Appl Biochem Biotechnol
DOI 10.1007/s12010-012-9791-8

Production of Triterpenoid Anti-cancer Compound Taraxerol in *Agrobacterium*-Transformed Root Cultures of Butterfly Pea (*Clitoria ternatea* L.)

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Received: 17 April 2012 / Accepted: 3 July 2012

Abstract Independent transformed root somaclones (rhizoclones) of butterfly pea (*Clitoria ternatea* L.) were established using explant co-cultivation with *Agrobacterium rhizogenes*. Rhizoclones capable of sustained growth were maintained under low illumination in auxin-free agar-solidified MS medium through subcultures at periodic intervals. Integration of T_L-DNA *rol*B gene in the transformed rhizoclone genome was verified by Southern blot hybridization, and the transcript expression of T_R-DNA *ags* and *man*2 genes was ascertained by reverse transcription polymerase chain reaction analysis. The major compound isolated and purified from the transformed root extracts was identified as the pentacyclic triterpenoid compound taraxerol using IR, ^{1}H-NMR, and ^{13}C-NMR spectroscopy. The taraxerol yield in cultured hairy roots, as quantified by HPTLC analysis, was up to 4-fold on dry weight basis compared to that in natural roots. Scanning of bands from cultured transformed roots and natural roots gave super-imposable spectra with standard taraxerol, suggesting a remarkable homology in composition. To date, this is the first report claiming production of the cancer therapeutic phytochemical taraxerol in genetically transformed root cultures as a viable alternative to in vivo roots of naturally occurring plant species.

Keywords *Agrobacterium rhizogenes* · *Clitoria ternatea* L. · Rhizoclones · ^{1}H-NMR · ^{13}C-NMR · HPTLC · Taraxerol

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Published online: 28 July 2012

Introduction

Over the past two decades, tremendous efforts have been made in the utilization of plant biotechnology to produce various bioactive secondary plant metabolites mostly through cell suspension cultures. However, success in this direction has been limited owing to certain technological problems associated with cell suspensions such as low level accumulation of secondary compounds, somaclonal variation accountable for biochemical and genetic instability of de-differentiated cells, and requirement of morphogenic differentiation for production of the organ-specific secondary metabolite(s). Since then, considerable interest has been addressed to transformed organ cultures, mainly root cultures, which are more stable, genetically and biochemically, compared to cell suspensions [1].

Plant transformation technology, precisely involving a soil bacterium *Agrobacterium rhizogenes*, has revolutionized the role of in vitro cultures in the biosynthesis of major secondary metabolites of pharmaceutical and therapeutic significance. *A. rhizogenes*, a phytopathogenic Gram-negative soil-dwelling bacterium, harbors characteristic root inducing plasmids (pRi) carrying an oncogenic T-region with two auxin biosynthetic genes—*iaa*M and *iaa*H—which, on transfer to plant genome through infection, cause the formation of genetically transformed roots exhibiting auxin autotrophy [1, 2]. The molecular events during transfer of T-DNA followed by its integration in the plant nuclear genome represent a unique signal transduction interplayed and shared by the participating plant and bacterial genetic systems involved through a transitory cell-to-cell association resembling conjugation. The level of transgene expression in plants not only varies with the site of integration but also depends on the transgene copy numbers. The plants upon infection with virulent strains of *Agrobacterium* develop the typical "hairy root" syndrome wherein fast-growing, negatively geotropic, highly branched adventitious roots emerge at the site of infection. These hairy roots, characterized by rapid biomass generating ability, high biosynthetic capacity coupled with genetic and biochemical stability, have been found to offer a considerably superior prospect for production of secondary metabolites, especially value-added phytochemicals, compared to the non-transformed roots or the de-differentiated cell cultures [3, 4].

Clitoria ternatea L. (butterfly pea) is a leguminous perennial twinner. In the Indian traditional system of medicine, the *Ayurveda*, the plant species ("Aparajita") is considered as a source of "Medhya" drug to improve intelligence and enhance memory function [5, 6]. It is also used in the treatment of chronic bronchitis, dropsy, goiter, leprosy, mucous disorders, sight weakness, skin diseases, sore throat, and tumors [7]. The roots possess laxative, diuretic, anthelmintic, and anti-inflammatory properties; they are useful in severe bronchitis, asthma, and fever [8, 9]. Phytochemical analysis of roots have revealed a wide range of secondary metabolites including two triterpenoids namely taraxerol and taraxerone [10, 11] and a flavonol glycoside 3,5,4′-trihydroxy-7-methoxyflavonol-3-*O*-β-D-xylopyranosyl-(1,3)-*O*-β-D-galactopyranosyl(1,6)-*O*-β-D-glucopyranoside [12]. Of these, taraxerol, a pentacyclic triterpenoid (Fig. 1), is the key compound possessing pharmacological value, including antimicrobial, antioxidant, anti-aging, antipyretic, anti-inflammatory, analgesic, sedative, insecticidal, and more importantly anti-tumor and anti-cancer properties [8, 13, 14]. Probably no other group of metabolites throughout the plant and animal kingdom, other than terpenoides, has such a remarkable diversity in structure and function [15]. Many terpenoids are produced in the cytosol via a route dependent on the mevalonic acid (MA; MA-dependent pathway) that probably had its origin during the early development of life on this planet. Other terpenoids are biosynthesized via a recently discovered pathway, a mevalonate-independent route [15–17], the

latter occurring in plastids (Fig. 1). Mevalonic acid is formed from three molecules of acetyl Co-A, but it leads the metabolism to different compounds from those of the acetate pathway. The mevalonic acid is transformed into five-carbon-containing isoprene units which, in the form of isopentenyl pyrophosphate and its isomer dimethylallyl pyrophosphate, combine to form a large number of terpenoid and steroid structures [18]. Perhaps taraxerol has been derived from MA-dependent pathway, precisely from squalene (C_{30}) as a probable precursor (Fig. 1).

In view of the proven pharmaceutical credentials of *C. ternatea*, modern biotechnological intervention could effectively exploit the plant species for medicinal purposes. As most of the active principles of pharmaceutical relevance are harbored in the roots of this plant species, in vitro root cultures could be the most appropriate source from which the useful secondary metabolites could be extracted in order to cater to the requirement of drug design and manufacture. *Agrobacterium*-transformed hairy root cultures of *C. ternatea* L. could serve as an effective and sustainable source of medicinally important phytochemicals, particularly taraxerol, needed as raw ingredients for manufacturing the selective drug types so as to target organ-specific cancers. To date, no report exists to demonstrate taraxerol production in transformed root cultures. This paper describes, for the first time, successful isolation, characterization, and quantification of taraxerol in transformed rhizoclones of *C. ternatea* vis-à-vis in vivo root system of the naturally occurring donor plant.

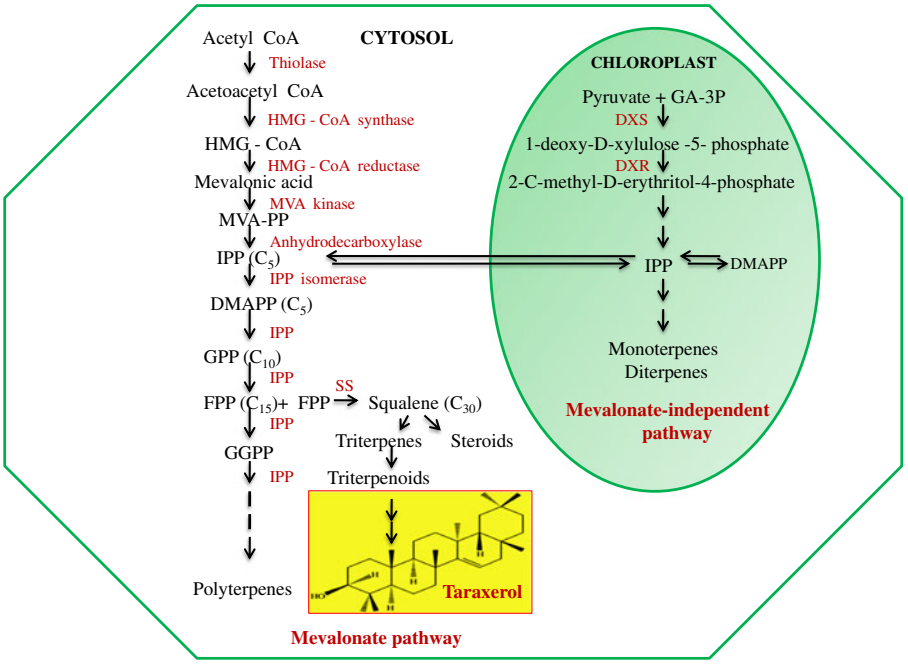

Fig. 1 Biosynthesis of taraxerol via mevalonic acid pathway in plant cell cytoplasm. *HMG-CoA* β-hydroxy β-methyl CoA, *MVA-pp* mevalonic acid-5pp, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranyl geranyl pyrophosphate, *SS* squalene synthase, *GA-3P* glyceraldehyde-3-phosphate, *DXP* 1-deoxy-D-xylulose-5-phosphate, *DXS* DXP synthase, *DXR* DXP reductoisomerase

Materials and Methods

Induction and Establishment of Hairy Root Cultures

A. rhizogenes Strains

The virulent agropine type *A. rhizogenes* strain A4T [19] and mannopine type strain 8196 [20], employed for transformation, had been resulted from transconjugation of pRiA4 [21] and pRi8196 into a pTi cured line of *Agrobacterium tumefaciens* C58, respectively. The strain LBA 9402 was a rifampicin-resistant derivative of NCPPB1855 and possessed an agropine-type Ri plasmid pRi1855 [22]. A4 and A4T strains were obtained as kind gift from Dr. D. Tepfer, Laboratoire de Biologie de la Rhizosphere, Institut National de la Recherche Agronomique, Versailles, Cedex, France, whereas the strains 8196 and LBA 9402 were generously donated by Dr. M. R. Davey, University of Nottingham, UK. The bacterial medium and culture conditions were in accordance with the published protocol [23].

Plant Material

Nodal explants were collected from a butterfly pea plant (*C. ternatea* L.) of blue-flowered variety (Fig. 2a) grown in the medicinal plant garden of the Department of Botany, Utkal University, Bhubaneswar (India). Following surface-disinfection nodes were inoculated in Murashige and Skoog (MS) [24] medium augmented with 1 mg l^{-1} ^{6}N-benzyladenine for developing in vitro shoot culture. The procedure for further subculture and in vitro maintenance of axenic shoots was as described by Barik et al. [25].

Fig. 2 **a** A blue-flowered variety of butterfly pea (*C. ternatea*) which served as the donor plant material. **b** Roots of **a**. **c** A root segment excised from A4T induced rhizoclone (HRT5E7; IFW 200 mg) and inoculated in MS0 (at 0 day) for determination of grow rate. **d** A well-proliferated A4T-derived rhizoclone (HRT5E7) in agar-solidified MS0 at 45 day under diffused illumination (10–15 μmol m^{-2} s^{-1} PFD). **e** A well-proliferated A4-derived rhizoclone (HRA2D3) in agar-solidified MS0 at 45 day under diffused illumination (10–15 μmol m^{-2} s^{-1} PFD). **f** A well-proliferated LBA9402-derived rhizoclone (HRL1A3) in agar-solidified MS0 at 45 day under diffused illumination (10–15 μmol m^{-2} s^{-1} PFD)

Infection and Co-cultivation

Explants (internodal segments and leaf explants from 4-week-old in vitro grown axenic shoot cultures) as well as those from the outdoor-grown donor plant, after surface disinfection, were subjected to transformation. The methodology of infection, co-cultivation, and establishment of transformed root cultures was adopted as per description in our previous report [23].

Molecular Characterization of Rhizoclones

Southern Blot Analysis

To confirm the integration of T-DNA into root genomic DNA, Southern blot analysis was performed (Southern 1975). Genomic DNA (5 μg) from fast-proliferating rhizoclones derived from independent transformation experiments were digested with 25 U each of *Nar*I and *Nde*I and resolved by electrophoresis on 0.8 % agarose gel before blot transfer to a nylon membrane was carried out. The membrane was pre-hybridized at 65 °C in 7 % SDS and 0.25 M $NaHPO_4$ and then hybridized with a DNA fragment (1.8 kb) containing *rol*B gene, as the probe which had been isolated from the Ri plasmid of A4T strain following double restriction with *Nar*I and *Nde*I and labeling with ^{32}P-dCTP using the method of Sambrook and Russell [26]. The hybridized blot was subjected to three washes in 20 mmol/l $NaHPO_4$ and 1 % SDS. The blot was exposed to X-ray film (Kodak) at −70 °C for 3 days.

RT-PCR Analysis

Total RNA was isolated from selected fast-growing root somaclones of *C. ternatea* using RNA isolation kit (Fermentas, Glen Burnie, MD, USA). Reverse transcription polymerase chain reaction (RT-PCR) was conducted using the Fermentas Revert First Strand Complementary DNA (cDNA) Synthesis Kit (Fermentas, USA) following the manufacturer's instructions. PCR amplification of *man*2 and *ags* genes in the cDNA products was carried out according to conditions described by Swain et al. [23]. Total RNA isolated from natural roots of the in vivo-grown plant or in vitro roots that had developed without *Agrobacterium* infection were subjected to RT-PCR under similar conditions to serve as a negative control. The housekeeping gene actin was used as a positive control (loading control) to indicate the amount of starter RNA reflecting the level of expression. The primer sequences were designed from the *Nicotiana tabacum* actin gene (GenBank accession no. X63603) as 5′-CGC GAA AAG ATG ACT CAA ATC-3′ and 5′-AGA TCC TTT CTG ATA TCC ACG-3′, which would be expected to generate a 533-bp product with cDNA. The primers for amplifying *ags* gene were 5′-CGG AAA TTG TGG CTC GTT GTG GAC-3′ and 5′-AAT CGT TCA GAG AGC GTC CGA AGT T-3′, expected to give a 1.6-kb product with cDNA. Another set of primers for amplifying the *man2* gene were 5′-GCGCATCCCGAGGCGATG-3′ and 5′-AGGTCTGGCGATCGCGAGGA-3′, expected to give a 512-bp product with cDNA. The PCR products were electrophoresed on 1.0 % agarose gels, detected by ethidium bromide staining, and photographed using the gel documentation system (Bio-Rad, USA).

Determination of Root Growth in Transformed Rhizoclones

After two successive sub-cultures, a single root segment (ca. 4–5 cm long) was excised from each of the transformed rhizoclone and transferred onto MS medium devoid of growth regulator supplement (MS0) and kept inside the culture room (25±1 °C) under diffused light

with a low PFD (10–15 μmol m^{-2} s^{-1}). The initial fresh weight (IFW; ca. 200 mg) was recorded at the time of inoculation. Root length and biomass of hairy roots were recorded at every 15-day interval up to 60 days (15, 30, 45, 60 days). These roots were taken out from the culture vessel, washed with double-distilled water, blotted dry, and weighed to determine the final fresh weight (FFW). Root growth index in terms of biomass gain over a culture period was calculated as (FFW−IFW)/IFW. In order to determine the time course for changes in growth and taraxerol content, fast-growing healthy clones derived from different *A. rhizogenes* strains were taken into observation.

Secondary Metabolite Analysis

Extraction of Transformed Roots

Transformed roots obtained from *Agrobacterium* treated cultures were properly washed with water and air dried at room temperature (25–30 °C) for 7 days. Dried samples (2 g) were powdered and extracted with dichloromethane–methanol (1:1) in a Soxhlet extractor for 48 h. The extract was filtered under vacuum and dried completely to a solid mass.

Thin-Layer Chromatography

Thin-layer chromatography (TLC) of the extract was carried out on pre-coated TLC plate and developed with different solvent systems like chloroform/methanol, *n*-hexane/ethyl acetate, *n*-hexane/acetone, benzene/ethyl acetate, etc. with varying proportions in a vapor-saturated TLC chamber containing the appropriate solvent system. Thereafter, the plates were sprayed with methanolic–sulfuric acid reagent (5 %) and heated at 105 °C to view the spots.

Isolation and Characterization of Taraxerol

For the separation of the compound from the extract, a crude column was set up. One gram of the extract was dissolved with chloroform–methanol (1:1) and adsorbed with silica gel. Then it was chromatographed over a glass column (5×130 cm, $D \times L$) set up vertically and packed up to three fourth of the column with silica (100–200 mesh). The column was eluted with solvents of increasing polarity from *n*-hexane to *n*-hexane–ethyl acetate mixtures (50 %). Fractions (each 250 mL) were collected and grouped based on their similar TLC pattern. Further, purification of the fractions and recrystallization from chloroform–methanol yielded a colorless amorphous powder, m. p. 140–142 °C; R_f 0.55 (hexane–ethyl acetate; 8:2, *v*/*v*); IR (KBr, in per centimeter): 3,445, 3,015, 1,617, 1,513, and 1,460; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ5.52 (dd, 1H, J=8.0 and 3.2 Hz, H-15), 3.20 (m, 1H, H-3), 1.90 (dd, 2H, H-16), 1.07 (H-27), 0.96 (H-23), 0.93 (H-29), 0.91 (H-24), 0.89 (H-26 and H-30), 0.80 (H-28), and 0.78 (H-25); ^{13}C-NMR ($CDCl_3$, 400 MHz): δ158.07 (C-14), 116.87 (C-15), 79.06 (C-3), 55.51 (C-5), 49.26 (C-18), 48.72 (C-9), 41.30 (C-19), 38.96 (C-8), 38.75 (C-4), 37.98 (C-1), 37.72(C-10), 37.70 (C-13), 37.55 (C-17), 36.66 (C-16), 35.78 (C-12), 35.10 (C-7), 33.68 (C-21), 33.34 (C-29), 33.08 (C-22), 29.91 (C-26), 29.81 (C-28), 28.79 (C-20), 27.99 (C-23), 27.13 (C-2), 25.90 (C-27), 21.30 (C-30), 18.78 (C-6), 17.49 (C-11), 15.44 (C-25), and 15.42 (C-24); MS (*m/z*): 426 [M+], 409, 302, 287, 218, 204, and 189.

HPTLC Analysis

The HPTLC system (Camag, Muttenz, Switzerland) consisted of a TLC scanner III with winCATS software, a Limomat V autosprayer connected to a nitrogen cylinder, a twin trough chamber (20×10 cm), a plate heater, a derivatization chamber, and a documentation unit Reproastar 3. The reagents used during the experiment were of analytical grade and obtained from the S.D Fine Chem. Ltd. (Mumbai, India), and HPTLC plates used were from Merck KgaA (Darmstadt, Germany).

For the separation of the compound from the extract, a crude column was set up. One gram of the extract was dissolved with chloroform–methanol (1:1) and adsorbed with silica gel. Then it was chromatographed over a glass column (5×130 cm, $D \times L$) set up vertically and packed up to three fourth of the column with silica (100–200 mesh). The column was eluted with solvents of increasing polarity from *n*-hexane to *n*-hexane–ethyl acetate mixtures (50 %). An accurately weighed root powder of normal root (1 g) and hairy root (0.5 g) were extracted with dichloromethane–methanol (1:1) in a Soxhlet extractor for 14 h. Extract solution was concentrated under vacuum, filtered, and finally made up to 25 mL in volumetric flask with methanol. The stock solution of pure isolated taraxerol was prepared by dissolving 2 mg in 100 mL of methanol (20 ng μL^{-1}).

The sample and standard solutions (pure isolated taraxerol) were applied onto the plate (10×10 cm aluminum-backed HPTLC plates pre-coated with a 0.2-mm layer of silica gel 60 F_{254}) as 6 mm band located 10 mm from bottom and 10 mm band gap using a Linomat 5 applicator mounted with a 100-μL syringe. For linearity experiment, the standard solution of concentration of 20 ng μL^{-1} was used and applied to the TLC plate in the concentration range of 50–300 ng. The volume of sample solution was applied to the TLC plate in order to get the concentration of taraxerol in the calibrated range. The applied HPTLC plates were developed in a Camag twin-trough glass chamber with the mobile phase of *n*-hexane/acetone (8:2, *v*/*v*) with chamber saturation time for 2 min, and the solvent front was allowed to reach a height of 74 mm. The taraxerol was quantified at its maximum absorbance of wavelength of 510 nm with the help of Camag TLC Scanner 3 and using winCATS software (version 1.4.2) in absorption–reflection scan mode after derivatization of the plates with methanolic–sulfuric acid reagent (5 %). The taraxerol content was determined in normal as well as in transformed roots from the scan intensity of diffusely reflected light using linear calibration via peak areas.

Statistical Analysis

All transformation experiments were set up in a completely randomized design. Each treatment consisted of 10 replicate jars. Experiments were repeated thrice. Data were analyzed using analysis of variance for a completely randomized design. Duncan's new multiple range test was used to separate the mean of significant effect [27].

Results

Induction of Rhizogenesis and Establishment of Transformed Root Cultures

Independent rhizoclones were established by individually culturing small hairy protuberances which had emerged at the inoculated sites. Such transformed root cultures were characterized by prolific hormone-independent growth, lack of geotropism, and vigorous

lateral branching. Most of them were plagiotropic (growing parallel to the culture medium) while some displayed a negative geotropism with a tendency to grow toward light and away from the culture medium (Fig. 2d–f). Among all the strains evaluated, A4T carrying pRiA4 was the most virulent resulting in the highest frequency transformation (ca. 85.8 % rhizogenesis). The hairy roots derived from A4T grew luxuriously with fast and extensive lateral branching up to 4–5 weeks resulting in the highest biomass at 45 days (rhizoclone HRT5E7, 9.2 g), whereas A4 and LBA9402 induced hairy roots grew at a relatively slow rate (rhizoclone HRA2D3, 6.2 g; rhizoclone HRL1A3, 2.9 g). During this period, rhizoclones which attained maximum biomass viz. HRT5E7, HRA2D3, and HRL1A3 reached root length of approximately 33.5, 22.4, and 9.2 cm, respectively. After 4–5 weeks, hairy roots gradually changed from white to brown and showed a decelerating growth rate associated with reduced branching ability irrespective of the strain type.

Molecular Confirmation of Integration and Expression of T_L-DNA and T_R-DNA Genes in Transformed Rhizoclones

The transformed nature of selected rhizoclones obtained from independent transformation events was evident by the Southern blot analysis which revealed strong hybridization signals demonstrating single-copy insertion of *A. rhizogenes* T_L-DNA *rol*B transgene into the genomic DNA of the putative transformed hairy root clones. *Nar*I-*Nde*I double digestion of genomic DNA from each of the tested rhizoclones generated an internal transgene fragment of 1.8 kb that hybridized with the probe DNA (Fig. 3). This confirmed the integration of the *A. rhizogenes rol*B ORF as part of the pRi T_L-DNA into the recipient plant genome via genetic transformation. In one of the rhizoclones (HRT7B5), the probe hybridized to an additional band (ca. 3.5 kb) which indicated the presence of an extra T-DNA insert in which one the restriction site adjacent to the T-DNA border was either mutated (deleted) or modified (truncated/methylated) perhaps owing to which it was not cleaved with the restriction enzyme. In contrast, no homology for the *rol*B transgene was detected for any of the non-transformed control roots as the *rol*B probe failed to hybridize to genomic DNA from natural roots of the in vivo-grown plant or in vitro roots that had developed without *Agrobacterium* infection (Fig. 3).

Further, to substantiate the transformed nature of rhizoclones, transcript expression studies of the opine genes, i.e., *ags* and *man*2 coding for agropine synthase and mannopine synthase, respectively, were carried out. RNA obtained from the independent transformed rhizoclones were subjected to RT-PCR analysis which revealed the presence of 1.6 kb or 512 bp band amplified from the cDNA products specifying *ags* or *man*2 gene, respectively, while cDNA from the non-transformed roots (from the naturally growing plant) showed no amplification (Fig. 4a, b).

Characterization of Taraxerol Using IR and NMR

The purified compound was obtained as a colorless amorphous powder from mixture of methanol–chloroform, m.p. 140–142 °C. The IR spectrum showed absorption band for hydroxyl group at 3,445 cm^{-1}, olefinic hydrogen at 3,015 cm^{-1}, and double bond at 1,617 cm^{-1}. The ^{1}H-NMR spectrum revealed the presence of an olefinic proton (H-15) which appeared as a double doublet at δ5.52. The significant signal for H-3 appeared at δ3.20 as a multiplet and a double doublet for H-16 appeared at δ1.90. There were eight tertiary methyl appeared as singlet at δ1.07, δ0.96, δ0.93, δ0.91, δ0.89, δ0.80, and δ0.78 for H-27, H-23, H-29, H-24, H-26 and H-30, H-28, and H-25, respectively.

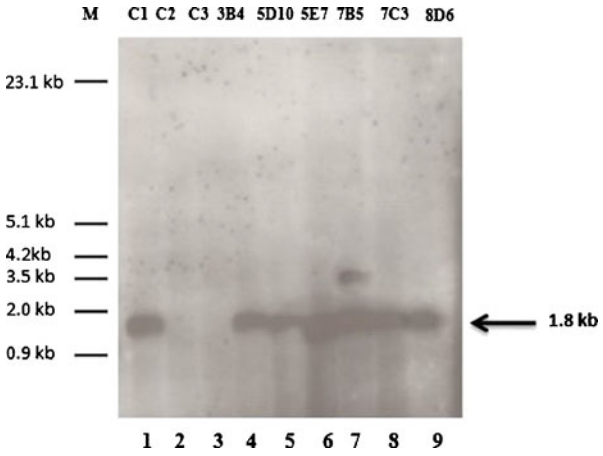

Fig. 3 Southern blot analysis showing T-DNA integration in genomic DNA of five selected rhizoclones. *Nar*I and *Nde*I digested genomic DNA was hybridized with the *rol*B gene probe. *M* lambda *Eco*RI/*Hin*dIII molecular DNA markers, *lane 1* A4 plasmid DNA (C1) (positive control), *lane 2* root DNA from a naturally occurring plant (C2), *lane 3* in vitro non-transformed roots (C3), *lanes 4–9* DNA from six randomly selected rhizoclones HRT3B4, HRT5D10, HRT5E7, HRT7B5, HRT7C3, and HRT8D6

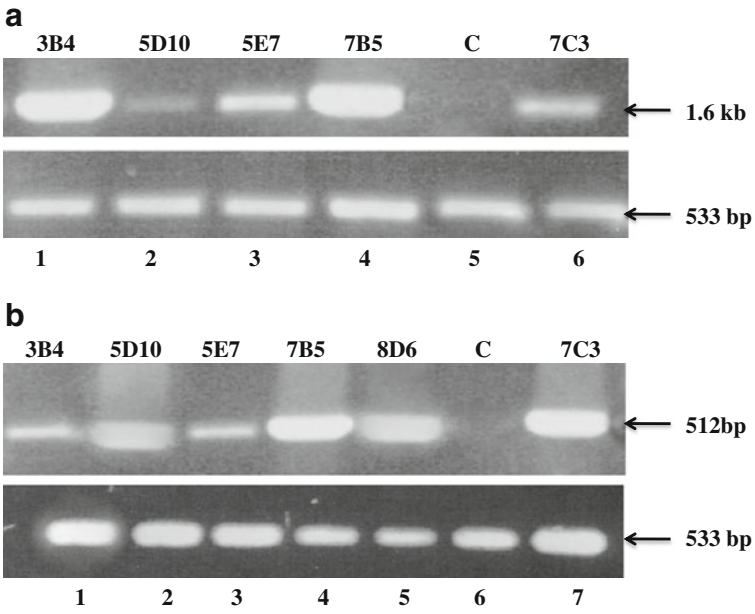

Fig. 4 RT-PCR analysis of the expression of the *ags* and *man*2 genes in selected transformed rhizoclones. **a** *Lane 5* cDNA-PCR product from a naturally growing plant (negative control), *lanes 1*, *2*, *3*, *4*, *6* cDNA-PCR product from five randomly selected transformed rhizoclones HRT3B4, HRT5D10, HRT5E7, HRT7B5, and HRT7C3. Transformed rhizoclones show an amplified band of 1.6 kb. The *lower panel* shows the amplification of actin used as a loading control. **b** *Lane 6* cDNA-PCR product from a naturally growing plant (negative control), *lanes 1*, *2*, *3*, *4*, *5*, *7* cDNA-PCR product from six randomly selected transformed rhizoclones HRT3B4, HRT5D10, HRT5E7, HRT7B5, HRT8D6, and HRT7C3. Transformed rhizoclones show an amplified band of 512 bp. The *lower panel* shows the amplification of actin gene used as a loading control

The ^{13}C-NMR spectrum confirmed 30 carbon signals in the molecule, of which there were eight methyls, nine methylenes, five methines, and eight quaternary carbons. An olefinic carbon resonance appeared at δ158.07 and 116.87 represents C_{14}–C_{15} double bond. The signal for the carbon C-3 which carries the hydroxyl group appeared at δ79.06. The mass spectrum exhibited a molecular ion peak at *m*/*z* 426 associated to the molecular formula as $C_{30}H_{50}O$ representing taraxerol. The experimental data were further confirmed in corroboration with reported literature [28].

Quantification of Taraxerol

The HPTLC method was applied with appropriate solvent system which gave high resolution and significantly different R_f values for the different compounds (Fig. 5a–c) including taraxerol in natural (Fig. 2b) and cultured transformed hairy roots of *C. ternatea*. Scanning of bands (R_f 0.53 individually at 420 nm) from transformed roots and natural roots gave super-imposable spectra with standard taraxerol suggesting a remarkable homology in composition (Fig. 5d). Six replicates for each determination were made, and the average content of taraxerol was calculated. Interestingly, there was a significant enhancement in the content of taraxerol to the tune of ca. 4-fold in genetically transformed hairy root cultures of *C. ternatea* (rhizoclone HRL1A3) compared to that observed for the naturally occurring variety.

Comparison of Taraxerol Production in Transformed Rhizoclones

From the above analysis data and the calibration curve, the maximum taraxerol yield in transformed roots and in vivo roots from a naturally grown plant was calculated to be 13.5 mg g^{-1} (rhizoclone HRL1A3) and 3.33 mg g^{-1}, respectively, on a dry-weight basis. The time course of hairy root growth and biomass generation vis-à-vis taraxerol production was investigated over a period of 15–60 days, and the results are presented in Fig. 6a, b. Of the four different strains of *A. rhizogenes* evaluated, A4T induced fastest-growing rhizoclones being endowed with highest biomass generation ability followed by A4 and LBA 9402 strains (Fig. 6a). The taraxerol production was enhanced exponentially after 15 days, and the maximum yield was recorded at 45 days beyond which there was no noticeable increase (Fig. 6b). This trend was evident regardless of *Agrobacterium* strains employed. The taraxerol content was inversely proportional to the biomass gain over a period of 45 days irrespective of the strains. Rhizoclones induced by LBA9402 strain grew at the slowest pace, albeit accumulated the maximum taraxerol content (13.5 mg g^{-1} d.wt. at 45 days; HRL1A3), whereas those incited by the A4T strain exhibited the fastest growth with the highest biomass but displayed the lowest taraxerol-yielding capacity (5.75 mg g^{-1} d.wt. at 45 days; HRT5E7; Table 1). A4 strain-induced root clones occupied an intermediate position with respect to biomass generation as well as taraxerol production (8.5 mg g^{-1} d.wt. at 45 days; HRA2D3; Table 1). It was interesting to note that the amount of taraxerol production in transformed root cultures which was the lowest among rhizoclones (HRT3B4; 4.35 mg g^{-1} d.wt.) was, albeit, higher than the naturally occurring roots of the donor plant (3.33 mg g^{-1} d.wt.; Fig. 6c).

Discussion

Fundamental to utilizing transformed hairy root cultures as a source of secondary metabolite(s) is the optimization of *Agrobacterium* × plant factors influencing transformation. Our earlier

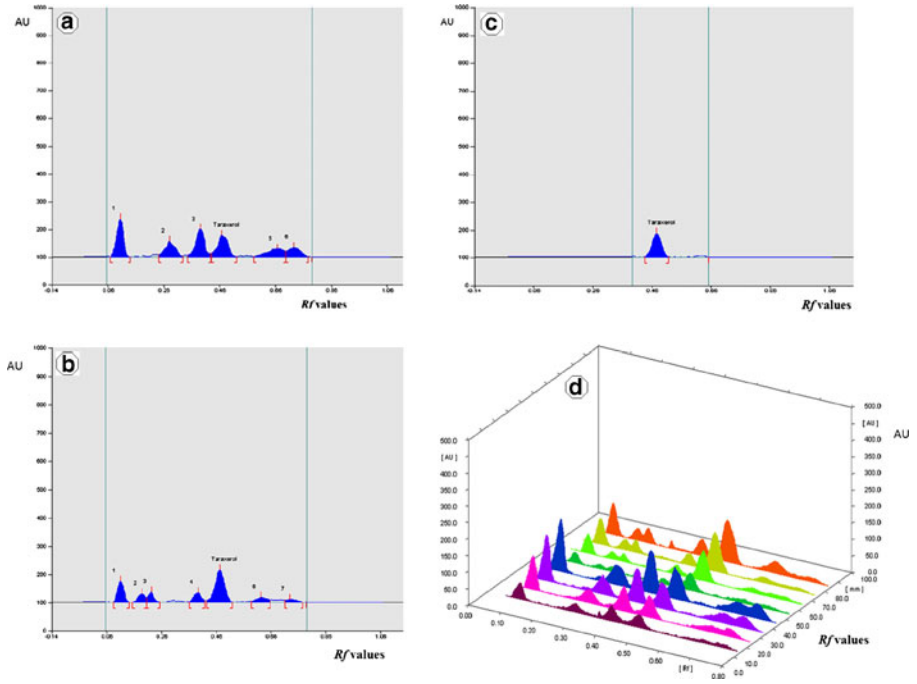

Fig. 5 HPTLC chromatogram of HRT5E7 confirming the presence of taraxerol. **a** Chromatogram of natural roots. **b** Chromatogram of hairy roots. **c** Chromatogram of the standard taraxerol. **d** Scanning of bands (R_f 0.53 individually at 420 nm) from cultured transformed root somaclone HRT5E7 and natural roots revealing superimposable spectra with standard taraxerol suggesting homology in composition

work [23] had identified several key parameters resulting in high throughput transformation of butterfly pea (*C. ternatea*) using *A. rhizogenes*. A4T was the most infectious among the strains employed. Internode segments were more responsive than leaves; outdoor-grown explants preferred to those from in vitro cultures. High-frequency transformation, resulting in up to 85.8 % rhizogenesis, was achieved using pre-pricked internodal explants for immersion (10 min) in *A. rhizogenes* (A4T) suspension grown overnight with acetosyringone (100 μM) to A_{660}=0.6, diluted to a density of 10^9 cells ml^{-1} and followed by 5-day co-cultivation [23].

Stable integration and expression of *Agrobacterium* pRi T-DNA genes are critically important to ensure sustained auxin-autotrophic growth and production of root-specific bioactive compounds, thereby accounting for successful application of transformed hairy root cultures. In the present study, the transformed nature of all the rhizoclones, obtained from independent transformation events, was demonstrated by employing the Southern blot analysis which revealed strong hybridization signals in favor of single-copy insertion of *Agrobacterium* T_L-DNA *rol*B transgene in the genomic DNA of the putative transformed hairy root clones. Likewise, the detailed integrative status of pRi T-DNA in transformed root cultures *Gentiana macrophylla* had been verified by Southern blotting [29]. Southern blot analysis was also carried out using a 750-bp digoxigenin-labeled *gus* fragment and GUS Plus fragment PCR amplified from pCAMBIA 1305 as a probe to identify the transgenic events [30, 31]. More importantly, in the present study, the transcript expression of the opine genes, i.e., *ags* and *man*2 coding for agropine synthase and mannopine synthase in selected rhizoclones, was confirmed by RT-PCR. In *Triphysaria versicolor*, the presence and expression of the transgene was similarly ascertained using genomic PCR, RT-PCR, and Southern hybridizations [31].

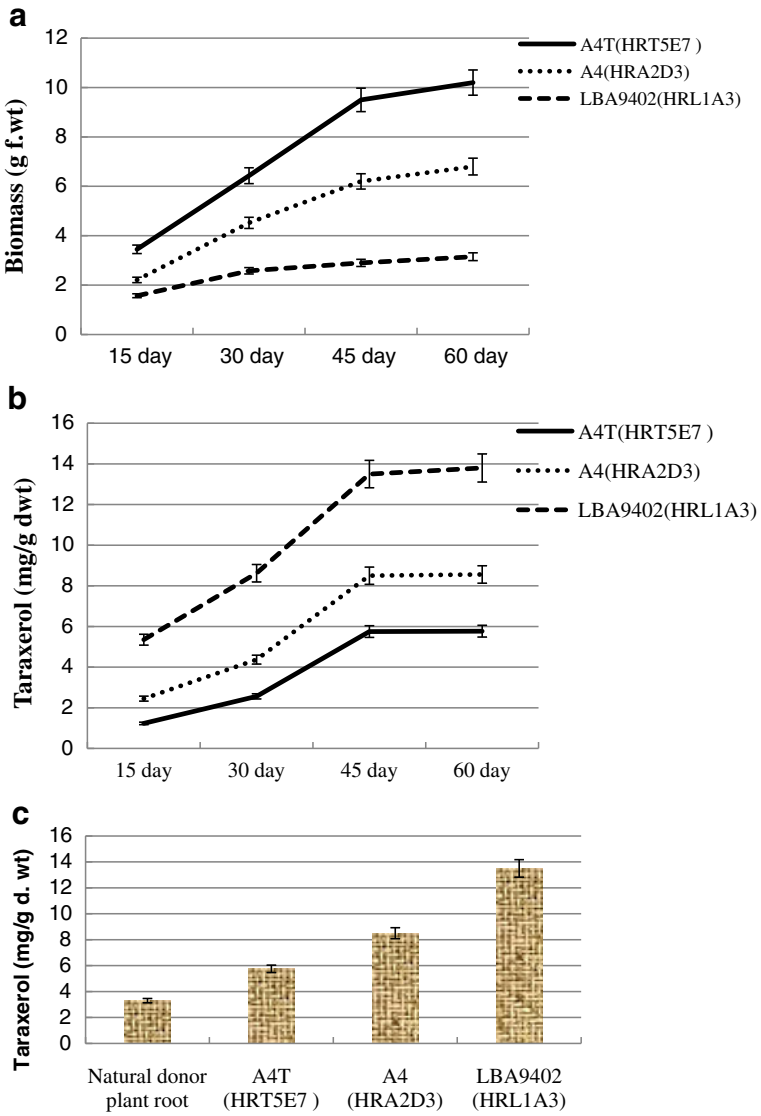

Fig. 6 **a** Relationship between growth rate (biomass gain) and taraxerol production of rhizoclones over a period of 15–60 days. **b** Comparative efficacy of *Agrobacterium* strains on taraxerol production vis-à-vis roots of the naturally occurring donor plant (**c**)

Herbal drugs, singularly and/or in combinations, contain myriad compounds in complex matrices in which no single active constituent is responsible for the overall efficacy in healthcare. This poses a challenge of establishing quality control standards for raw materials and the standardization of finished herbal drugs. Currently, there exists a common practice in natural product analysis of selecting one or more markers for purposes of identification and quality assessment. Estimation of the marker content in *Ayurvedic* botanicals is of utmost significance in evaluating the phytochemical utility of the source plant species. Hence, with the overall goal of producing an easily recoverable enriched fraction of secondary

Table 1 Evaluation of growth rate (biomass gain) and taraxerol production in selected rhizoclones

Strain	Rhizoclone	Root length (cm)	Root growth index	Taraxerol content (mg/g d. wt.)
LBA9402	HRL1A2	8.3f	7.12j	10.8abc
	HRL1A3	9.2f	13.92i	13.5a
	HRL1B3	7.7f	7.67j	9.2bcd
	HRL1C4	5.8f	9.75ij	12.0ab
A4	HRA1A2	18.5e	25.0fgh	8.2cde
	HRA1A5	20.3de	26.5efg	7.3cde
	HRA2B4	20.7de	28.22ef	7.7cde
	HRA2D3	22.4cde	30.17e	8.5cde
A4T	HRT3B4	26.6bcd	35.25cd	4.35g
	HRT5D10	28.7abc	37.5bc	4.5f
	HRT5E7	33.5a	46.5a	5.75de
	HRT7B5	32.3ab	40.15b	5.5ef

Mean values within column with different letters are significantly different ($p \leq 0.05$; Duncan's new multiple range test)

metabolites of pharmaceutical relevance, the transformed root culture of *C. ternatea* was evaluated for taraxerol content using HPTLC. The major phytoconstituents found in *C. ternatea* are two pentacyclic triterpenoids, namely taraxerol and taraxerone [10], of which the former is the key bioactive principle shown to be exhibiting a number of pharmacological activities [32, 33]. The fast-proliferating renewable hairy root cultures as a source of taraxerol from *C. ternatea* may be used as an alternative means for sustainable production of this pharmaceutically important compound.

Interest in hairy root cultures has mainly been manifested in studies on secondary metabolic production and metabolic engineering and has been well reviewed in literature [3, 4, 34–37]. Reports from the early 1980s indicated that roots of several species transformed by *A. rhizogenes* were capable of rapid growth in vitro in defined nutrient medium without requiring exogenous phytohormones [38, 39]. Since then, attempts have been made to utilize hairy root cultures for generation of stable transgenic plants [40, 41], secondary metabolite production, gene analysis, and studies on more fundamental understanding of plant–pathogen interactions [42]. Hairy root cultures have several properties that have prompted their use in plant biotechnological applications. Their fast growth, short doubling time, ease of maintenance, and ability to synthesize a range of chemical compounds and proteins offer additional advantages over plant cell suspension cultures as a viable source for production of valuable secondary metabolites and novel proteins. They are viewed as a renewable source of root-derived phytochemicals that are useful as pharmaceuticals, cosmetics, and food additives. Transformed roots of many medicinal plants have been widely studied for in vitro production of secondary metabolites such as bacopa saponins from *Bacopa monnieri* [43], glycyrrhizic acid from *Glycyrrhiza glabra* [44], salidroside from *Rhodiola sachalinensis* [45], tylophorine from *Tylophora indica* [46], anthraquinones in *Rubia cordifolia* [47], tanshinone from *Salvia miltiorrhiza* [48], ajmalicine and ajmaline from *Rauvolfia serpentina* [49], and ginsenoside from *Panax ginseng* [50]. Similarly several terpenoids, namely phytoecdysteroids, forsokolin, sesquiterpene, and taxon, were reportedly isolated from hairy root cultures of *Ajuga reptans* [51], *Coleus forskohlii* [52], and *Leontopodium alpinum* [53], respectively. Secondary metabolites are accumulated in both hairy roots and culture medium; for instance, glucosinolates accumulated in *Brassica rapa* [54],

alkaloids in *Catharanthus roseus* [55], and flavonoids in *Glycyrrhiza uralensis* [56]. In a few cases, some metabolites accumulated more in the medium than in the cells [54]. For example, in hairy root cultures of *G. uralensis*, up to 98 % of the total licochalcone and 94 % of total flavonoids were secreted into the culture medium [56]. In *Arachis hypogaea* hairy root culture, over 90 % of the total resveratrol, arachidin-1, and arachidin-3 were accumulated in the medium [57].

Hairy roots are unique in their ability to maintain genetic and biosynthetic stability on long-term basis. The hairy root cultures of many plant species have been widely studied for in vitro production of secondary metabolites, which they synthesize over successive generations without losing the biosynthetic steadiness [34, 58–61]. Besides, hairy root cultures are usually capable of producing the same compound(s) of identical chemistry found in wild-type roots of the naturally occurring parent plant without loss of structural integrity and/or quantity or concentration of the product, which is frequently observed in callus or cell suspension cultures [62, 63]. Often, such transformed root cultures offer a valuable source of root-derived phytochemicals which they synthesize at a higher or comparable level to that found in in vivo roots [29, 64–68]. Interestingly, in the present study, we were able to achieve up to 4-fold enhancement in taraxerol production in transformed hairy root cultures of *C. ternatea* in comparison with that in natural roots of the donor plant.

Hairy root culture represents a well-established experimental system which provides many insights into root-specific metabolism and regulation. In recent years, elicitation, precursor feeding, cell permeabilization, and trapping of the molecules released in the liquid medium have been projected as efficient ways to increase the productivity of hairy roots [4]. Exciting possibilities with hairy roots via biotransformation, metabolic engineering, phytoremediation, and scaling up of the culture process using custom-built bioreactors to render them an attractive industrial tool have been discussed [4, 69]. In addition, hairy root cultures are now being exploited not only for the production of secondary metabolites but also for isolation and activity study of different enzymes and therapeutic proteins. Production of recombinant proteins in plant cell or organ cultures and their secretion into the plant cell culture medium simplify the purification procedure and increase protein yield. This was corroborated with the experiment in which the sweet-tasting protein thaumatin I was expressed and successfully secreted from tobacco hairy root cultures [70]. Similarly, the effect of *A. rhizogenes* T-DNA on nicotine content and the pattern of antioxidative enzymes, peroxidase and superoxide dismutase, in hairy roots and regenerated plants of *N. tabacum* were investigated [71]. Besides, many secondary metabolites having herbicidal and pesticidal activity were reported from hairy root cultures. For instance, hairy roots of *Fagopyrum tataricum* (tartary buckwheat) have been shown to produce allelochemicals that have herbicidal activity [72]. Similarly, azadirachtin, a well-known biopesticide, was produced in leaf-derived hairy roots of *Azadirachta indica* [73].

In our investigation, four different *A. rhizogenes* strains—A4T, A4, LBA9402, and 8196—were evaluated to ascertain their ability to transform *C. ternatea* and produce taraxerol in resulting hairy root cultures. Previously, other studies have also reported the differential efficiency of various *A. rhizogenes* strains in promoting the induction, growth, and secondary metabolite production of hairy roots. For example, different *A. rhizogenes* strains affected the growth rate, saponin production, and the ratio of different astragalosides in transgenic root cultures of *Astragalus mongholicus* [67]. The strains of *Agrobacterium* also influenced the development, the growth rate, and the tropane alkaloid production in transformed root cultures of *Hyoscyamus muticus* [74, 75]. Hairy root cultures of *G. macrophylla* were established by infecting with four *A. rhizogenes* strains, and each hairy root lines showed different response regarding growth and production of secoiridoid glucoside gentiopicroside in transformed hairy

root cultures [29]. Clearly, the selection of an effective *Agrobacterium* strain for efficient production of secondary metabolites from transformed root cultures is highly dependent on the candidate plant species, and this needs to be determined empirically.

The present study has demonstrated that transformed root cultures proved efficient at par with in vivo roots from naturally grown donor plants with respect to production of taraxerol—a key pentacyclic triterpenoid responsible for pharmacological activities needed for prevention and cure of a range of diseases including cancer. Establishment of renewable transformed root cultures of *C. ternatea*, as a result of the present investigation, would not only provide a direct sustainable source of therapeutic phytochemicals of medicinal significance such as taraxerol but could also facilitate germplasm conservation of dwindling medicinal plant species, in general, which are incessantly uprooted, their roots being over-exploited for pharmaceutical purposes. In addition, such type of investigations could also pave new avenues of research in understanding evolutionary inter-relationships between pathogenic bacteria and host plants, particularly in unraveling further intricacies of the hitherto intriguing signal transduction pathway being interplayed between *Agrobacterium* and dicotyledonous plant species.

Acknowledgments Funding support by the Department of Science & Technology (Biotechnology), Government of Orissa (India) through a major research project is gratefully acknowledged. We thank Dr. Sagar K. Mishra, Assistant Professor, University Department of Pharmaceutical Sciences, Utkal University, Bhubaneswar (India) for helpful technical discussions in relation to taraxerol identification and quantification. We also wish to thank Dr. Jatindra K. Nayak, Professor of English, Utkal University for critically reading the manuscript and making useful changes in the text language.

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