Plant Cell Tiss Organ Cult (2017) 130:117–130
DOI 10.1007/s11240-017-1208-5

ORIGINAL ARTICLE

In vitro regeneration, *Agrobacterium*-mediated transformation, and genetic assay of chalcone synthase in the medicinal plant *Echinacea pallida*

Hsin-Mei Wang[1,2] · Shih-Tong Jeng[2] · Kin-Ying To[1]

Received: 20 December 2016 / Accepted: 24 March 2017 / Published online: 1 April 2017

Abstract In vitro plant regeneration was established in *Echinacea pallida*, a plant that is commonly used as a folk medicine to treat the common cold, fevers, inflammation and so on. Conditions for callus induction, lateral root and shoot regeneration were determined. Subsequently, two vectors pCHS and pOSAG78, carrying different selection marker genes resistant to kanamycin and hygromycin, respectively, were independently used to transform leaf explants of *E. pallida* using an *Agrobacterium*-mediated method. Genomic PCR analysis confirmed the presence of the transgene and selection marker gene in obtained transgenic lines. Southern hybridization indicated that the T-DNA insertion in some transgenic *E. pallida* was single copy. Among them, transformants carrying *Petunia* chalcone synthase (CHS) were selected for further study. CHS is a key enzyme in the biosynthesis of diverse flavonoids including anthocyanin pigmentation. Here, we analyzed the roles and compared the gene expression of two clusters of *CHSs, EpaCHS-A* and *EpaCHS-B* (*EpaCHS-B1* and *EpaCHS-B2*), isolated from *E. pallida*. Two of the genes, *EpaCHS-A* and *EpaCHS-B1*, were abundantly expressed in petals, whereas *EpaCHS-B2* was expressed at high levels in leaves. The expression of *EpaCHSs* remained constant in leaves and roots of *Petunia CHS* transformants, while *EpaCHS-B2* expression was changed in flowers of transgenic plants. The biosynthesis of caffeic acid derivatives, cichoric acid and caftaric acid, was increased in leaves and roots of CHS transformants, respectively, while the amount of echinacoside in roots of transgenic plants was decreased. This is the first report on genetic engineering of *E. pallida*. The information contained herein can be used as a tool for further study of the biological pathways and secondary metabolism of specific compounds from medicinal *Echinacea* species.

Keywords *Agrobacterium* · Caffeic acid derivatives (CADs) · Chalcone synthase (CHS) · *Echinacea pallida* · High-performance liquid chromatography (HPLC) · Metabolic engineering · Real-time PCR

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzyladenine
CADs	Caffeic acid derivatives
CaMV	Cauliflower mosaic virus
CHS	Chalcone synthase
DC	3,6-Dichloro-*o*-anisic acid
Epa	*Echinacea pallida*
GA	Gibberellin
GA_3	Gibberellin A_3
GUS	β-Glucuronidase
HPLC	High-performance liquid chromatography
hptII	Hygromycin phosphotransferase II
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
MS	Murashige and Skoog (1962)
NAA	α-Naphthaleneacetic acid
nos	Nopaline synthase

Electronic supplementary material The online version of this article (doi:10.1007/s11240-017-1208-5) contains supplementary material, which is available to authorized users.

✉ Kin-Ying To
kyto@gate.sinica.edu.tw

[1] Agricultural Biotechnology Research Center, Academia Sinica, Taipei, Taiwan

[2] Institute of Plant Biology and Department of Life Science, National Taiwan University, Taipei, Taiwan

nptII Neomycin phosphotransferase II
OSAG *Oncidium* senescence-associated gene

Introduction

Echinacea species have been used to treat the common cold, headaches, fevers, inflammation, and used against infections and for stimulation of various immune cells for hundreds of years in North America and Europe (Barrett 2003). Among the dozen or so species that make up the *Echinacea* genus, three species, namely *Echinacea purpurea* (also known as purple coneflower), *Echinacea pallida* (also known as pale purple coneflower) and *E. angustifolia* (also known as narrow-leaf coneflower), are commonly used as medicinal plants. These three species share similar morphological characteristics and pharmacological use. Products made from *E. purpurea, E. pallida* and *E. angustifolia* are sold commercially as herbal supplements; however, there are significant differences in the chemical composition of these species (Binns et al. 2002; Laasonen et al. 2002; Barrett 2003; Barnes et al. 2005; Mistríková and Vaverková 2007). Many years are required to breed inbred lines and some species are self-incompatible (Ison et al. 2014). Clonal micropropagation is an approach that is capable of producing large numbers of genetically similar, disease-free individual plants with superior characteristics in a short period of time and limited space. This approach also has potential for the development of techniques to facilitate phytochemical production of high quality and study new methods of biosynthesis (Abbasi et al. 2007a, b). Previous reports have described hairy root culture and genetic engineering of *E. purpurea* (Koroch et al. 2002; Liu et al. 2006), but there have been few reports on strategies about genetic engineering of *E. pallida*.

Conventional propagation of *Echinacea* includes seed germination and protocols for optimum growth (Zheng et al. 2006; Maria et al. 2012; Qu and Widrlechner 2012), whereas in vitro tissue culture of *Echinacea* provides rapid multiplication and genetic modification (Harbage 2001; Wang and To 2004). Regeneration of *Echinacea* can occur by organogenesis and/or somatic embryogenesis. Plant growth regulators (PGRs) play a key role in adjusting tissue differentiation in vitro (Koroch et al. 2003; Jones et al. 2007). In most studies, the presence of 6-benzyladenine (BA) was essential for shoot organogenesis in *Echinacea* species. Murashige and Skoog (MS; Murashige and Skoog 1962) medium with different combinations of BA, kinetin and indole-3-butyric acid (IBA) was effective in inducing shoot organogenesis from intact leaves and the medium containing 3,6-dichloro-*o*-anisic acid (DC) or 2,4-dichlorophenoxyacetic acid (2,4-D) affected somatic embryogenesis from hypocotyls of *E. pallida* (Lakshmanan et al. 2002). The BA and α-naphthaleneacetic acid (NAA) combination was used for regeneration of plantlets via indirect shoot organogenesis from the leaf explants of *Echinacea* (Koroch et al. 2003; Sauve et al. 2004; Wang and To 2004). MS supplemented with BA (26.6 μM) and NAA (0.11 μM) achieved the optimum shoot regeneration frequency (63%) and was associated with a high number of shoots per explant of *E. pallida* (Koroch et al. 2003).

All species of *Echinacea* are grown as medicinal and ornamental plants (McKeown 1999; Walck et al. 2002). Flavonoids are a class of plant secondary metabolites and flavones and flavonols are used to change color hues. Chalcone synthase (CHS) is the first committed step of flavonoid biosynthesis. In *E. purpurea*, biosynthesis of caffeic acid derivatives (CADs) and anthocyanins, which are derived from flavonoids were increased in hairy root cultures grown in the light (Abbasi et al. 2007b). Regulation of *CHS* genes was thus designed to change the flavonoid biosynthesis of *E. pallida*. In this study, two vectors pCHS (carrying expression cassettes for selection marker *nptII* conferring kanamycin resistance and *Petunia CHS*) and pOSAG78 (carrying expression cassettes for *OSAG78* from *Oncidium* orchid, selection marker *hptII* conferring hygromycin resistance and *GUS* reporter gene) were employed independently to transform *E. pallida* using an *Agrobacterium*-mediated method. The vector pCHS bearing the *Petunia CHS* gene, which is capable of increasing the expression of exogenous *CHS*, was introduced into *E. pallida* and the interactions of the three *Echinacea CHS* genes were analyzed. *OSAG78* in another vector pOSAG78 encodes a patatin-like protein isolated from senescent flowers in *Oncidium* Grower Ramsey (Lin et al. 2011). Patatin-like proteins have lipase and phospholipase A activities. Previously, transgenic *Arabidopsis* plants overexpressing *OSAG78* showed higher lipase activity and altered phenotypes including smaller leaves, rounder flowers and later flowering (Lin et al. 2011). Here pOSAG78 was chosen to investigate (1) whether hygromycin can be used as a selection agent in *E. pallida*; and (2) whether GUS expression in vector pOSAG78 can be visualized in transgenic *E. pallida* plants. We did not focus on the function and effect of overexpressing *OSAG78* on transgenic *E. pallida* plants. The results of this study may provide an alternative method for the study of the biological pathways and secondary metabolites in *Echinacea*. The transformation system of *E. pallida* established provides the possibility of investigating the effect of expression of various genes, and may contribute to the successful modification of medicinal plants for higher yield of natural products.

Materials and methods

Plant materials and culture conditions

Seeds of *E. pallida* were purchased from Johnny's Selected Seeds (Maine, USA), surface sterilized according to a previously published procedure (Wang and To 2004) and transferred to a basal nutrient medium (MS) containing MS salts (Murashige and Skoog 1962), 3% sucrose and 0.7% agar. The pH value of the medium was adjusted to 5.7. Seeds were incubated at 25 °C with a 16-h illumination (100 μmol m^{-2} s^{-1}) for germination. Leaves from in vitro germinated seedlings were used as the source of explants to generate the shoot multiplication and in vitro explants were used to establish plant regeneration and the *Agrobacterium*-mediated transformation system.

Plant regeneration from in vitro leaf explants

For callus induction and shoot initiation, the leaves were cut into small pieces (0.5 × 0.5 cm^2) onto a callus induction Ref medium (MS salts; 30 g l^{-1} sucrose; 0.4 mg l^{-1} thiamine; 100 mg l^{-1} *myo*-inositol; 0.02 mg l^{-1} NAA; 6 mg l^{-1} BA; 0.8% Bacto-agar; pH 5.7) in the dark for 3 weeks and then transferred to sub-culture shoot induction N0 medium (MS salts; 30 g l^{-1} sucrose; 0.004 mg l^{-1} NAA; 0.8 mg l^{-1} BA; 0.8% Bacto-agar; pH 5.7) in a 22 °C growth chamber with a photoperiod of 8-h light and 16-h darkness. Shoots about 4–5 cm long were transferred to MS medium supplemented with a low concentration of IBA and GA_3, alone or in combination, for 8 weeks. The numbers of roots per shoot were recorded after 16 weeks of culture. For acclimatization, the plantlets were transferred to pots in a mist chamber for 2 weeks and then moved to the greenhouse at Academia Sinica, Taipei, Taiwan.

Plasmid construction

The plasmid pCHS contains a full-length cDNA encoding *Petunia* chalcone synthase (*PhCHS*; 1170 bp) gene and a neomycin phosphotransferase II (*nptII*; 795 bp) gene driven by the cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthase (*nos*) promoter, respectively (Wang and To 2004). In addition, pOSAG78 was constructed by carrying a full-length cDNA of *Oncidium* senescence-associated gene (*OSAG78*; 1224 bp) (Lin et al. 2011) alone downstream of the CaMV 35S promoter and then cloned into binary vector pCAMBIA1301 which contains a hygromycin phosphotransferase II (*hptII*) gene driven by the CaMV 35S promoter. The pCHS confers kanamycin resistance whereas the pOSAG78 confers hygromycin resistance (Supplementary Fig. S1). These vectors were transformed individually into the *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Gene Pulser II, Bio-Rad) and bacteria were prepared for transformation as previously described in AB/kanamycin medium (Wang and To 2004).

Agrobacterium-mediated transformation

Leaves from in vitro explants were cultured in Ref medium for 2 days. The 2-day pre-cultured leaves were transferred into the *Agrobacterium* suspension (OD_{600} = 0.8–1.0) containing 100 μM acetosyringone (AS; Fluka Chemika, Switzerland), and soaked for 1 h, and then blotted dry on sterile filter paper to remove excess bacterial suspension. The infected explants were incubated Ref-co medium (50 g l^{-1} glucose in Ref medium was substituted by 30 g l^{-1} sucrose and supplemented with 100 μM AS) for 5 days in the dark. After co-cultivation, explants were washed by shaking in a Ref solution containing 200 mg l^{-1} timentin (Duchefa, The Netherlands) for inhibition of *Agrobacterium*. The explants were blotted dry and transferred onto selection medium containing Ref medium or N0 medium supplemented with 200 mg l^{-1} timentin and 50 mg l^{-1} kanamycin (for pCHS vector) or 10 mg l^{-1} hygromycin (for pOSAG78 vector) for approximately 2 weeks. For the pre-selection phase, after co-cultivation, leaf explants were cultured on Ref medium supplemented with 200 mg l^{-1} timentin for 2 weeks, and then selection was carried out as detailed above. Afterwards, putative transformants were sub-cultured in N1 medium (MS salts; 30 g l^{-1} sucrose; 0.004 mg l^{-1} NAA; 0.2 mg l^{-1} BA; 0.8% Bacto-agar; pH 5.7; antibiotics), transferred to rooting medium and then grown in pots.

Molecular analysis of putative transformants

DNA was extracted from the leaf using the CTAB method (Michiels et al. 2003) and PCR-amplification was carried out using primer pairs: Kana-5′/Kana-3′, GUS-5′/GUS-3′, and Hygro-5′/Hygro-3′ (Supplementary Table S1) to detect the kanamycin resistance gene, the *GUS* gene, and the hygromycin resistance gene, respectively. GUS staining of the leaves and roots of the transformants OSAG78 was carried out as previously described (Wang et al. 2015).

Southern blotting

For transformant analysis, genomic DNA (15 μg) was digested with *Eco*RI (New England BioLabs, MA, USA) at 37 °C overnight. After electrophoresis on a 0.8% agarose gel in TAE buffer, the gels were denatured and blotted onto Hybond-N+ nylon membrane (GE Healthcare, UK). The non-radioactive DIG-labelled 35S-pro-CHS probe was prepared by PCR with 35S pro-5′/CHS-R1 (Supplementary Table S1) as primers and plasmid pCHS as template; the non-radioactive DIG-labelled Hygro probe was prepared

by PCR with Hygro-5′/Hygro-3′ (Supplementary Table S1) as primers and plasmid pOSAG78 as template. For endogenous *CHS* gene identification in *E. pallida*, 15 µg genomic DNA of wild-type was digested by restriction enzymes *Bsc*GI, *Spe*I, *Nde*I, *Alf*II, *Ban*I, *Nco*I, *Nsi*I, *Eco*RV, *Hin*dIII or *Sca*I (New England BioLabs, MA, USA), and hybridization with the probes CHS-A and CHS-B1 was carried out using primer pairs comprising CHSA-5′/CHSA-3′ and CHSB1-5′/CHS1-3′ (Supplementary Table S1) based on information from *EpaCHS-A* and *EpaCHS-B1* (Supplementary Fig. S2), respectively. Conditions for probe preparation, membrane hybridization and detection were as previously described (Wang and To 2004).

Total RNA isolation, reverse transcription (RT)-PCR and real-time PCR

RNA was extracted using the Trizol method (Invitrogen, http://www.invitrogen.com) according to the manufacturer's protocol and the concentration was determined by the A_{260} measured using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). RT reaction was performed using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany). Each reaction consisted of 150 ng RNA amplified for 35 cycles. *E. pallida* actin gene served as an internal control. Specific primer sets for amplification of the full-length *Petunia CHS* cDNA and *Oncidium OSAG78* cDNA are listed in Supplementary Table S1. cDNA synthesis was performed by the M-MLV Reverse Transcriptase and RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Carlsbad, CA, USA). The real-time PCR reaction was carried out using KAPK SYBR FAST qPCR Kit (KAPABIOSYSTEMS, Boston, USA) and the reaction was performed on an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Three biological replicates were used for quantification, and data analysis was performed on an ABI PRISM 7500 thermocycler. The ratios relative to the lowest value, fold-change $=2^{-\Delta(\Delta C_T)}$, are shown as the relative expression level. Based on the isolated sequences of *EpaCHS* genes, the PCR fragments for detecting *EpaCHS-A, EpaCHS-B1* and *EpaCHS-B2* were in positions 855–1027, 1–228, and 296–486 of Supplementary Fig. S2, respectively.

Cloning of *CHS* genes in *E. pallida* and phylogenetic analysis

To investigate *CHS* genes in *E. pallida*, degenerate primers were designed based on homologous sequences from the National Center for Biotechnology Information (NCBI). The candidate sequences were amplified using the SMART RACE cDNA amplification kit (Clontech, Mountain View, CA) and sequences were assembled by ContigExpress of Vector NTI Suite 9.0 (Invitrogen, Carlsbad, CA) to obtain full-length cDNA encoding *E. pallida* chalcone synthase. The expected size from start codon and stop codon was gel-purified (Qiagen, Valencia, CA), cloned into the pGEM-T Easy vector (Promega, WI, USA), and transformed into *E. coli* JM109 competent cells. Plasmid DNA was isolated and then sequenced. Nucleotide sequences encoding chalcone synthase in *E. pallida* were submitted into GenBank with the following accession numbers: EpaCHSA, KY081676; EpaCHSB1, KY094647; EpaCHSB2, KY094648. For phylogenetic analysis, protein sequences of selected chalcone synthases were obtained from GenBank. The accession numbers are: Petunia CHSA, AAF60297; Petunia CHSB, CAA32732; Petunia CHSD, CAA32733; Petunia CHSG, P22927.2; Petunia CHSJ, CAA32737; Antirrhinum CHS, CAA27338; Arabidopsis CHS, NP_196897; Dahlia CHS1, BAK08887; Dahlia CHS2, BAK08888; Gerbera CHS1, CAA86218; Gerbera CHS2, CAA86219; Gerbera CHS3, CAA86220; Ipomoea CHSA, AAB62591; Ipomoea CHSB, AAC49030; Pea CHS1, CAA44933; Pea CHS2, CAA44934; Pea CHS3, CAA44935. A neighbor-joining phylogenetic tree based on the alignment of the polypeptides was constructed with the ClustalW program (Vector NTI Suite 9.0; Invitrogen, Carlsbad, CA) and calculated with MEGA software 6.0 by maximum likelihood with bootstrap resampling of 1000 replicates (Tamura et al. 2011).

High-performance liquid chromatography and mass spectrometry analyses

Phenolic acids (caftaric acid, cichoric acid, and echinacoside) in extracts of *E. pallida* were prepared with 60:40 methanol/water (Brown et al. 2010). Tissues (100 mg) were extracted with 1 ml of extraction solvent, and each sample was mixed with 1 ml of 10:90 DMSO/methanol containing 3 µg luteolin-7-*o*-glucoside (Extrasynthese, Genay Cedex, France) as an internal standard. High-performance liquid chromatography (HPLC) analysis was conducted using an Agilent 1200 Chemstation HPLC system and a C18 reverse-phase column (YMC-Pack Polymer C18, YMC, Japan) as described (Wang et al. 2015). Phenolic compounds were monitored at 332 nm, and the mobile phase consisted of 0.1% formic acid in H_2O (A) and acetonitrile (B), and separations were performed using the following solvent gradient: 0 (10% B), 2 (15% B), 12 (30%B), 20 (65% B), 27 (80% B), 32 (90% B), and 50 min (10% B) (Pellati et al. 2012). The injection volume was 20 µl. The fractions of caftaric acid, cichoric acid and echinacoside were collected using HPLC and further analyzed by LC–MS (Pellati et al. 2012; Thomsen et al. 2012).

Results and discussion

In vitro plantlet regeneration

At present, even though there are several micropropagation strategies available to *Echinacea* manufacturers (Harbage 2001; Lakshmanan et al. 2002; Sauve et al. 2004), only a few integrated regeneration programs are successful in *E. pallida* (Lakshmanan et al. 2002; Koroch et al. 2003). In the initial shoot regeneration experiment, leaves were excited from in vitro-grown plantlets, cut into small pieces, and cultured on MS medium supplemented with 0.02 mg l^{-1} NAA and 6 mg l^{-1} BA in a growth chamber at 25 °C under a photoperiod of 16-h illumination (100 µmol m^{-2} s^{-1}) and 8-h darkness for 1 month. As a result, frequency of 60% leaf discs producing shoots was scored; and it is consistent with a high number of shoots per explant as previously described (Koroch et al. 2003). The efficiency of root induction resolves the same problem in whole-plant regeneration of *E. pallida* micropropagation (Harbage 2001; Lakshmanan et al. 2002; Koroch et al. 2003). Of the several combinations tested, MS medium supplemented with MS vitamins, 0.2 mg l^{-1} IBA and 3 mg l^{-1} GA_3 was the most effective for root regeneration from explants. Roots were developed and callus production at the basal cut end of shoots was inhibited (Table 1). Moreover, the percentage of rooting reached 78–89% of all the explants and at least 3 roots per plantlet were observed after 16 weeks of rooting treatment. Generally, the average number of roots per plantlet was over five (Fig. 1) and more than 90% of the plantlets propagated through tissue culture survived acclimation. Auxins induce plant rooting and regulate root growth (Overvoorde et al. 2010; Saini et al. 2013; Perianez-Rodriguez et al. 2014; Chandler and Werr 2015), but auxin is not necessary for root organogenesis in in vitro regeneration of *Echinacea* species (Harbage 2001). In addition, even a very low concentration of auxins inhibits *Echinacea* root induction. Presence of IBA above 1 µM caused considerable callus production at the basal cut end of shoots, and it is likely that the auxin-induced callus at the stem base might have inhibited root production (Lakshmanan et al. 2002). However, it was reported that medium without regulator as well as medium with IBA at concentrations from 0.49 to 9.8 µM resulted in no significant difference in the efficiency of rooting in *E. pallida* (Koroch et al. 2003).

The physiological functions of GA in root growth have been discussed for *Arabidopsis* (Tanimoto 2012; Mauriat et al. 2014) and crosstalk between indole-3-acetic acid (IAA) and GA has been demonstrated in the signaling system (Fu and Harberd 2003; Gou et al. 2010). Supplementation of GA_3 was reported to induce increases in secondary metabolite production and also affected cell viability and root morphogenesis of *E. purpurea* in hairy root cultures (Abbasi et al. 2012). *E. purpura* with a fibrous root system was easily rooted without auxins in vitro; however, rooting response of both *E. pallida* and *E. angustifolia* in taproot systems in the field was poor (Harbage 2001; Koroch et al. 2003).

Table 1 Effects of IBA or GA on *E. pallida* root initiation

Medium[a]	Rooting (%)	Callus (%)
VG	68	21
VI	37	21
VGI	78	28
GI	89	45
G	55	5

Shoots about 4–5 cm long were used for each medium (1 shoot per tube and approximately 19 explants per treatment) for a period up to 8 weeks

[a]Medium (MS salts; 3% sucrose; 0.72% agar and pH 5.7) supplemented with MS vitamin (V), 0.2 mg l^{-1} indole-3-butyric acid (I), or 3 mg l^{-1} gibberellic acid (G)

Agrobacterium-mediated transformation

Transformation via leaf organogenesis has been described in many species (Dayal et al. 2003; Husaini 2010; Van et al. 2010; Liu et al. 2011; Verma and Mathur 2011). Using leaf tissue as an explant source in transformation for further secondary metabolism investigation provides a convenient approach for non-destructive selection and avoids the outcrossing reproductive nature of the genus from seed or seedling explants (Koroch et al. 2003). In our transformation experiment, we found that *E. pallida* leaf explant is very sensitive to *Agrobacterium* infection and pre-culture was found to improve the survival rate of regenerated shoots under selection medium. Moreover, selection agents interfered with regeneration of *E. pallida* transformants (Table 2; Fig. 2a, b), and there was a significant effect on percentage of survival shoots, causing an increase of 11–23% of leaf discs regenerating shoots with a 14-day pre-selection phase, while the explants rarely regenerated shoots when subjected to selection pressure immediately after co-cultivation. When the inoculated leaves were pre-selected on shoot regeneration medium, they showed tiny brown calli (Fig. 2c, d), and some calli and shoots became necrotic on different selective media, while putative transgenic shoots further elongated (Fig. 2e–h). These shoots regenerated into plantlets on rooting medium and were then grown in pots (Fig. 2i, j). Pre-incubation before co-cultivation and pre-selection of explants effectively minimized such inhibition which was not observed in the transformation of *E. purpurea* (Wang and To 2004) and tomato (Van et al. 2010). Increases in the efficiencies of transformation using pre- and/or post-agroinfection have been reported in

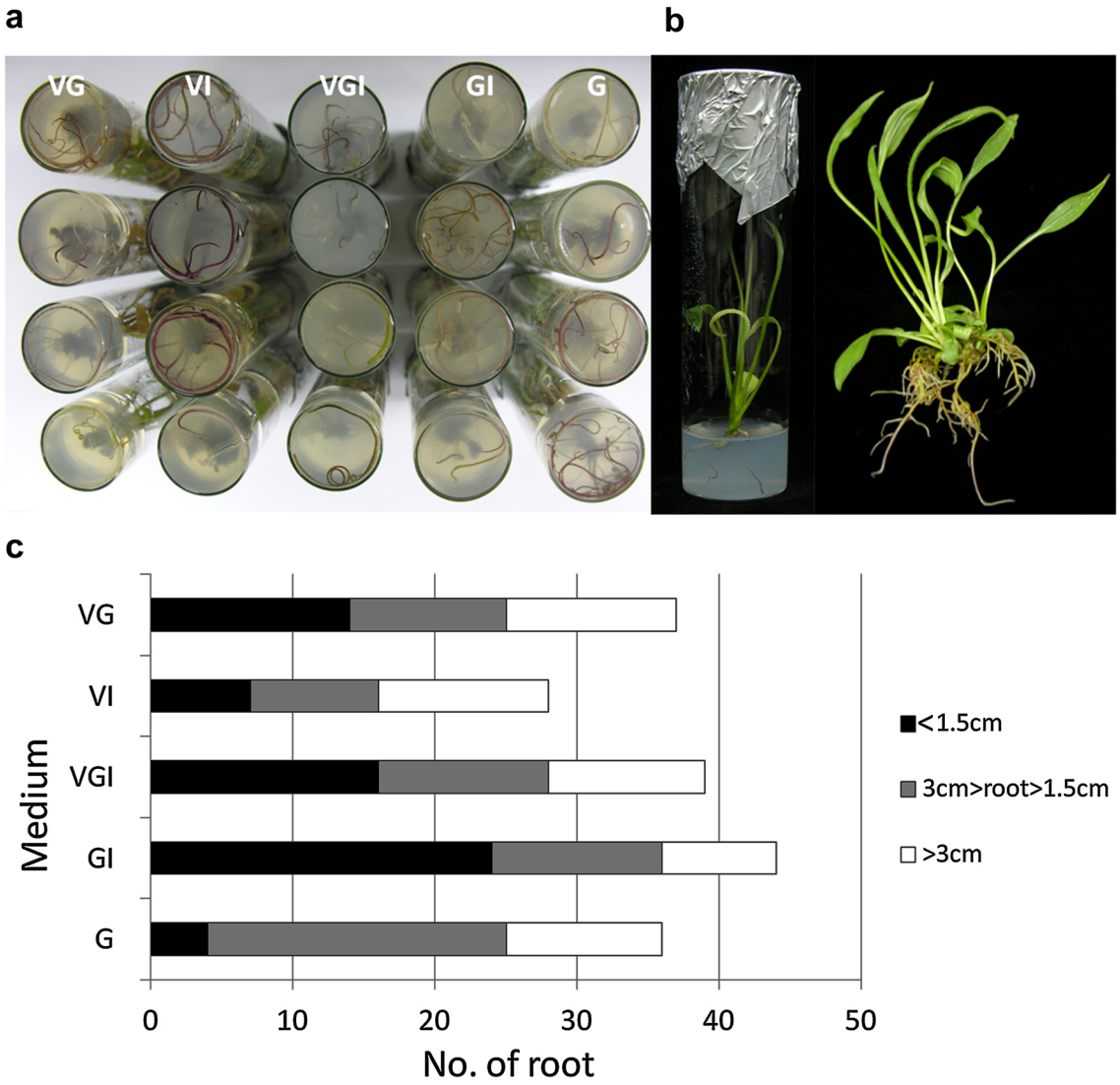

Fig. 1 Rooting and whole-plant regeneration of *E. pallida*. **a** Photograph from the bottom of tubes showing induction and growth of multiple roots from single shoot explant. **b** In vitro healthy plantlet. **c** Five plantlets were used for root length analysis under medium treatments at 16 weeks after transplantation

strawberry (Husaini 2010), petunia (Liu et al. 2011), orchid (Chai et al. 2002), *Catharanthus roseus* (Verma and Mathur 2011), and *Brassica napus* (Ovesná et al. 1993; Cardoza and Stewart 2003). In this study, the percentage of shoot regeneration under kanamycin-containing medium from *E. pallida* was 24% (Table 2) which is lower than for *E. purpurea* (47%; Wang and To 2004).

Selectable marker genes have been pivotal in identifying and selecting transformed progeny. Plant transformation technologies require the use of different marker genes to monitor the multi-genic traits of transgenic plants by the multi-step transformation. In our studies, we tested the most commonly used antibiotics, kanamycin and hygromycin (Miki and McHugh 2004). It is commonly found that some selection systems are more effective in certain plant species and regeneration systems than others. An example is the lower efficiency of kanamycin resistance as a selection system in cereals than in dicots (Twyman et al. 2002). *E. pallida* has lower shoot regeneration frequency with hygromycin (Table 2), and transgenic lines OSAG78-4 and OSAG78-6 had retarded shoot regeneration.

Characterization of transgenic plants

PCR amplifications of DNAs from CHS transformants carrying kanamycin-resistant *nptII* gene were considered to be transgenic positive plants (Fig. 3a). In parallel, Southern blot analysis was carried out to estimate transgenic copy

Table 2 Effects of pre-selection phase on differentiation of *E. pallida* transformants on selective regeneration media containing different levels of antibiotics

Vector/gene/antibiotic	No. of leaf disc	No. of callus	No. of embryo and shoot	No. of survival shoot under antibiotic (% of plantlets)
No pre-selection[a]				
pBI121/*CHS*/kana	279	63	10	1 (0.36)
pCAMBIA1301/*OSAG78*/hygro	675	1	ND	0 (0)
Pre-selection[b]				
pBI121/*CHS*/kana	121	108	99	29 (23.97)
pCAMBIA1301/*OSAG78*/hygro	128	121	51	15 (11.72)

Tissue explants (approximately 12 leaf discs per Petri dish) were excised from 4 to 5 cm long shoots for a period up to 3 months

ND not determined

[a]No pre-selection: after co-cultivation for 5 days, leaf discs were transferred onto callus induction (Ref) medium or shoot induction (N0) medium supplemented with 200 mg l^{-1} timentin and 50 mg l^{-1} kanamycin (for pCHS vector) or 10 mg l^{-1} hygromycin (for pOSAG78) for 2 weeks

[b]Pre-selection: between co-cultivation and selection, leaf discs were cultured on callus induction (Ref) medium or shoot induction (N0) medium supplemented with 200 mg l^{-1} timentin for 2 weeks

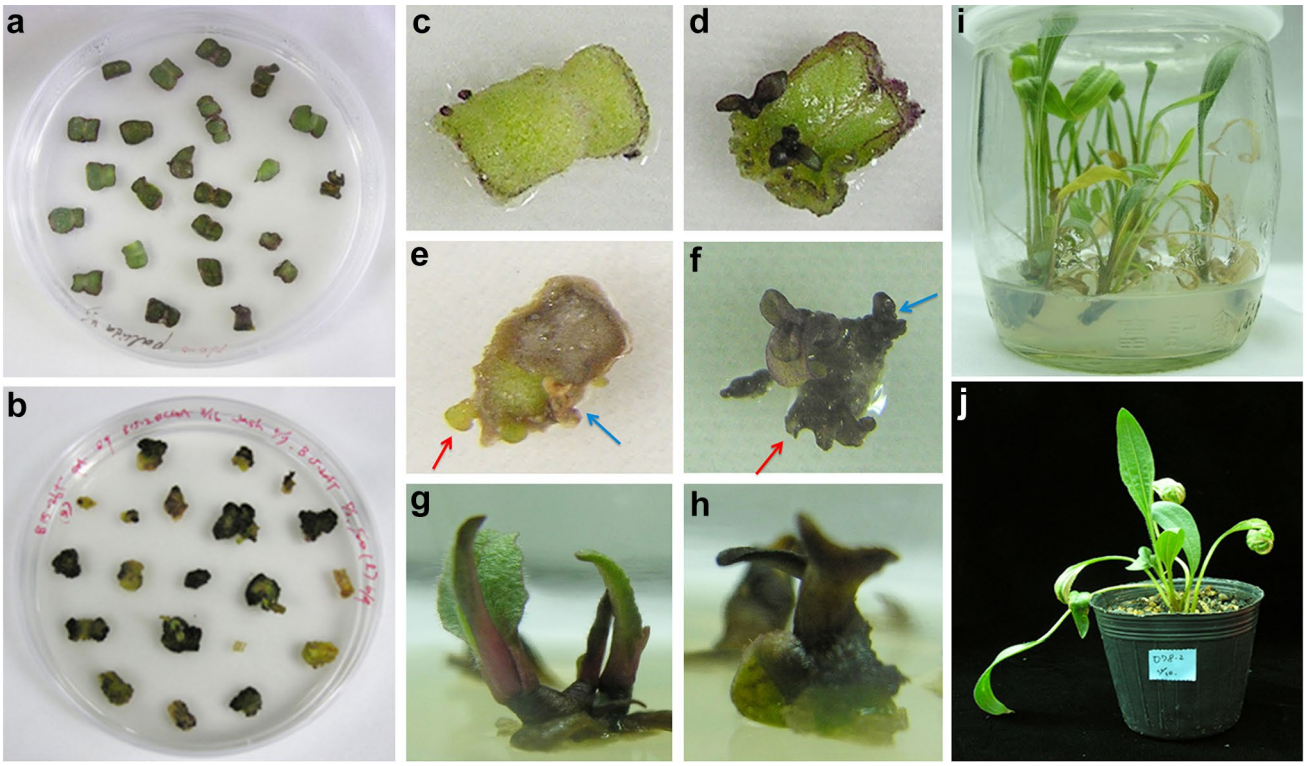

Fig. 2 *Agrobacterium*-mediated transformation of the medicinal *E. pallida*. A typical no pre-selection plate (**a**) and a pre-selection plate (**b**). Formation of tiny calli (**c**) and shoot buds (**d**), *brown in color*. **e, f** Putative transformants (*red arrow*) and non-transformants (*blue arrow*) showed green shoot buds and shoot necrosis, respectively, in selection medium containing antibiotics. **g** An enlarged photo showing transformants with healthy development of shoots and leaves. **h** An enlarged photo showing non-transformants with brown necrotic shoots that finally died. Transgenic plantlets grown in vitro (**i**) and transferred to a pot (**j**)

number and demonstrated that all transgenic plants were independent integration events (Fig. 3b). Transgenic CHS-3, CHS-4 and CHS-5 were suggested to be single copy while CHS-2 and CHS-7 had multiple copies. However, only *nptII* gene conferring kanamycin resistance could be detected in CHS-1 in which *Petunia CHS* was lost as

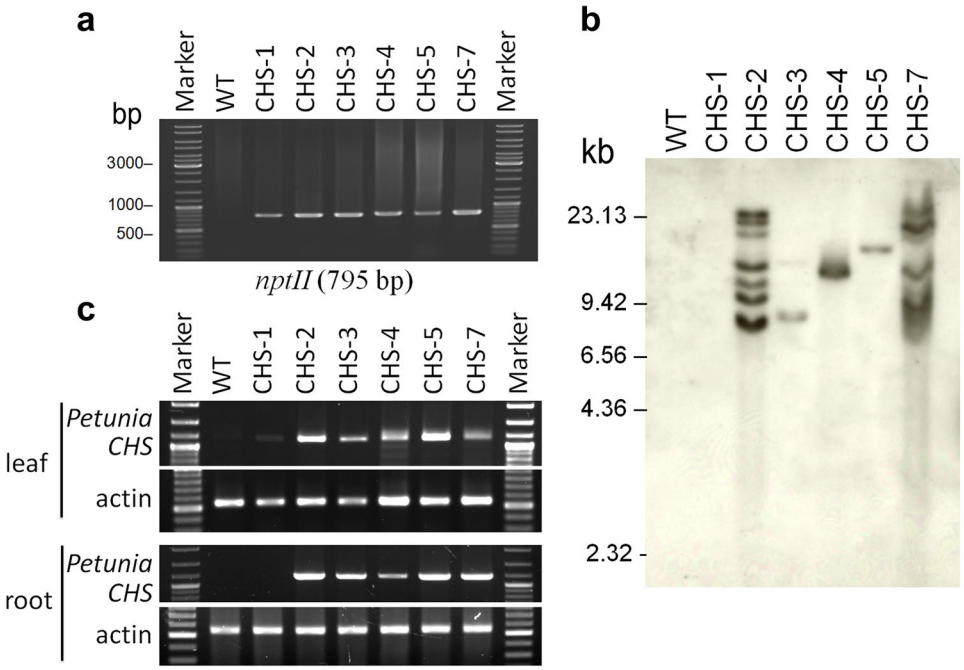

Fig. 3 Characterization of CHS transgenic plants. **a** Genomic PCR analysis of transgenic plants. The kanamycin resistance gene (*nptII*) was detected by PCR with primer set Kana-5′/Kana-3′. Vector construction and primer information are presented in Fig. S1 and Table S1, respectively. One microgram of DNA isolated from the extended leaves was used for genomic PCR. **b** Southern blot of six individual transgenic plants. Eighteen micrograms of DNA was digested with *Eco*RI and probed with DIG-labeled PCR product containing 35S promoter and *Petunia CHS* cDNA. **c** Expression patterns of *PhCHS* in the *E. pallida* leaf and root. Primer information is presented in Table S1

revealed by Southern blot analysis. Thus, in this study, the transformation efficiency from *E. pallida* was calculated as 4% (5/121) which is similar to *E. purpurea* (3.4%; Wang and To 2004). Genome-walking was further used to characterize the plant/T-DNA insertion sites in the transgenic CHS-3, CHS-4 and CHS-5. The genomic sequence in the T-DNA integration site in transformants had no similarity to nucleotide databases and EST databases (Supplementary Fig. S3). We found that 160 bases were deleted in a flanking sequence in CHS-3, and a reverse recombination was detected in the left border of CHS-5. In addition, the flanking sequences which were isolated from CHS-4 could not be confirmed by wild-type plants (data not shown). It seems complicated by this transformation process.

For transformant OSAG78, the reporter *GUS* gene and the hygromycin-resistant *hptII* gene were detected in genomic PCR analysis (Fig. 4a), and Southern blot analysis was also observed. A single hybridization band was revealed in OSAG78-1 and OSAG78-2; three hybridization bands were detected in OSAG78-5 (Fig. 4b). However, no *GUS* product of PCR amplification was detected in OSAG78-1 (Fig. 4c). Moreover, the large deletion of a transforming vector has been reported from transgenic plants (Wu et al. 2006; Wang et al. 2015). Truncation of T-DNA results in loss of transgene function, as was the case in our study for CHS-1 and OSAG78-1, in which only the selection marker was detected (Figs. 3, 4a). In addition, herb, floricultural plant, wheat, triticale, orchid and barley transgenic plants have been shown to carry incomplete T-DNAs after *Agrobacterium*-mediated transformation (Bartlett et al. 2008; Hensel et al. 2012; Tsai et al. 2012; Wang et al. 2012, 2015).

Histochemical staining for GUS activity produced blue coloration in the tissues of OSAG78-2 and OSAG78-5 but not OSAG78-1 (Fig. 4c). Unfortunately, transformants CHS-6, OSAG78-3, OSAG78-4 and OSAG78-6 did not survive due to pathogen infection.

Expression of foreign genes in transgenic plants was analyzed by RT-PCR (Figs. 3c, 4d). *Petunia CHS* was highly expressed in the leaves and roots of all transgenic lines except CHS-1 in which *Petunia CHS* was lost (Fig. 3b, c). The expression of *OSAG78* was only observed in OSAG78-2; its expression was not detectable in transgenic lines OSAG78-1 and OSAG78-5 (Fig. 4d). The expression of *OSAG78* was not detected, but GUS was highly expressed in OSAG78-5 (Fig. 4c, d).

Isolation and comparison of *E. pallida CHS* genes

In this study, we cloned *CHS*-like genes by conserved regions from the NCBI. Three copies of the *EpaCHSs* genes were found: *EpaCHS-A, EpaCHS-B1*, and *EpaCHS-B2* (Supplementary Fig. S2) and the presence of multiple *CHS* genes in the *E. pallida* genome was analyzed by Southern blotting (Fig. 5). The *CHS* multigene family has been divided into two subgroups according to the degree of nucleotide identity in the open reading frames (Durbin et al. 2000; Tuteja et al. 2004; Wan et al. 2011). Based on the homology sequences, one gene forming the original CHSA, and three DNA fragments producing the second duplicate subgroup were obtained

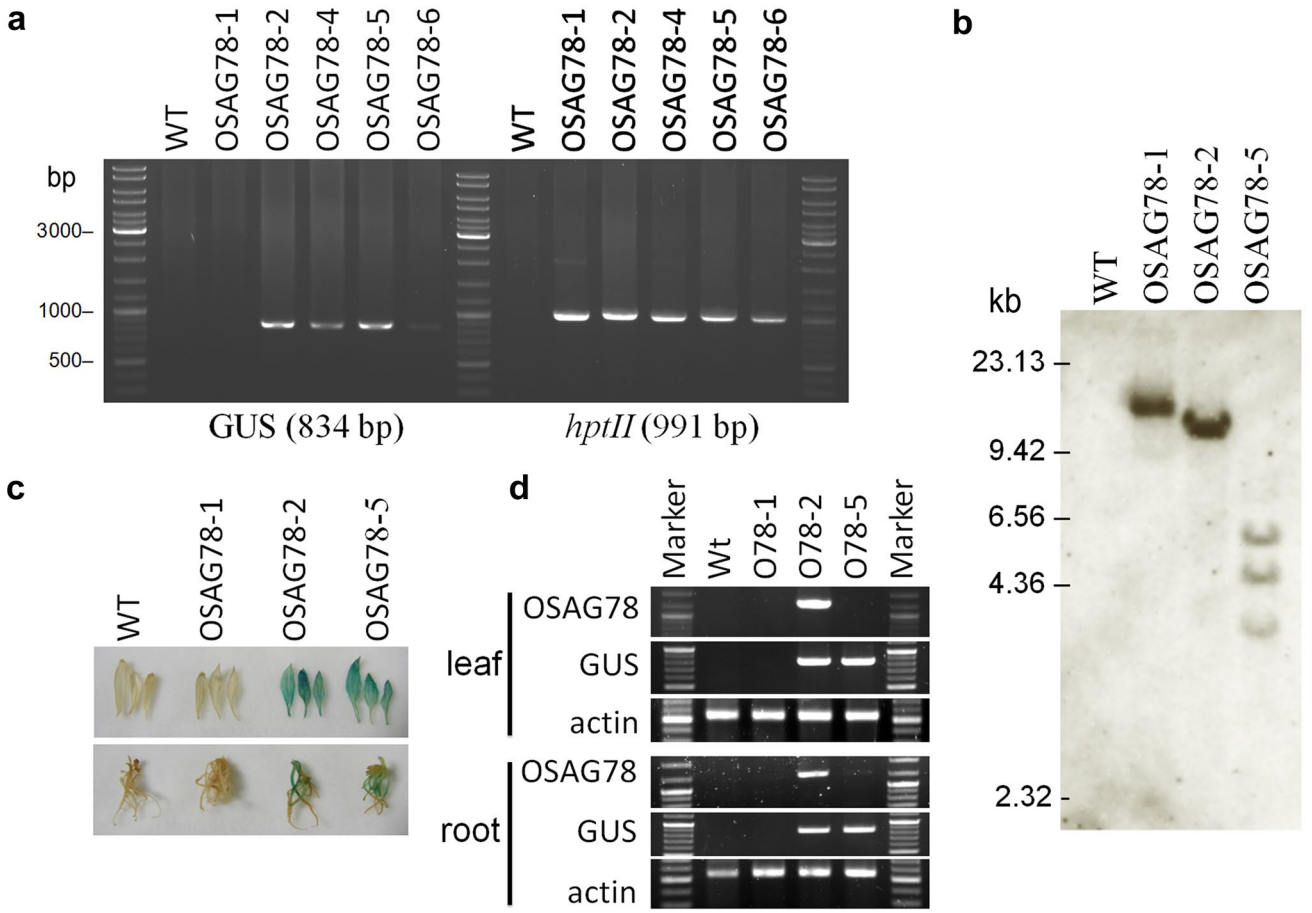

Fig. 4 Characterization of OSAG78 transgenic plants. **a** Genomic PCR analysis of transgenic plants. The *GUS* gene and hygromycin resistance gene (*hptII*) were detected by PCR with primer sets GUS-5′/GUS-3′ and Hygo-5′/Hygo-3′, respectively. **b** Southern blot of three individual transgenic plants. Eighteen micrograms of DNA was digested with *Eco*RI and probed with DIG-labeled PCR product of *Oncidium OSAG78* cDNA. **c** GUS stains of leaves and roots. **d** Expression patterns of *Onc. OSAG78* in *E. pallida*

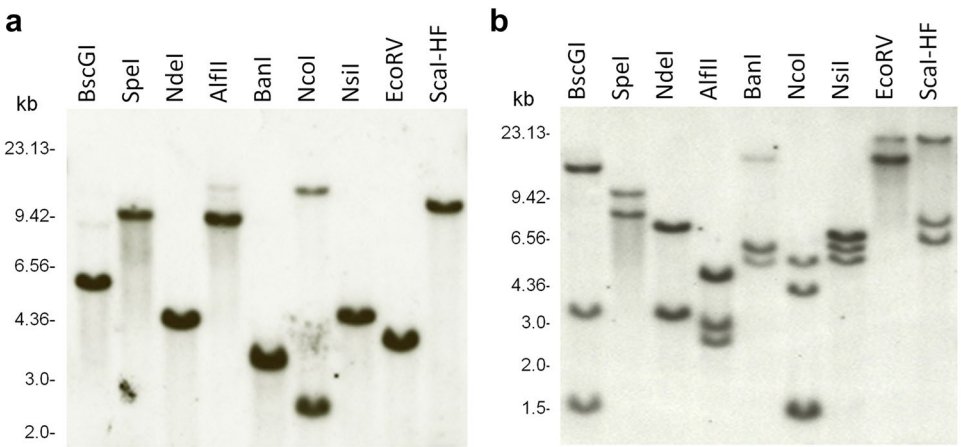

Fig. 5 Southern blot analysis of the *CHS* genes in *E. pallida*. Genomic DNA of wild type was digested by restriction enzymes and hybridized with the *E. pallida* CHS-A probe (**a**) and the CHS-B1 probe (**b**)

by the digestion of different restriction enzymes (Fig. 5). *EpaCHS-A* with 389 amino acids was classified as cluster A, and a total of 1366 base pairs (bp), including 1170 bp of exon and 196 bp of the single intron, were sequenced. The other two cDNA fragments *EpaCHS-B1* and *EpaCHS-B2* encoded 398 amino acids, with 86.7%

nucleotide similarity belonging to the *CHSB* subgroup. They exhibited 88.8% protein sequence identity. The two exons of the *EpaCHS-B1* gene are 188 and 1009 bp in length, respectively. They are separated by a 1070 bp intron. The *EpaCHS-B1* and *EpaCHS-B2* sequences were 68.6 and 66.9% identical to that of *EpaCHS-A*, respectively. Nucleotide sequences have been submitted into GenBank with accession numbers as follows: *EpaCHS-A*, KY081676; *EpaCHS-B1*, KY094647; *EpaCHS-B2*, KY094648. The sequence analysis suggested that more copies of *CHS* exist in the *E. pallida* genome.

The protein sequences of *EpaCHS-B1* and *EpaCHS-A* shared 78.4% identity, whereas the *EpaCHS-B2* and *EpaCHS-A* protein sequences shared 74.5% identity (Supplementary Fig. S4). The phylogenetic tree analysis (Supplementary Fig. S5) based on the chalcone synthase amino acid sequences revealed two notable characteristics. First, *Echinacea* CHS falls into *Dahlia* CHS of the Helianthodae subtribe. Second, the protein sequences of EpaCHSA and petunia CHSA encoded 389 amino acids, with 83.1% identity, and were therefore more similar than the CHSB subgroup (Supplementary Figs. S4, S5). The EpaCHSB1 and EpaCHSB2 protein sequences shared 81.5 and 77.3% identity to petunia CHSA, respectively. We used quantitative real-time PCR to confirm *EpaCHS* expression in various tissues, and the results indicated that *EpaCHS-A* and *EpaCHS-B1* are abundantly expressed in petals, whereas expression of *EpaCHS-B2* was strongly detected in leaves (Fig. 6).

The *CHS* family with a single intron is commonly found in the *CHS* genes of most angiosperms except for *Antirrhinus majus* (Sommer and Saedler 1986). The *CHS* genes of *E. pallida* contain an intron which splits a cysteine codon at a conserved position, and have identical amino acids at four chemically reactive residues (Cys164, Phe215, His303, Ans336 from *Medicago sativa*) of a well-known CHS-related enzyme (Wan et al. 2011). The CHS enzyme is encoded by a small multigene family in plants and most of the duplicated genes may have sub-functionalization and tissue-specificity (Durbin et al. 2000; Ohno et al. 2011; Wan et al. 2011). There was 78.4% identity between CHSA1 and CHSB1 in *E. pallida*, and the CHSB subgroup exhibited 88.8% identity (Supplementary Fig. S4). However, *EpaCHS-A1* and *EpaCHS-B1* are abundantly expressed in petals, and *EpaCHS-B1* and *EpaCHS-B2* have been shown to be regulated in different tissue (Fig. 6). In addition, *EpaCHS-B2* had Ile inserted before The194, but Ile254 was lost as an active site residue in CHS2 of *M. sativa* (Supplementary Fig. S4). These conservative changes may change the structure and chemical properties of the protein, and differentially regulate anthocyanin and flavone synthesis (Wang et al. 2007; Ma et al. 2009; Ohno et al. 2011). The different expression patterns of two homologous *EpaCHSBs* in tissues of *E. pallida* imply these two chalcone synthase paralogs possibly play distinct roles in different tissues.

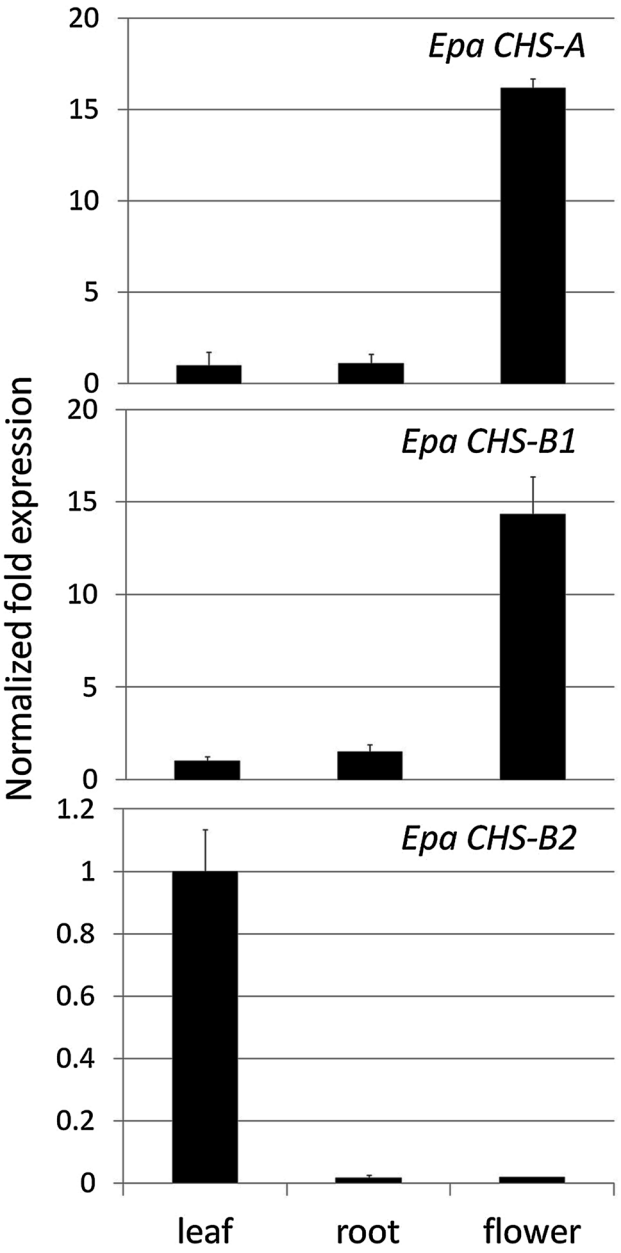

Fig. 6 Expression of *EpaCHS-A, EpaCHS-B1*, and *EpaCHs-B2* in roots, leaves, and flowers of *E. pallida*

EpaCHS expression and metabolite analysis in CHS transgenic plants

Transgenic plants were grown to maturity and no phenotypic changes were observed. In the current study, a change in the distribution of secondary metabolite main products

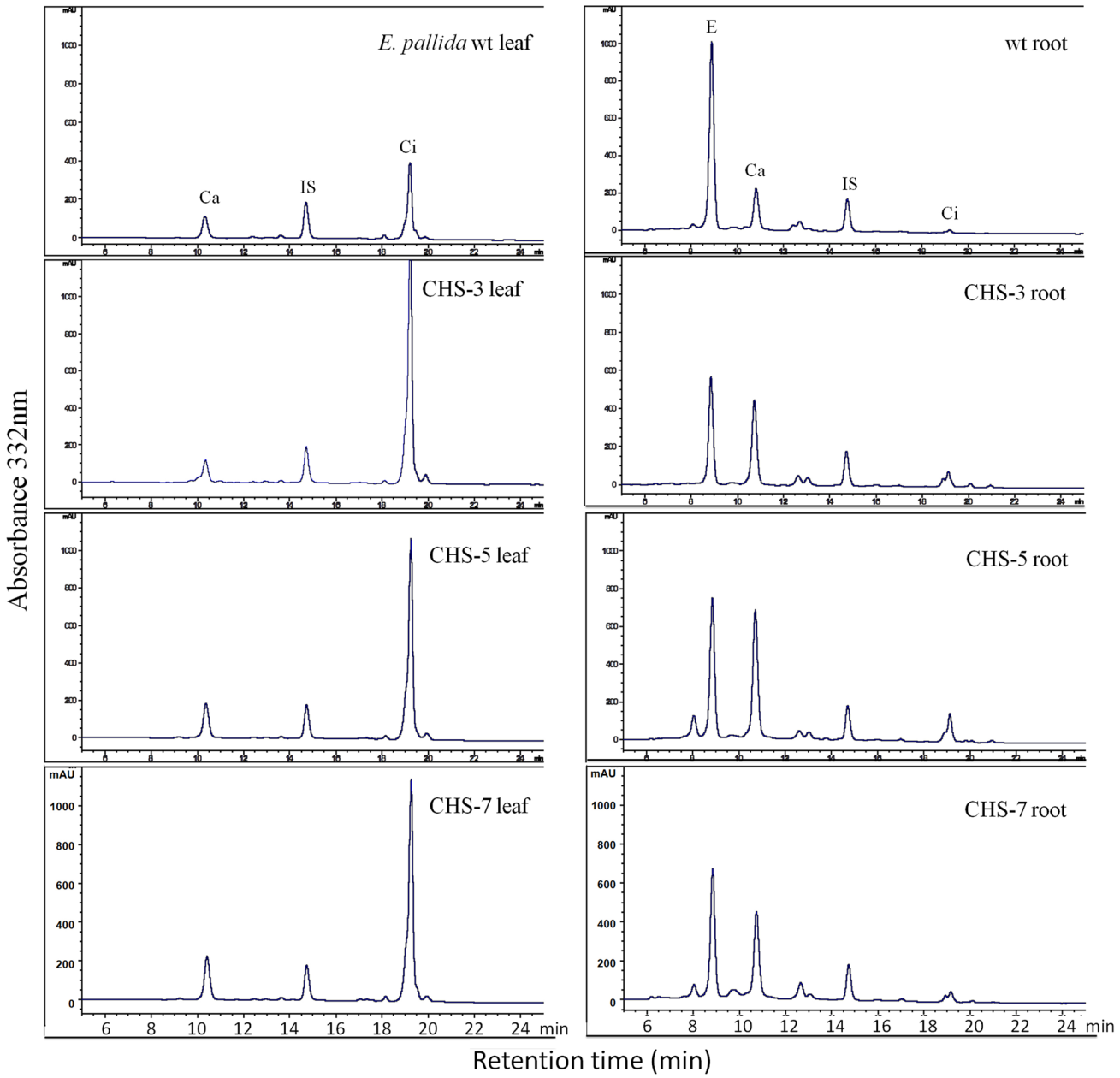

Fig. 7 HPLC analyses of phenolic compounds in the roots of WT and transgenic CHS plants. The relative content of caftaric acid (Ca), echinacoside (E), and cichoric acid (Ci) to the internal standard luteolin-7-*O*-glucoside (IS) is shown

was observed in the leaves and roots of transgenic CHS-3, CHS-5 and CHS-7 by HPLC (Fig. 7), and there were no significant differences in expressions of *EpaCHS* in these tissues by quantitative real-time PCR (Supplementary Fig. S6). Furthermore, real-time PCR showed that *EpaCHS-B2* which was expressed in leaves was present at dramatically increased levels in the flower of CHS-5, and *Petunia CHS* was reduced (Fig. 8). The expression pattern in which *EpaCHS-B2* was increased and *Petunia CHS* decreased was also revealed in transgenic CHS-7 (Fig. 8). Therefore, *EpaCHS-B2* and *Petunia CHS* may interact with each other in CHS-5 and CHS-7. The caffeic acid derivatives (CADs) are responsible for the flavonoid biosynthesis in secondary metabolism of *E. pallida*. To study the effects of the CHS transgene on CAD biosynthesis by *Petunia CHS* gene transformation, we analyzed the content of leaf and root caftaric acid, echinacoside and cichoric acid by HPLC. Biosynthesis of CADs including cichoric acid and caftaric acid was

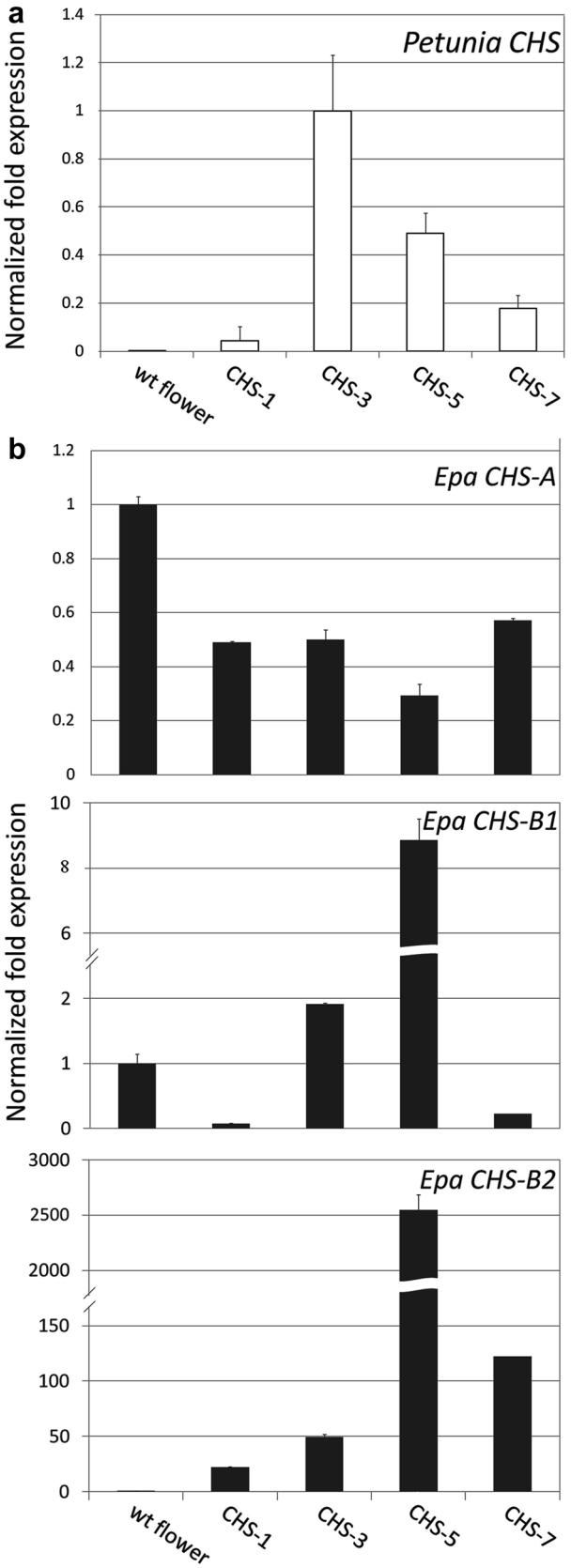

Fig. 8 Expression patterns of *CHS* in the flower of *E. pallida*. **a** ▸ Expression patterns of *PhCHS* in the *E. pallida* flower. **b** Expression levels of *EpaCHS* in the flower of wild-type and CHS lines were analyzed by real-time PCR. Data were normalized against the expression levels of the *actin* gene, and the ratios of reactions from the flower of wild-type plant (wt flower) were designated a value of one to determine the relative ratios of other reactions

increased in leaves and roots of CHS transformants, respectively, while the amount of echinacoside in roots of transgenic plants was decreased in comparison to that in the WT (Fig. 7). The flavonoid biosynthesis of 4-coumaroyl-CoA and CADs biosynthesis of caffeic acid begins with coumaric acid precursors via the generation of the phenylalanine and tyrosine pathway (Brenda 2001; Jia et al. 2015). However, the amounts of cichoric acid and caftaric acid were increased in *Petunia* CHS transformants. These peaks in cichoric acid and caftaric acid purity from transgenic CHS plants were further determined by HPLC–ESI-MS (Table 3), and were confirmed to be a single compound. The interaction between the flavonoid pathway and CAD biosynthesis in *Echinacea* species has not been elucidated. These results may indicate that CADs are responsible for the flavonoid biosynthesis in secondary metabolism of *E. pallida* by exogenous *CHS* gene transformation.

Conclusion

In this study, conditions for callus induction and plant regeneration from leaf explants of in vitro-grown *E. pallida* were determined. Subsequently, two vectors pCHS and pOSAG78, carrying different selection marker genes, were independently used to transform leaf explants of *E. pallida* using an *Agrobacterium*-mediated method. The transgenic plants obtained were confirmed and characterized. Among them, transformants carrying *Petunia CHS* were further selected to study the function of foreign *CHS* in *E. pallida*, which contains at least three copies of endogenous *CHS*. Furthermore, HPLC analysis coupled with mass spectrometry identification revealed that two CADs, cichoric acid and caftaric acid, were increased in leaves and roots of CHS transformants, respectively, whilst the amount of echinacoside in roots of transgenic plants was decreased. There are relatively few studies of genetically engineered *Echinacea* (Abbasi et al. 2007a). To our best knowledge, this is the first report regarding metabolic engineering in this medicinal plant. This study successfully produced transgenic *E. pallida* plants with monitored plant secondary metabolites. This procedure may improve genetic engineering of *Echinacea* species.

Table 3 MS spectral data for identification of phenolic compounds in *E. pallida*

Compound	t_R min^{-1}	[M + H] + (m/z) found	[M + H] + (m/z) reported	Fragment ions (m/z) found
Echinacoside	7.14	1572, 785	1571[a], 785[ab]	623
Caftaric acid	7.66	623, 311	623[a], 311[ab]	179, 149
Cichoric acid	16.85	947, 473	947[a], 473[ab]	311, 293[a], 179[b], 149

[a]Based on references by Pellati et al. (2012)

[b]Based on references by Thomsen et al. (2012)

Acknowledgements The authors would like to express thanks to Miranda Loney for critical reading and editing of the manuscript. We also thank Dr. Chin-Yu Lin for LC-MS identification and the Metabolomics Core Facility in Academia Sinica for technical assistance. This work was supported by Academia Sinica of Taiwan to Dr. Kin-Ying To.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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