduction

Saussurea involucrata Kar. et Kir. ex Maxim. has been widely used in Chinese medicine because of its effect in promoting blood circulation and anti-inflammation, and its analgesic functions (Li et al., 1980). The main bioactive chemical constituents of this plant are syringin, *S. involucrata* polysaccharides, umbelliferone, and flavonoids (Wang et al., 1986; Zheng et al., 1993; Han, 1995; Zhao and Wang, 2003). The content of total flavonoids has been established as the criterion for quality control (Wang et al., 1996). Among the flavonoids investigated (such as apigenin (**5**), jaceoside, hispidulin, quercetin and rutin), apigenin (**5**) (5,7,4′-trihydroxyflavone) is of particular interest (Fig. 1). Studies on apigenin (**5**) have demonstrated that it can inhibit the development of several types of cancers (Yin et al., 1999; McVean et al., 2000; Wang et al., 2000) as well as its anti-inflammatory effects (Liang et al., 1999; Gupta et al., 2001). These properties are responsible for a growing demand for *S. involucrata* and its flavonoid extracts. Supply of this medicinal plant is presently only from the wild populations, since field cultivation of this plant remains unsuccessful. In the wild, *S. involucrata* grows only on snowy mountains at altitudes of 4000–5000 m, and now this valuable plant species is under enormous pressure for survival. Production of the desired flavonoids by means of chemical synthesis could be an alternative source, only

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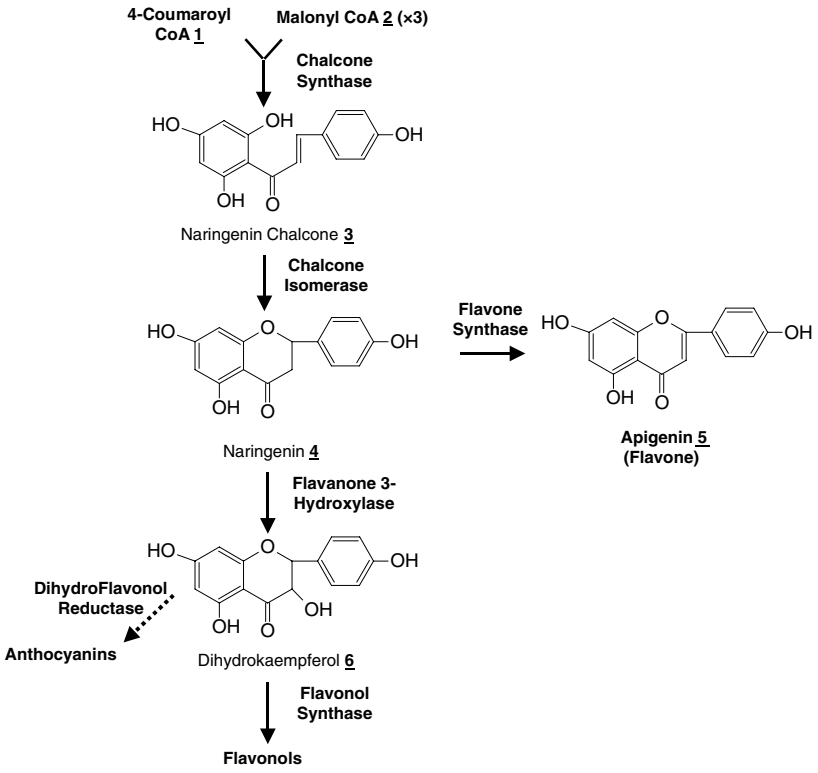

Fig. 1. The flavonoid pathway.

it will be too costly for commercial application. Still another option is to produce the bioactive compounds by in vitro culture systems (Kieran et al., 1997), but cell lines tend to accumulate only low levels of secondary metabolites and are genetically unstable (Rhodes et al., 1990). Our goal has been to establish an in vitro system for *S. involucrata* with satisfactory efficiency of flavonoids accumulation in general and apigenin biosynthesis in particular.

Hairy roots systems have attracted much attention by their great potential for producing important pharmaceuticals (Hamill et al., 1987; Yoshikawa and Furuya, 1987). Hairy roots are normally formed when the T-DNA of the root-inducing plasmid (pRi) resided in *Agrobacterium rhizogenes* is integrated into the genome of a plant (White and Nester, 1980). Hairy root cultures are characterized by rapid growth, frequent branching and, more importantly, the ability to synthesize the same compounds as the intact plants. However, in most cases the yields of specific metabolites are so low that commercial exploitation is not possible. Metabolic engineering could be a useful approach to boost accumulation of targeted metabolites. We have chosen chalcone isomerase (CHI; EC 5.5.1.6) to start our investigation in this direction. CHI is one of the key enzymes of the flavonoid pathway (Fig. 1). Specifically, CHI naringenin converts chalcone (**3**) into (2*S*)-naringenin (**4**). Although this conversion can occur spontaneously, CHI catalyzes this conversion by 10^7-fold faster (Bednar and Hadcock, 1988). In addition, CHI controls the preferred formation of biologically active (2*S*)-flavanones as well (Jez et al., 2000; Jez and Noel, 2002). In support of the notion that CHI plays critical roles in flavonoid metabolism, Reuber et al. (1997) reported that a barley mutant of CHI defection showed a dramatic reduction of flavonoid levels in the primary leaf. In a more recent study, Kim et al. (2004) described that inactivation of CHI in onion resulted in a significantly reduced amount of quercetin. Alternatively, Muir et al. (2001) documented that overexpression of petunia *chi* in tomato, was responsible for up to 78-fold increases in fruit peel flavonols, mainly in the form of quercetinglycosides. These examples suggest that CHI is a very good candidate for genetic manipulation.

In the present work, the *chi* gene from *S. medusa* was overexpressed in hairy roots of *S. involucrata* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The growth rates, as well as apigenin (**5**) and total flavonoid production capacity, of the engineered hairy root lines were compared with those of wild-type hairy roots.

2. Results and discussion

2.1. Root transformation and establishment of hairy root lines

A. rhizogenes strain R1601 carrying pRiA4 and the binary vector pCHI (Fig. 2(a)) was able to transform *S. involucrata* roots. Hairy roots were formed 2–3 weeks of

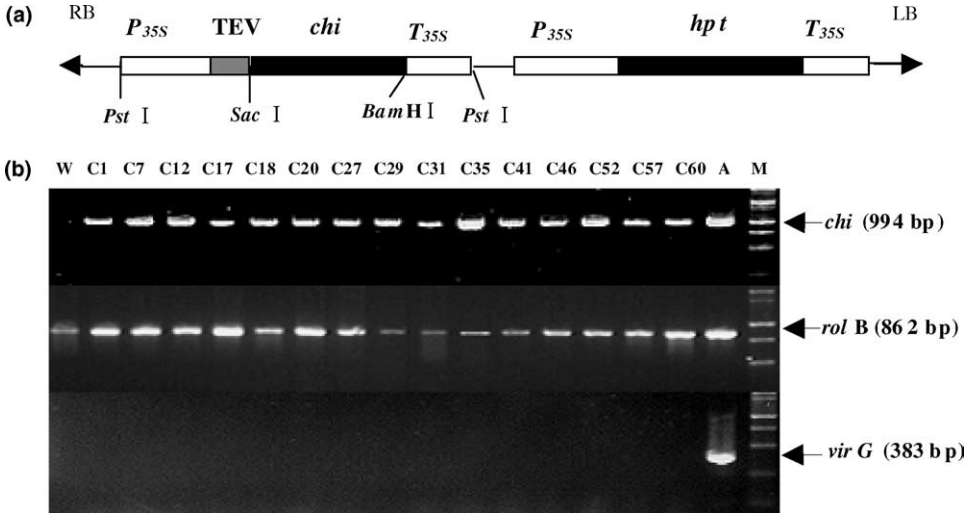

Fig. 2. The pCHI plasmid used in transformation and PCR analysis of transgenic hairy root lines: (a) schematic representation of the *p*CHI construct. P_{35S}, CaMV 35S promoter; TEV, tobacco etch virus translational enhancer sequence; *chi*, *S. medusa* chalcone isomerase gene; T_{35S}, CaMV 35S terminator; *hpt*, hygromycin phosphotransferase gene; (b) representative PCR analyses for the *chi*, *rol*B and *vir*G genes in transgenic hairy root lines. W, wild-type hairy root induced by *A. rhizogenes* strain R1601; Cn, transgenic hairy root lines induced by *A. rhizogenes* strain R1601 with the binary vector pCHI ("*n*" indicates the line number); A, pRi from *A. rhizogenes* strain R1601; M, DNA marker (DGL-2000).

inoculation on over 80% of the root explants to constitutively express the *Saussurea medusa chi* gene. Such a high transformation efficiency with *A. rhizogenes* strain R1601 was unusual, as in our previous experiments on *S. medusa* with strains R1000, A4 and LBA9402 transformation rates never exceeded 5% (Zhao et al., 2004).

About 10–15 d after hairy roots' emergence, individual root clones were pre-selected in 1/2 MS medium supplemented with 20 mg L^{-1} hygromycin for at least five generations. Cefotaxime was added to the medium at 300 mg L^{-1} until the bacteria were eliminated. The resultant hairy root lines were then subcultured for 5–6 weeks in hormone-free, 1/2 MS medium. In all cases, some transgenic roots turned brown and aged considerably faster than wild-type root cultures (transformed by wild-type *A. rhizogenes* strain R1601 carrying only pRiA4). These brown roots were discarded and only those that showed a good growth capacity were maintained for further characterization. Altogether, 15 *chi*-transformed lines were maintained; they were designated C1, C7, C12, C17, C18, C20, C27, C29, C31, C35, C41, C46, C52, C57, and C60.

The presence of *chi*, *rol*B and *vir*G genes in the hairy roots' genomes was confirmed by PCR (Fig. 2(b)). All selected transgenic lines gave two bands, one at 994 bp and another at 862 bp. The former corresponded to the *S. medusa chi* fragment and the latter to the *rol*B fragment. The coexistence of the *chi* and *rol*B genes confirmed that our root transformation was achieved as desired, by both the pCHI and pRiA4 plasmids. Wild-type hairy roots gave only one band corresponding to the *rol*B gene, which is a proof of transformation by the pRiA4 only. Hairy root lines obtained through transformation with *A. rhizogenes* strain R1601 carrying an empty pCAMBIA1301 vector (without *chi*), which were used as a second control, also gave a band corresponding to *rol*B. Neither *chi*-transformed root lines nor control hairy roots carried the *vir*G gene, which is present outside the T-DNA region of the Ri-plasmid; thus the possibility of *A. rhizogenes* R1601 contamination of hairy root cultures was ruled out.

Considerable variations in growth capacity among individual lines were observed after the 5-week cultivation (Fig. 3). For example, line C46 achieved twofold higher fresh weight than line C52. Such variations were also observed in both groups of control hairy root lines. As previously reported for a few other hairy root systems (Jouhikainen et al., 1999; Moyano et al., 2003; Palazón et al., 2003; Zhang et al., 2004), differences in growth capacity were quite common, since each clone arised from an independent transformation event, depending on the presence of certain T-DNA genes of the Ri-plasmid, mainly the *aux*1 gene, in the genome of the root line obtained, growth could be very different.

There was some indication that *chi*-transformed hairy roots senesced faster than control hairy roots. Maximum biomasses (dry weight) were achieved at 24–28 days and 30–35 days in *chi*-transformed and control hairy root lines (data not shown), respectively.

2.2. Apigenin (**5**) production

The *chi*-transformed hairy roots of *S. involucrata* displayed a flavonoid spectrum similar to that of control hairy roots, as judged from HPLC analyses (Fig. 4). As mentioned in earlier reports, the profiles of secondary products are often conserved while, sometimes, concentrations of some compounds may alter (Park and Facchini, 2000; Palazón et al., 2003). In our system, there was a wide range of apigenin (**5**) content among the *chi*-transformed root lines,

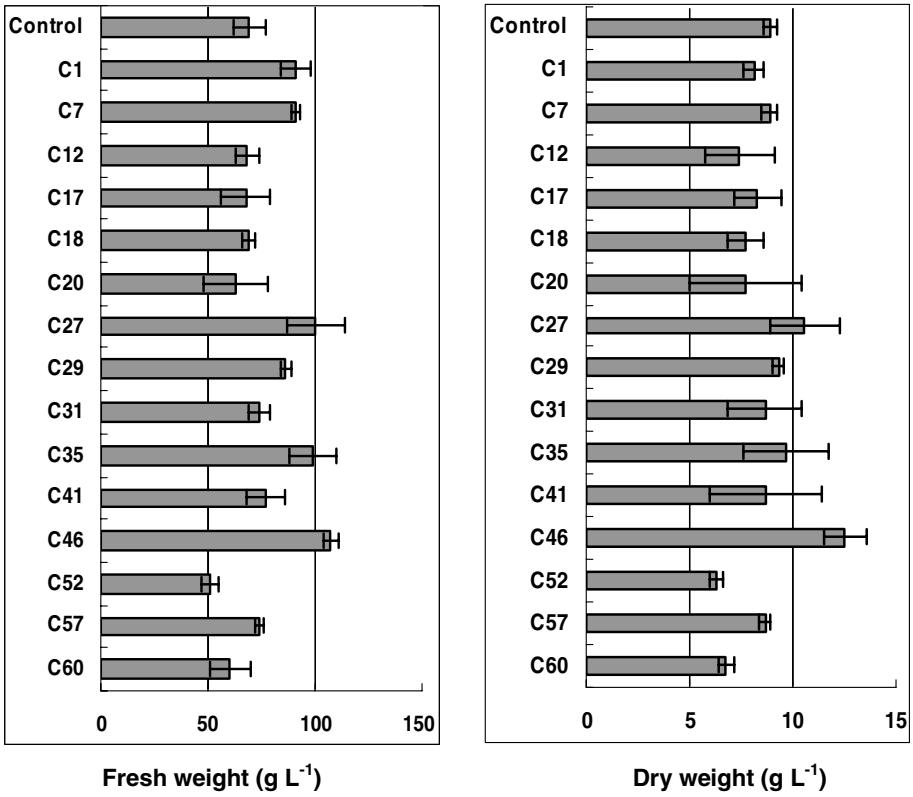

Fig. 3. Fresh and dry weights (g L^{-1}) of *chi*-transformed hairy root lines at the end of the culture period (35 d). The value for each line is the mean of three to five determinations ±SD, and the value for control represents the mean of five individual wild-type hairy root lines ±SD.

from 3.2 mg L^{-1}(line C31) to 32.1 mg L^{-1} (line C46). These lines were divided into three groups according to their performance in apigenin production: best- (lines C17 and C46), moderate- (lines C1, C12, C18, C20, C27, C29, C35, C41, C52, C57 and C60), and low-performing (lines C7 and C31). It was exciting that the majority of these lines accumulated higher amounts of apigenin (**5**) than even the best apigenin-producing wild-type lines did ($p < 0.05$), except for the low-performance lines (lines C7 and C31) (Fig. 5(a)). Furthermore, a comparison on total flavonoids production indicated that, in spite of the considerable variations observed, the majority of the *chi*-transformed lines had significantly higher flavonoids than the control lines ($p < 0.05$) (Fig. 5(b)). Essentially same results were obtained from empty vector lines as wild-type lines (not shown). These observations strongly suggest that CHI is a key enzyme in the flavonoid pathway, and up-regulating CHI leads to enhanced biosynthesis of flavonoids. This conclusion is in agreement with an earlier report for tomato overexpressing petunia *chi* (Muir et al., 2001).

2.3. Expression of chi and enzyme activity

To establish a possible relationship between the constitutive *chi* overexpression and the capacity of the hairy root lines to synthesize apigenin, *chi* gene transcript levels and CHI activity were studied in both control and *chi*-transformed hairy roots. Two lines from each of the three categories, best-, moderate-, and low-performing, were chosen from *chi*-transformed hairy roots for RT-PCR. Wild-type hairy roots and empty vector hairy roots were also examined. It was shown that the *S. medusa chi* gene was transcribed in all transformed hairy root lines of *S. involucrata* tested, but the transcripts were detected as different richness (Fig. 6 (a)). No detectable difference was found between wild-type hairy roots and empty vector hairy roots and, thus, only results for wild-type hairy roots were presented. In addition, the CHI enzymatic activity in these root lines was measured simultaneously, and a good correlation was observed between *chi* transcript levels and enzymatic activities. The strongest *chi* expression and high CHI activity were observed in the best-performing lines (C17 and C46), intermediate expression and activity in moderate-performing lines (C20 and C41), and low expression and activity in the low-producing lines (C7 and C31) (Fig. 6). Combining results on apigenin production (above) and CHI activity, it becomes clear that the two are positively correlated, confirming the pivotal role of *chi* in the regulation of apigenin synthesis.

In summary, the present study provides an effective approach to efficiently increase the end products of secondary metabolic pathways by appropriate genetic engineering strategies. With the *S. medusa chi* in particular, which is an important gene in the flavonoid pathway, its overexpression in *S. involucrata* hairy roots did lead to enhanced apigenin biosynthesis.

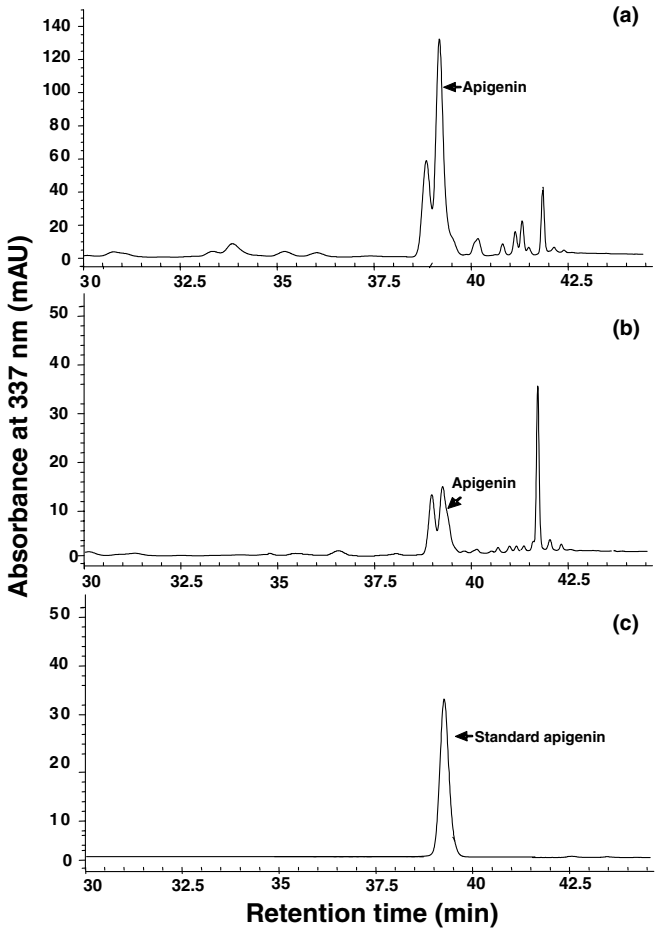

Fig. 4. Chromatograms recorded at 337 nm: (a) *chi*-transformed root line C46; (b) a wild-type root line; (c) standard sample of apigenin (**5**).

3. Experimental

3.1. Construction of the chi binary expression vector

The *chi* gene from *S. medusa* was amplified from the plasmid pTripIEx2. The latter contained the complete *chi* cDNA that was cloned from the Red callus line of *S. medusa* in our laboratory (Jin et al., 2004). The *chi* gene coding sequence thus obtained was digested with *Sac* I and *Bam*H I, and then subcloned into pRTL2 between the CaMV 35S promoter linked to translational enhancement TEV leader sequence and the CaMV 35S terminator. The resultant construct, designated pRTL2-*chi*, was digested by *Pst* I and a 2236-bp fragment containing the complete *chi* expression cassette was obtained. This latter cassette was cloned into the pCAMBIA1301 vector (Cambia, Australia) carrying the hygromycin phosphotransferase gene (*hpt*) driven by the CaMV 35S promoter. The new binary vector was referred to as pCHI (Fig. 2(a)).

The binary vector pCHI was mobilized by electroporation into the armed *A. rhizogenes* strain R1601 containing genes conferring resistance to kanamycin and rifampin (Mozo and Hooykaas, 1991). Recombinant clones of *A. rhizogenes* were selected with 100 mg L^{-1} kanamycin, 50 mg L^{-1} hygromycin and 50 mg L^{-1} rifampin. Positive clones as determined by PCR and enzymatic digestion for the presence of the *chi* gene were used to transform *S. involucrata* roots.

3.2. Root transformation and hairy root culture

Genetic transformation of root explants from *S. involucrata* was carried out following the method used to transform *S. medusa* (Zhao et al., 2004). Briefly, micropropagated shoots of *S. involucrata* were rooted on 1/2 MS solid medium supplemented with 2.7 μM NAA. Root sections of about 10 mm in length were excised from these plants and were co-cultivated with the recombinant *A. rhizogenes* carrying pCHI at 25 °C for 2 d in the dark. Next, the roots were incubated on 1/2 MS solid medium supplemented with 20 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime for 4 weeks.

Hairy roots developed at cut ends, which normally took place 2–3 weeks in culture, were excised and selected on 1/2 MS solid or liquid medium supplemented with 20 mg L^{-1} hygromycin. Cefotaxime was added to the medium at 300 mg L^{-1} until the bacteria were eliminated. Root cultures were grown at 25 °C on a rotary shaker (90 rpm) under a 12/12-h (light/dark) photoperiod (45 mmol photons m^{-2} s^{-1}, with Osram 40-W fluorescent tubes) and routinely subcultured every 35–42 d. After a minimum of five generations in liquid culture, rapidly growing roots were used to establish hairy root lines. To generate a hairy root line, about 80 mg (fr. wt) root sections of 20 mm were transferred to 30 ml 1/2 MS liquid medium in a 100-ml Erlenmeyer flask. As a control, hairy root lines were established by transforming roots with wild-type *A. rhizogenes* strain R1601 (wild-type control lines). A second control was also included by transforming roots with *A. rhizogenes* strain R1601 carrying a pCAMBIA1301 vector (empty vector control lines). Culture conditions were the same as for *chi*-engineered hairy root lines described above.

3.3. Transgene confirmation by PCR

The *S. medusa chi* gene and the *A. rhizogenes rol*B gene in *chi*-transformed hairy roots were examined by PCR. Genomic DNA samples were obtained from both *chi*-engineered hairy root lines and wild-type hairy roots using the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990). A primer pair of 5′-CCCACTATCCTTCG-3′ and 5′-GCATGAGAGCTCTGGGCACACAGATGATTT-3′ was designed to amplify a 994-bp fragment for the *S. medusa chi* gene (GenBank accession no. AF509335). A second pair of primers, 5′-TACTGCAGCAGGCTTCATGCA-3′ and 5′-GCTTTCCCGACCAGAGACTG-3′, would amplify an 862-bp fragment of the *rol*B gene (Slightom et al., 1986). The *rol*B gene is located in the T-DNA region of pRiA4 plasmid, and thus can be used as a marker for transformation with this plasmid. In addition, primers (5′-GGCTTCGCCAACCAATTTGGAGAT-3′ and 5′-TTTTGCTCCTTCAAGGGAG-

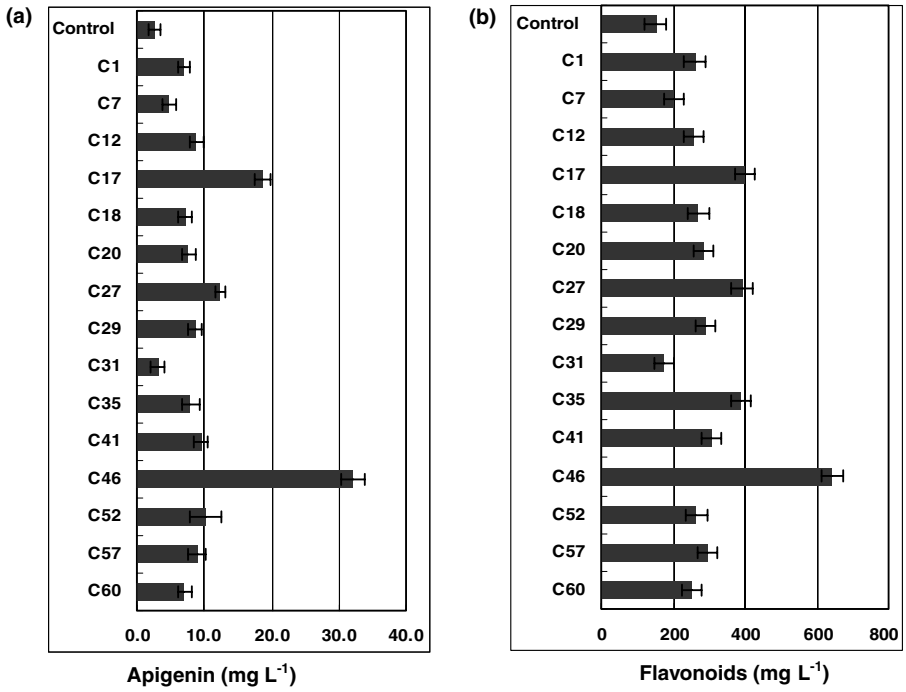

Fig. 5. Apigenin (**5**) and total flavonoids (mg L^{-1}) production by *chi*-transformed hairy root lines at the end of the culture period (35 d). The value for each line is the mean of three to five determinations ±SD, and the value for control represents the mean of five individual wild-type hairy root lines ±SD.

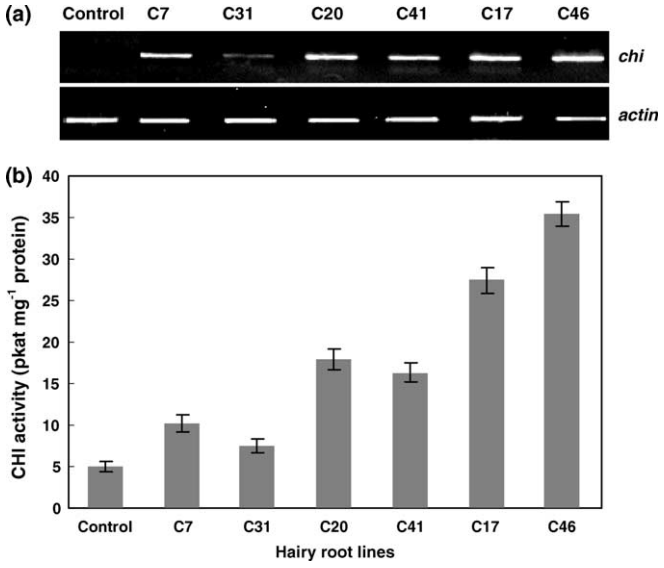

Fig. 6. Analysis of *chi* gene expression and CHI activity: (a) RT-PCR analysis of the *chi* gene. Data are shown for control, lines C7, C31, C20, C41, C17 and C46. The *actin* gene was used as a loading control; (b) analysis of enzyme activity of hairy root lines. The value for each line is the mean of five determinations ±SD, and the value for control represents the mean of five wild-type hairy root lines ±SD.

GTGCC-3′) for detecting the *vir*G gene outside the T-DNA of pRiA4 were also tested to eliminate the possibility of *A. rhizogenes* contamination of the hairy root cultures. Each PCR mixture contained 200 ng plant genomic DNA (or 100 pg Ri plasmid DNA), 400 μM each primer, 200 μM dNTPs, 1.0 unit *Taq* DNA polymerase (TaKaRa) and 2.5 μl 10× buffer in a total volume of 25 μl. Conditions for the *chi* amplification were as follows: initial denaturation at 94 °C for 4 min, followed by 30 cycles of amplification (94 °C 1 min, 56 °C 1 min and 72 °C 45 s) and 10 min at 72°C. For *rol*B and *vir*G, PCR was carried out in the following conditions: initial denaturation at 94 °C for 4 min, 30 cycles of amplification (94 °C 1 min, 53 °C 1 min, and 72 °C for 1 min), and 72 °C for 10 min. PCR products were analyzed on 1.0% agarose gels.

3.4. Analyses of apigenin (*5*) and total flavonoids

For all established root lines, roots were collected at 3, 7, 14, 21, 28, 32, 35 and 42 d after inoculation. Following filtration, roots were washed with 10 ml of distilled water and then dried at 60 °C to a constant weight. Root samples (1.5 g dry wt) were ground and extracted with EtOH–H_2O (30 ml, 7:3, v/v) at 25 °C for 24 h. The extracts were cleared by centrifugation at 13,000*g* for 20 min. Analysis of total flavonoids content in the *chi*-transformed roots of *S. involucrata* were performed following the protocol of Guan et al. (1995). Wild-type hairy roots were examined as a control. Apigenin (**5**) levels were determined using an Agilent 1100 system (Agilent, USA) with a Zorbax 300 SB-C18 reversed-phase analytical column (250 × 4.5 mm, particle size 4 μm). A photodiode array detector (Agilent) was used to record online spectra (from 190 to 400 nm) of compounds eluted from the column. In each case, 5 μl sample was separated at constant ambient temperature using a gradient of MeOH in 0.1% orthophosphoric acid, at a flow rate of 1.0 ml min^{-1}: 5–20% linear in 5 min, 20–30% in 7 min,

30–45% in 20 min and 45–70% in 10 min, followed by a 10 min washing with MeOH–H_2O (7:3) in 0.1% orthophosphoric acid. Peak purity, identification, and integration were carried out on Agilent Chemstations software A.10.02. Peak was monitored at 337 nm. The detection limit of this system was 0.05 μg ml^{-1} extract, corresponding to ~5 mg kg^{-1} dry wt *S. involucrata* hairy roots.

3.5. Gene expression analysis by RT-PCR

Total RNA was isolated using Trizol (Invitrogen, USA) from the hairy root lines after 3 weeks in culture. Reverse transcription was performed with 1 μg total RNA using a Promega® reverse transcription kit. An aliquot from the reaction mixture was subjected to PCR with two pairs of primers, one pair was used to amplify the internal standard actin gene for a loading control, the other pair (5′-GATAAAGCCATTCCGTCAC-3′ and 5′-ACTCGATTACTGTTTGTGCC-3′) was used to amplify the *S. medusa chi* gene. PCR was carried out in the following conditions: initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C 45 s, 50 °C 45 s and 72 °C 1 min, and terminated after extension at 72 °C for 10 min. All products were examined on 1.5% agarose gels.

3.6. CHI enzyme activity assay

CHI activity in *chi*-transgenic and control hairy root lines was measured with the method employed by Fouché and Dubery (1994) and Jez and Noel (2002). Hairy roots cultured 3 weeks were used. First, fresh hairy roots (2 g) were collected from the culture and washed with distilled water and homogenized in 6 ml 120 mM potassium phosphate buffer (pH 8.0), containing 3 mM EDTA, 20 mM β-mercaptoethanol, 2% hydrated polyclar AT, and 100 μM jodoacetic acid, followed by centrifugation at 12,000*g* for 30 min. The protein content of samples was determined by the Bradford method (Bradford, 1976).

The standard CHI assay was performed at 25 °C by monitoring the isomerization of naringenin chalcone (**3**) at 390 nm using a Beckman DU-640 spectrophotometer. The reaction mixture contained the following in a final volume of 1 ml: 50 mM Tris–HCl buffer (pH 7.6) containing 1% EtOH, 100 μM narigenin chalcone (**3**) (4,2′,4′,6′-tetrahydroxychalcone), and an appropriate amount of the enzyme. The rate of spontaneous cyclization of the substrate was determined beforehand and was subtracted from the rate in the presence of enzyme. Naringenin chalcone (**3**) was synthesized from naringenin (**4**) according to the procedure of Moustafa and Wong (1967) and naringenin (**4**) was purchased from Herbfine (Nanchang, China).

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References

Bednar, R.A., Hadcock, J.R., 1988. Purification and characterization of chalcone isomerase from soybeans. J. Biol. Chem. 263, 9582–9588.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Doyle, J.J., Doyle, J.L., 1990. Isolation of plant DNA from fresh tissue. Focus 12, 13–15.

Fouché, S.D., Dubery, I.A., 1994. Chalcone isomerase from *Citrus sinensis*: purification and characterization. Phytochemistry 37, 127–132.

Guan, J.Y., Wang, W.W., Ma, M.T., 1995. Investigation of technological preparation on extracts of *Saussurea involucrata*. J. Shenyang Pharma. Univ. 12, 209–211.

Gupta, S., Afaq, F., Mukhtar, H., 2001. Selective growth-inhibitory, cell-cycle deregulatory and apoptotic response of apigenin in normal versus human prostate carcinoma cells. Biochem. Biophys. Res. Commun. 287, 914–920.

Hamill, J.D., Parr, A.J., Rhodes, M.J.C., Robins, R.J., Walton, N.J., 1987. New routes to plant secondary products. Biotechnology 5, 800–804.

Han, S.L., 1995. Study on the effect of four chemical constituents against cancer of *Sausurea involucrata*. Aibian Jibian Tubian 7, 80–83.

Jez, J.M., Noel, J.P., 2002. Reaction mechanism of chalcone isomerase. J. Biol. Chem. 277, 1361–1369.

Jez, J.M., Bowman, M.E., Dixon, R.A., Noel, J.P., 2000. Structure and mechanism of the evolutionarily unique plant enzyme chalcone isomerase. Nat. Struct. Biol. 7, 786–791.

Jin, Z.P., Zhao, D.X., Qiao, C.L., Fu, C.X., 2004. Construction of cDNA library from the callus of *Saussurea medusa*. Chin. Bull. Bot. 21, 61–65.

Jouhikainen, K., Lindgren, L., Jokelainen, T., Hiltunen, R., Teeri, T.H., Oksman-Caldentey, K.-M., 1999. Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208, 545–551.

Kieran, P.M., MacLoughlin, P.F., Malone, D.M., 1997. Plant cell suspension cultures: some engineering considerations. J. Biotechnol. 59, 39–52.

Kim, S., Jones, R., Yoo, K.S., Pike, L.M., 2004. Gold color in onions (*Allium cepa*): a natural mutation of the chalcone isomerase gene resulting in a premature stop codon. Mol. Genet. Genom. 272, 411–419.

Li, G.H., Liu, F., Zhao, R.C., 1980. Studies on pharmacological actions of *Saussurea involucrata*. Kar. et Kir. ex Maxim. Acta Pharmacol. Sin. 15, 368–369.

Liang, Y.C., Huang, Y.T., Tsai, S.H., Lin-Shiau, S.Y., Chen, C.F., Lin, J.K., 1999. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 20, 1945–1952.

McVean, M., Xiao, H.Y., Isobe, K.I., Pelling, J.C., 2000. Increase in wild-type p53 stability and transactivational activity by the chemopreventive agent apigenin in keratinocytes. Carcinogenesis 21, 633–639.

Moustafa, E., Wong, E., 1967. Purification and properties of chalcone-flavanone isomerase from soya bean seed. Phytochemistry 6, 625–632.

Moyano, E., Jouhikainen, K., Tammela, P., Palazón, J., Cusidó, R.M., Piñol, M.T., Teeri, T.H., Oksman-Caldentey, K.-M., 2003. Effect of *pmt* gene overexpression on tropane alkaloid production in transformed root cultures of *Datura metel* and *Hyoscyamus muticus*. J. Exp. Bot. 54, 203–211.

Mozo, T., Hooykaas, P.J.J., 1991. Electroporation of megaplasmids into *Agrobacterium*. Plant Mol. Biol. 16, 917–918.

Muir, S.R., Collins, G.J., Robinson, S., Hughes, S., Bovy, A., Ric de Vos, C.H., van Tunen, A.J., Verhoeyen, M.E., 2001. Overexpression of

petunia chalcone isomerase in tomato results in fruits containing increased levels of flavonols. Nat. Biotechnol. 19, 470–474.

Park, S.U., Facchini, P.J., 2000. *Agrobacterium rhizogenes*-mediated transformation of opium poppy, Papaver somniferum L., and California poppy, *Eschscholzia californica* Cham., root cultures. J. Exp. Bot. 51, 1005–1016.

Palazón, J., Moyano, E., Cusidó, R.M., Bonfill, M., Oksman-Caldentey, K.-M., Piñol, M.T., 2003. Alkaloid production in *Duboisia* hybrid hairy roots and plants overexpressing the *h6h* gene. Plant Sci. 165, 1289–1295.

Reuber, S., Jende-Strid, B., Wray, V., Weissenböck, G., 1997. Accumulation of the chalcone isosalipurposide in primary leaves of barley flavonoid mutants indicates a defective chalcone isomerase. Physiol. Plant. 101, 827–832.

Rhodes, M.J.C., Robins, R.J., Hamill, J.D., Parr, A.J., Hilton, M.G., Walton, N.J., 1990. Properties of transformed root cultures. In: Charlwood, B.V., Rhodes, M.J.C. (Eds.), Secondary Products from Plant Tissue Culture: Proceedings of the Phytochemical Society of Europe, vol. 30. Clarendon, Oxford, pp. 201–225.

Slightom, J.L., Durand-Tardif, M., Jouanin, L., Tepfer, D., 1986. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. Identification of open reading frames. J. Biol. Chem. 261, 108–121.

Wang, H.K., Lin, Z.D., He, K., Wan, S.W., 1986. Studies on the chemical constituents of *Saussurea involucrata*. Yao Xue Xue Bao 21, 680–682.

Wang, B.F., Lu, J., Guan, J.Y., 1996. The extraction of *Saussurea involucrata* injection. Chin. Pharm. J. 31, 299–301.

Wang, W.Q., Heideman, L., Chung, C.S., Pelling, J.C., Koehler, K.J., Birt, D.F., 2000. Cell-cycle arrest at G_2/M and growth inhibition by apigenin in human colon carcinoma cell lines. Mol. Carcinogen. 28, 102–110.

White, F.F., Nester, E.W., 1980. Hairy root: plasmid encodes virulence in *Agrobacterium rhizogenis*. J. Bacteriol. 141, 1134–1141.

Yin, F., Giuliano, A.E., Van Herle, A.J., 1999. Signal pathways involved in apigenin inhibition of growth and induction of apoptosis of human anaplastic thyroid cancer cells. Anticancer Res. 19, 4297–4303.

Yoshikawa, T., Furuya, T., 1987. Saponin production by cultures of *Panax ginseng* transformed with *Agrobacterium rhizogenes*. Plant Cell Rep. 6, 449–453.

Zhang, L., Ding, R.X., Chai, Y.R., Bonfill, M., Moyano, E., Oksman-Caldentey, K.-M., Xu, T.F., Pi, Y., Wang, Z.N., Zhang, H.M., Kai, G.Y., Liao, Z.H., Sun, X.F., Tang, K.X., 2004. Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc. Natl. Acad. Sci. USA 101, 6786–6791.

Zhao, L., Wang, X.L., 2003. Research on chemical composition, pharmacology and its clinic application of *Saussurea involucrata*. J. Southwest National Coll. 29, 424–428.

Zhao, D.X., Fu, C.X., Chen, Y.Q., Ma, F.S., 2004. Transformation of *Saussurea medusa* for hairy roots and jaceosidin production. Plant Cell Rep. 23, 468–474.

Zheng, R.L., Liu, G.S., Xing, G.X., Jia, Z.J., Du, M., Tan, L.Q., 1993. Free radical scavenging and antifatigue activities of *Saussurea involucrata* polysaccharides. Acta Pharmacol. Sin. 14 (Suppl.), 47–49.