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Agrobacterium rhizogenes mediated transformation of *Scutellaria baicalensis* and production of flavonoids in hairy roots

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Abstract

Using different explants of *in vitro* seed grown *Scutellaria baicalensis* Georgi plantlets, hairy roots were induced following inoculation of *Agrobacterium rhizogenes* strains A_4GUS, R1000 LBA 9402 and ATCC11325. The A_4GUS proved to be more competent than other strains and the highest transformation rates were observed in cotyledonary leaf explant (42.6 %). The transformed roots appeared after 15 - 20 d of incubation on hormone free Murashige and Skoog medium. Growth of hairy roots was assessed on the basis of total root elongation, lateral root density and biomass accumulation. Maximum growth rate was recorded in root:medium ratio 1:100 (m/v). Hairy root lines were further established in Gamborg B_5 medium and the biomass increase was maximum from 15 to 30 d. PCR, Southern hybridization and RT-PCR confirmed integration and expression of left and right termini-linked Ri T-DNA fragment of the Ri plasmid from A_4GUS into the genome of *Scutellaria baicalensis* hairy roots. GUS assay was also performed for further integration and expression. All the clones showed higher growth rate them non-transformed root and accumulated considerable amounts of the root-specific flavonoids. Baicalin content was 14.1 - 30.0 % of dry root mass which was significantly higher then that of control field grown roots (18 %). The wogonin content varies from 0.08 to 0.18 % among the hairy root clones which was also higher than in non-transformed roots (0.07 %).

Additional key words: baicalin, genetic transformation, medicinal plants, wogonin.

Introduction

Scutellaria baicalensis Georgi (*Lamiaceae*) is widely distributed in different regions of Russia, Japan, Korea and Mongolia. High biological activity of huang-qin root extract is determined by the presence of almost 70 flavonoids such as baicalin, baicalein, wogonin, wogonin 7-*o*-glucuronide, oroxylin A, and oroxylin A 7-*o*-glucuronide (Wang *et al*. 1994). Out of these, baicalin and wogonin are the most studied components (Zhou and Gu 1991, Jang *et al*. 2003). They are antioxidants and free radical scavengers (Shieh *et al*. 2000) and possess anti-allergic, anti-bacterial, anti-HIV, anti-hepatitis-B and anti-tumor activities. The natural plant population of this extremely important medicinal plant is over exploited for pharmaceutical preparations and therefore *in vitro* production as an alternative source has been attempted (Yamamoto *et al.* 1986, Morimoto *et al.* 1995). However, the cultures tend to be genetically unstable and synthesize very low levels of useful secondary metabolites (Rhodes *et al*. 1990). In contrast, *Agrobacterium rhizogenes* mediated hairy root cultures exhibit stable and fast growth rate comparable to cell suspension culture, genetic and biochemical stability and increased production of secondary compounds (Giri and Narasu 2000). Moreover, roots can also be successfully cultured in large-scale bioreactors (Flores *et al.* 1999, Sévon and Oksman-Caldentey 2002). These transformed hairy root, can be produced by inoculation of plants with agropine strains of *A. rhizogenes* containing a T-DNA, divided into two regions, T_L and T_R (Huffman *et al.* 1984). The T_L region of Ri plasmid contains eighteen open reading frames

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Abbreviations: ags - agropine synthase; B5 medium - Gamborg B_5 medium; FM- fresh mass; HPLC - high performance liquid chromatography; MS medium - Murashige and Skoog medium; NT- non-transformed; PCR - polymerase chain reaction; Ri - root-inducing plasmid; T_L- left-terminus DNA; T_R - right-terminus DNA; YEB - yeast extract broth.

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(ORF) (Slightom *et al*. 1986). ORFs 10, 11, 12 and 15 represent the root locus, *i.e.*, *rolA, rolB, rolC* and *rolD*, respectively. Root loci (*rol*) have been found to be essential for hairy root induction (White *et al*. 1985, Jouanin *et al.* 1987). Among these *rol*B plays central and most important role while *rolA, rolC* and *rolD* promote the root formation synergistically (Aoki and Syono 1999). The T_R-DNA of the agropine-type Ri plasmid contains genes that are homologous to the *iaa*M and *iaa*H genes of the Ti T-DNA (Huffman *et al.* 1984). The agropine synthase (ags) gene responsible for opine biosynthesis in the transformed tissue are also located in T_R region of Ri plasmid (Binns and Tomashow 1988). The roots produced by subsequent transfer of T-DNA have been recognized as potential alternative sources of many secondary compounds in medicinally important plants as the genetic transformation does not affect the natural root synthetic capacities (Flores and Medina-Bolivar 1995). The potential of hairy root of *Scutellaria baicalensis* have been investigated several time with respect to production of root specific flavones and flavonoids (Nishikawa *et al.* 1999, Zhou *et al.* 1997, Kuzovkina *et al.* 2001) but no data available on morphology and growth characteristics of hairy roots, time course of accumulation of biologically active compounds (baicalin and wogonin) and establishment of hairy root liquid culture. The aim of the present investigation was the transformation of *Scutellaria baicalensis* by four different *Agrobacterium rhizogenes* strains, establishment of hairy roots cultures, and stable integration of T_L-DNA and T_R-DNA and expression in the plant genome. The accumulation of flavonoids baicalin and wogonin in established hairy roots was also studied.

Materials and methods

Bacterial strains: *Agrobacterium rhizogenes* strains A_4GUS, R1000, LBA 9402 (Pompani *et al.*1983) and ATCC11325 (Riker *et al.* 1930) were used to determine the transformation efficiency. Agropine-type strain A_4GUS harbors $pRiA_4$ plasmid with GUS construct integrated into T_L region between the *rol*C and *rol*D genes (Tepfer and Casse-Delbart, 1987). GUS construct contains *uidA* sequence under the 70S promoter (enhancer-doubled 35S CaMV promoter), followed by NOS polyadenilation sequence. *A. rhizogenes* strain R1000 is an *A. tumefaciens* strain of C_{58} chromosomal background that have been cured of Ti plasmid and has had Ri plasmid of $pRiA_4$b conjugated in to it (Moore *et al.* 1979, White *et al.* 1985). Plasmid $pRiA_4$ of R1000 contains does *uidA* sequence in present study. Bacterial strain A_4GUS, R1000 and LBA9402 were shaken at 28 °C in liquid YEB medium (Vervliet *et al.* 1975) supplemented with 100 mg dm^{-3} neomycin, 50 mg dm^{-3} neomycin and 50 mg dm^{-3} rifampcine, respectively, whereas ATCC11325 was activated on YMB medium (Hooykas *et al.* 1975) for 3 d followed by shaking at 28 °C in liquid YMB liquid medium in dark. Activated bacterial cultures were collected by centrifugation for 10 min and resuspended in MS (Murashige and Skoog 1962) liquid medium containing 50 μm aceto-syrengone. Bacterial cultures were shaken on for 4 h at 28 °C in rotatory shaker at 120 rpm before inoculation.

Plant material and hairy root induction: Hypocotyl, cotyledonary leaf, cotyledonary petiole (1-week-old) and young leaf (2-week-old) *in vitro* grown plants of *Scutellaria baicalensis* Georgi were pre-incubated on half-strength MS liquid media for 4 h and inoculated with different bacterial strains of *A. rhizogenes* (A_4GUS, R1000, LBA9402 and ATCC11325) for hairy root induction. Pre-incubated explants were cut into small pieces and infected with the bacterial cultures separately (A_{600} = 0.8 - 1.0) for 20 min. Explants were blotted dry on sterile filter-paper and co-culture onto hormone free MS media containing 50 μm aceto-syringone. After 48 h of co-culture explants were rinsed with sterile water, blotted dry and transferred onto hormone free MS media containing 500 mg dm^{-3} cefotaxime (*Shanghi Sangon*, Shanghi, China). After 8 d, hairy roots appeared on cut ends of explants; they were detached and cultured onto fresh 1/2 strength MS media containing 500 mg dm^{-3} cefotaxime at 23 ± 1°C under dark. Hairy root cultures were transferred to fresh media every 3 weeks and maintained as separate independent clones.

Growth study and initiation of hairy root liquid culture: The growth of root clones transformed by *A. rhizogenes strain* A_4GUS on solid B_5 medium was studied. Roots excised from intact non-transformed (NT) *in vitro* plantlets were used as controls since the uninfected controls did not produce roots. Four root tips [approx. 3 cm long; 0.2 g fresh mass (FM)] of each clone, harvested from approximately 3-week-old cultures were transferred to the same medium (20 cm^3) in 9-cm Petri dishes and cultured for 35 d. Three Petri dishes were used for each clone. Total root elongation was recorded after 35 d. The number of lateral branches (number of roots per centimeter on the primary roots) was recorded and expressed as the lateral root density. The roots from each sample were washed with sterile water, blotted dry and the fresh mass (FM) determined.

To determine the effect of inoculum : medium ratio (1:50, 1:100, and 1:150 m/v), fastest growing hairy roots (200 mg) of clone emerged from A_4GUS strain were transferred into MS, White's (White 1963), B_5 (Gamborg *et al.* 1968) and half strength MS + B_5 vitamin liquid media containing 500 mg dm^{-3} cefotaxime on rotatory shaker (90 rpm) at 27 °C in dark. Data were taken after 10 and 15 d of culture. To time course for biomass accumulation (FM) an increase in FM was recorded at 5-d intervals for 5 weeks. The concentration of cefotaxime was gradually lowered and finally omitted after 8 weeks.

GUS histochemical assay: The GUS expression in transformed (A_4GUS and R1000) and non-transformed roots was determined histochemically several times over a period of 1 year according to Jefferson (1987). The roots were placed in enough X-GLUC (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium salt; *AMRESCO*, Ohio, USA) solution and incubated at 37 °C for 12 h followed by washing with sterile water and examine under light microscope for detection of blue spots.

DNA extraction and PCR amplification: Total genomic DNA from the different clones of hairy roots was extracted by CTAB DNA isolation method (Khanuja *et al.* 1999) with minor modifications and subjected to PCR analysis. Plasmid DNA from *Agrobacterium rhizogenes* strains was isolated by alkaline lysis methods (Sambrook *et al.* 1989). Approximately 250 ng of genomic DNA was used as a template for PCR. DNA fragments were amplified using 2.5 U of Taq DNA polymerase (*Shanghi Sangon*) in a final volume of 0.03 cm^3. In order to show the integration of T-DNA of *Ri* plasmid in the transformed roots, segments from both the T_L-DNA (*rol*B gene) and T_R-DNA (*ags* gene) regions were amplified. The pair of gene-specific primers used for amplifying *rol*B (780 bp) gene on the T_L-DNA region were: 5'-ATG-GAT-CCC-AAA-TTG-CTA-TTC-CTT-CCA-CGA-3' and 5'-TTA-GGC-TTC-TTT-CTT-CAG-GTT-TAC-TGC-AGC-3' and primer pair 5'-CGG-AAA-TTG-TGG-CTC-GTT-GTG-GAC-3' and 5'-AAT-CGT-TCA-GAG-AGC-GTC-CGA-AGT-T-3', was used to amplify *ags* gene segment (1.6 kb) of the T_R-DNA region. Non-transformed *S. baicalensis* root DNA was used as a negative control for PCR analysis. The amplification protocol for 780 bp *rol*B fragment was: a 3-min melting at 94 °C followed by 30 cycles of a 50 s melting at 94 °C, a 1 min annealing at 53°C and a 1 min elongation at 72 °C and final elongation for 10 min at 72 °C. Amplification conditions for 1.6 kb T_R (*ags*) fragment was a 3-min melting at 94 °C followed by 36 cycles of a 50 s melting at 94 °C, a 50 s annealing at 53 °C and a 50 s elongation at 72 °C and final elongation for 10 min at 72 °C. The pair of primers specific to the *vir*D1 sequence of *A. rhizogenes* used was: 5'-ATG-TCG-CAAGGA-CGT-AAG-CCC-3' and 5'-GGA-GTC-TTT-CAG-CAT-GGA-GCA-3'. Amplification of *vir*D1 gene (450 bp) was attempted to confirm the absence of bacteria in transformed root tissue. The amplification protocol for the *vir*D1 was: a 5 min melting at 95 °C followed by 25 cycles of a 1 min melting at 95 °C, a 1 min annealing at 52 °C, a 2 min elongation at 72 °C, and final elongation for 5 min at 72 °C. Besides histochemical GUS assay PCR amplification of *uid*A gene was also performed, on hairy root lines and non-transformed control roots to confirm the integration of *uid*A gene in transformed line that fail to react with GUS stain. Primers used for amplification of 697 bp fragment of *uid*A gene were 5'-TTA-TCT-CTA-TGA-ACT-GTG-CGT-CA-3' and 5'-TTG-GAC-ATA-CCA-TCC-GTA-ATA-A-3'. The amplification condition consisted of 1 min melting at 94 °C followed by 30 cycles of a 45 s melting at 94 °C, a 1 min annealing at 57 °C and a 2 min elongation at 72 °C and final elongation for 10 min at 72 °C. PCR products were analyzed by electrophoretic separation on 1 % agarose gels (m/v) in 0.5× TBE buffer and staining with ethidium bromide.

Southern hybridization analysis: Hairy roots clones were subjected to Southern analysis. The DNA isolated from untransformed root was used as a negative control. Southern blot analysis was performed as per Sambrook *et al.* (1989). Root DNA (10 µg) and 10 pg plasmid DNA was digested with *Bam*HI (*TaKaRa Biotechnology*, Beijing, China) and fractionated on a 1.0 % (m/v) agarose gel. The DNA was then transferred to total blot N+ nylon membranes (*AMRESCO*) by capillary transfer for 20 h. The probe for *rolB* genes were generated by PCR method described above and purified by *TaKaRa* agarose Gel DNA purification kit *ver2.0*. Purified PCR products were labeled with-α[^{32}P]-dCTP by using *TaKaRa* random priming kit according to manufacturer's instruction. Hybridization was carried out at 42 °C for 16 h. The membrane was washed with 50 cm^3 pre-warmed buffer: *1*) twice with 2× SSC+ 0.1 % (m/v) sodium dodecyl sulphate (SDS) at 42 °C, *2*) twice with 1× SSC+ 0.1 % SDS, at 42°C, *3*) twice with 0.5× SSC+ 0.1 % SDS at 65 °C, and *4*) twice with 0.1× SSC + 0.1 % SDS at 65 °C for 10 min each. The blots were exposed to *Kodak X-Omat* film at -70 °C for 4 d.

Reverse polymerase chain reaction (RT-PCR): Total RNA was extracted from hairy root clones by CTAB and PVP method (Salzman *et al.* 1999). Approximately 0.6 µg total RNA of each hairy root line was subjected to RT-PCR analysis, according to manufacturer's instruction, by using one step RT-PCR kit (*M-MuLV* provided by *Shanghi Sangon*). The pair of gene-specific primers used for 780 bp fragment of *rol*B gene on the T_L-DNA region were: 5'-ATG-GAT-CCC-AAA-TTG-CTA-TTC-CTT-CCA-C GA-3' and 5'-TTA-GGC-TTC-TTT-CTT-CAG-GTT-TAC-TGC-AGC-3'. RT-PCR condition for 780 bp *rol*B fragment was: a 37 °C for 30 min followed by 3 min melting at 94 °C followed by 30 cycles of a 50 s melting at 94 °C, a 1 min annealing at 53 °C and a 1 min elongation at 72 °C and final elongation for 10 min at 72 °C. RNA sample of each line was tested for the presence of contaminating DNA by PCR amplification with same primer pair and Taq-DNA polymerase. The amplified fragments were separated using electrophoresis on 1 % agarose gel with DNA marker and visualized with ethidium bromide staining under UV radiation.

Total alkaloid extraction and high-performance liquid chromatography analysis: In order to determine the time course for production of flavonoids baicalin and wogonin in dry root sample, we selected the fast-growing clone (HQ-16) and analyzed in B_5 liquid medium at 1:100 inoculum:medium ratio. Three replicates of each culture type were removed for analysis every 5 d. All 6 selected

root clones was also subjected to alkaloid analysis at the day when maximum alkaloid accumulation was determined. Hairy roots were carefully cleaned and dried in a hot air oven at about 40 °C for three days. Extraction of total alkaloid and HPLC analysis for flavonoids, baicalin and wogonin was carried out by the method of Yang *et al.* (2002) with minor modifications. Analysis was performed on reverse-phase HPLC (*Shimadzu, LC-4A*, Japan) with C_{18} column (*Water Co*., USA). The mobile phase (methanol:water:acetic acid 41:59:0.2) was pumped at a flow rate of 1.0 cm^3 per min. The cycle time of analysis was about 45 min. The compound was identified on the basis of its retention time and the comparison of UV spectra (254 nm) with the authentic standard. Quantification was repeated three times for each root line.

Results and discussion

Establishment of hairy root cultures: Hairy roots appeared from deeply wounded sites after 15 - 26 d of inoculation from different explants with A_4GUS, d R1000 and LBA9402 strains of *A. rhizogenes*. Hairy roots, in contrast to untransformed roots, grow autonomously in PGR-free medium showing a loss of gravity response. No adventitious root was formed from control explants. Strain ATCC11325 failed to induce roots from any of the explants used (Table 1) whereas the transformation efficiency with LBA9402 was very low compare to other strains. However R1000 and A_4GUS strains showed different level of transformation frequency and A_4GUS proved to be more competent than R1000. The highest transformation rates were observed in cotyledonary leaf with strain A_4GUS (42.6 %), with strain R1000 23 %, LBA9402 7.4 %, and 15, 17 and 26 d, respectively, was necessary for root emergence. Hypocotyl also proved to be susceptible explant to A_4GUS (26.4 %) and R1000 (8.7 %). Though A_4GUS strain proved to be susceptible to all the explants used, besides cotyledonary leaf and hypocotyl the transformation rate was low. Influence of bacterial strains on transformation frequency has been documented earlier in different plant species (Zehra *et al.* 1999). Strains R1000 and A_4GUS behave differently in present study because they have different chromosomal virulence genes (see Materials and methods). It was observed that most hairy roots emerged from the deep wounded sites rather than mere scratching but this phenomenon was not investigated further. It was hypothesized that the cell which contain high contents of auxin and sucrose are ideal targets for hairy root induction (Nilsson and Olsson 1997). Since the phloem cells, positioned deep in plant organs, are suppose to have high sucrose and auxin contents, they could be the target of *A. rhizogenes*. This might be the possible explanation of the above observation.

Table 1. Frequency of hairy root induction at the site of infection on different explants by different *Agrobacterium rhizogenes* strains in *Scutellaria baicalensis*. Means ± SE, n = 30. No response was found with ATCC11325 strain.

Strain	Explant	Hairy roots [%]	Root emergence [d]
A4GUS	hypocotyl	26.4 ± 2.0	18 ± 1.3
	cotyl. leaf	42.6 ± 3.0	15 ± 1.2
	cotyl. petiole	8.4 ± 0.6	23 ± 1.2
	young leaf	6.7 ± 0.4	25 ± 0.9
R1000	hypocotyl	8.7 ± 0.4	19 ± 0.5
	cotyl. leaf	23.3 ± 1.6	17 ± 0.7
	cotyl. petiole	0	-
	young leaf	0	-
LBA9402	hypocotyl	0	-
	cotyl. leaf	7.4 ± 0.8	26 ± 1.6
	cotyl. petiole	0	-
	young leaf	0	-

Table 2. GUS activity in transformed and non-transformed (control) roots by histochemical GUS assay and PCR amplification in GUS negative explants.

Strain	Number of explants	GUS positive after 7 d	after 2 months	PCR [%]
A_4GUS	72	58	32	100
R1000	5	0	0	0
Control	10	0	0	0

GUS histochemical assay: All the lines induced by A_4GUS were subjected to GUS histochemical assay to determine the potential of *Scutellaria baicalensis* to react with GUS stain (Table 2). At least 3 root tips of each line were stained after 7 and 60 d. Later, all the lines failed to react with GUS stain, were subjected to PCR analysis for *uid*A gene. Blue spots were observed only in 58 out of 72 lines tested after 7 d. However, the intensity of blue stain varied from faint to intense (Fig. 1). Root tip and central cylinder showed the highest intensity of coloring in different root. Interestingly the number of GUS positive lines reduced to 32 after 60 d of testing. However, all the tested 10 lines which failed to GUS reaction showed positive PCR amplification of *uid*A fragment. These results indicated the silencing of GUS gene during the course of growth and tissue development. Roots emerged from R1000 did not show blue coloring. Control root did not show intrinsic GUS like activity indicated that there is no endogenous GUS activity present in the root tissue of *Scutellaria baicalensis* and *uid*A gene can be used as marker in further studies related to *Scutellaria baicalensis* transformation experiments.

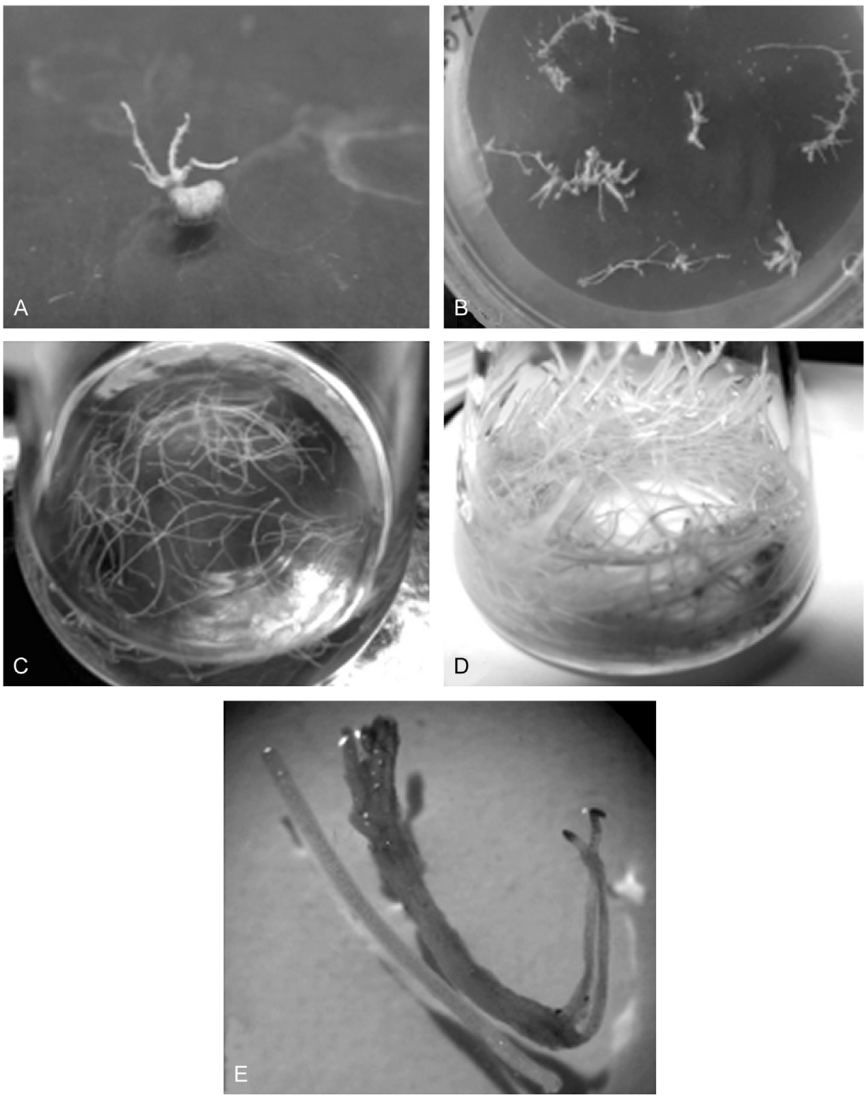

Fig. 1. Hairy root induction in *Scutellaria baicalensis*. *A* - Hairy root induction in infected explants, *B* - hairy root in semi solid culture medium, *C* and *D* - liquid cultures of hairy roots, *E* - one week old hairy roots showing positive GUS staining.

Molecular analysis of transgenic hairy roots: Initially all lines produced by A_4GUS and R1000 were maintained (Fig. 1). Further study was carried out on hairy root clones of A_4GUS only as both R1000 and A_4GUS strains contain same pRiA4 plasmid and the transformation rate of A_4GUS was found higher than R1000. Randomly selected 6 lines of A_4GUS were subjected to molecular analysis for confirmation of transformation event, growth dynamics and chemical analysis of root alkaloids.

T-DNA of agropine type of Ri plasmids consists of T_L-DNA and T_R-DNA, which separated by 16 - 18 kb non-transferred DNA sequence (White *et al.* 1985, De Paolis *et al.* 1985). Approximately 19 - 20 kb size of T_L-DNA integrates into plant genome (Taylor *et al.* 1985). In contrast, T_R-DNA has been found to vary between 8 kb and 20 kb (Slightom *et al.*1986).

Randomly selected 6 lines of hairy root were subjected to PCR analysis. The integration of T-DNA region was confirmed by showing the presence of the 780 bp *rol*B segments and T_R region by the 1.6 kb agropine synthase (*ags*) gene (Fig. 2*A*,*B*). PCR results showed the integration of T_L-DNA and T_R-DNA in all the clones except non-transformed root. However, several reports indicated the possibility of either integration of T_L-DNA or T_R-DNA or both (Batra *et al.* 2004, Guivarch *et al.* 1999) but we found integration of both in all tested lines. Integration of T_L-DNA region was further confirmed by amplification of *uid*A gene (integrated between *rol*C and *rol*D at T_L-DNA region). No amplification of either gene fragments was achieved from non-transformed root DNA. Presence of residual contaminating bacteria was checked by PCR analysis using *vir*D1 specific primer sequence. No amplification was achieved in any hairy root samples. However, positive control showed amplification of

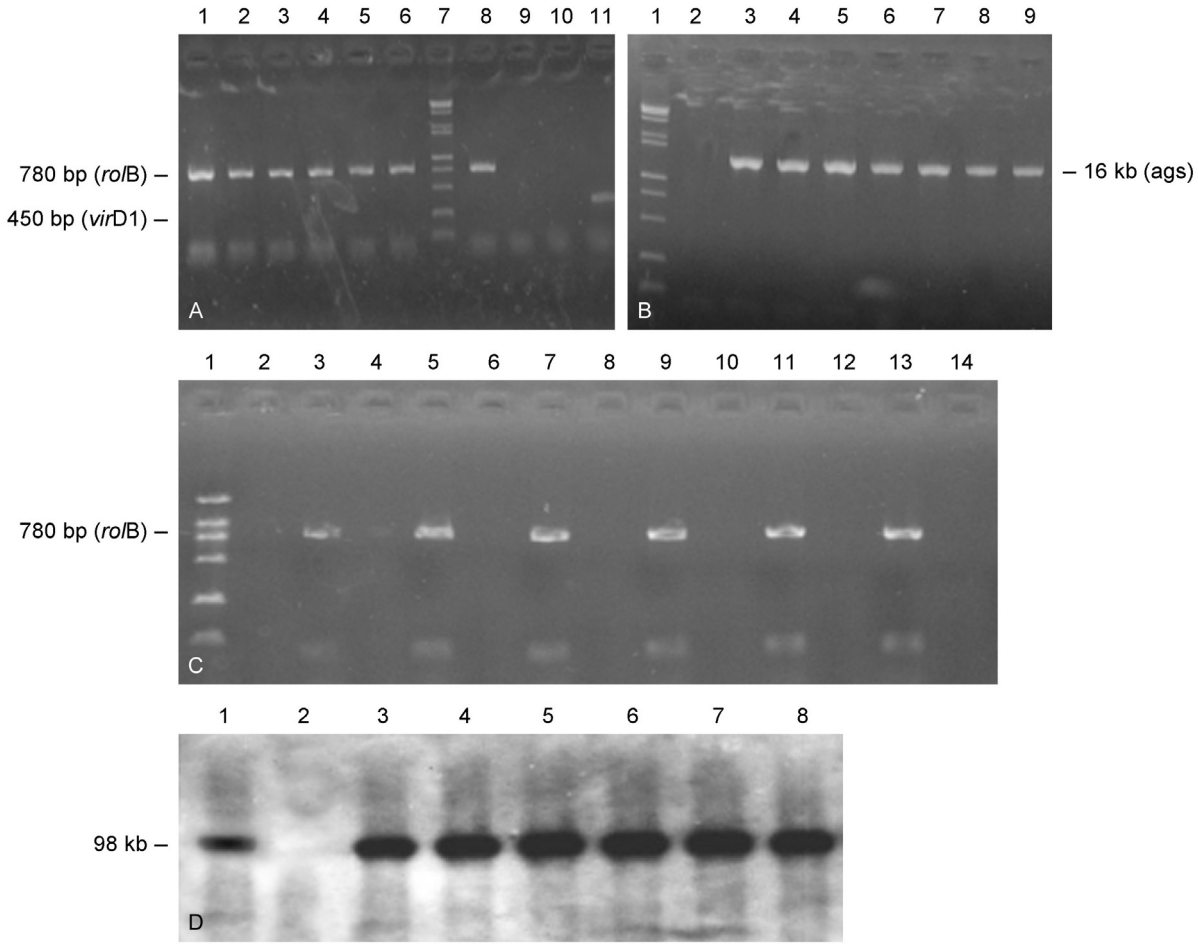

Fig. 2. PCR and RT-PCR analysis of genomic DNA/RNA isolated from *Scutellaria baicalensis* hairy roots. *A* - PCR analysis showing the DNA marker in *lane 7*, *lanes 1 - 6* showing the presence of *rol*B gene in clones HQ-1, HQ-8, HQ-11, HQ-13, HQ-16, HQ-20 respectively, *lane 8* positive control (pRiA$_4$Gus plasmid), *lane 9* non-transformed plant DNA, *lanes 10, 11* sample representative of *vir*D1 in hairy root DNA of HQ-1 and plasmid DNA. *B* - PCR analysis showing the DNA marker in *lane 1*, *lane 2* non-transformed root DNA, *lanes 3 - 8* presence of *ags* genes in HQ-1, HQ-8, HQ-11, HQ-13, HQ-16, HQ-20, *lane 9* positive control (pRIA4Gus plasmid) *C* - RT-PCR analysis of RNA; *lane 1* DNA marker, *lane 2* RNA from non-transformed roots, *lanes 3, 5, 7, 9, 11, 13* HQ-1, HQ-8, HQ-11, HQ-13, HQ-16, HQ-20 showing *rol* B expression (one step RT-PCR amplification of *rol*B), *lanes 4, 6, 8, 10, 12, 14* PCR amplification of DNAase treated RNA samples showing absence of DNA contamination. *D* - Southern blot analysis. *Bam*H1 digested DNA hybridized to probe *rol*B (T_L); *lane 1* positive control (pRiA4 plasmid), *lane 2* DNA of non-transformed root (negative control), *lanes 3 - 8* hairy root DNA of HQ-1, HQ-8, HQ-11, HQ-13, HQ-16, HQ-20.

expected 450 bp *vir*D1 fragment (Fig. 2*A*; HQ-1 clone showing representative sample).

Integration of T-DNA of Ri plasmid was further confirmed by Southern blot analysis (Fig. 2*D*). DNA of all the lines previously subjected to PCR analysis was digested with *Bam*H1 and separated on 1.0 % (m/v) agarose gel. Plasmid DNA was taken as positive control and DNA of non-transformed root as negative control. Following hybridization with PCR generated *rolB* (T_L-DNA) probes, the DNA of independent clones of produced expected band of 9.8 kb whereas no hybridization signal was recorded in non-transformed root DNA. Hairy roots results from the integration of root loci (*rol*), present at T_L-DNA, in plant genome (White *et al.* 1985, Jouanin *et al.* 1987). The expression of *rol*B is reported to interfere with plant morphogenesis resulting in morphological anomalies and adventitious rooting (Schmulling *et al.* 1988). Results obtained from RT-PCR assay further confirm the integration and expression of *rol*B gene (T_L region) in all transformed lines whereas no amplification was seen non-transformed sample (Fig. 2*C*). However, we did not realize the difference in expression level of *rol*B gene in different samples. No amplification signals in any sample (with Taq-DNA polymerase) confirm that RNA samples were free from DNA. The growth pattern of *rol*B-induced root is characterized by fast growth, high branching and plagiotropism (Capone *et al.* 1989b). The meristem-inductive role of *rol*B has been shown *in planta*, and it has been confirmed that *rolB*-induced meristem are initially indeterminate (Koltunow *et al.* 2001). According to most recent model the auxin-inducible nuclear protein plays key role in

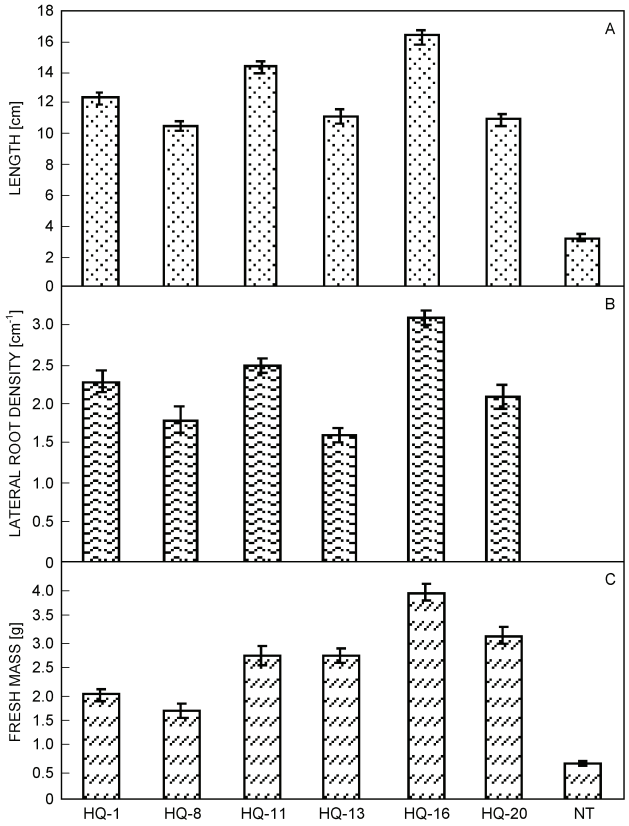

Fig. 3. Growth of *Scutellaria baicalensis* hairy root clones and non-transformed root culture after 35 d of culture on semi-solid culture media in Petri dishes. Total root length (A), lateral root density (number of roots, *B*) and fresh mass (*C*). Means ± SE, $n = 10$.

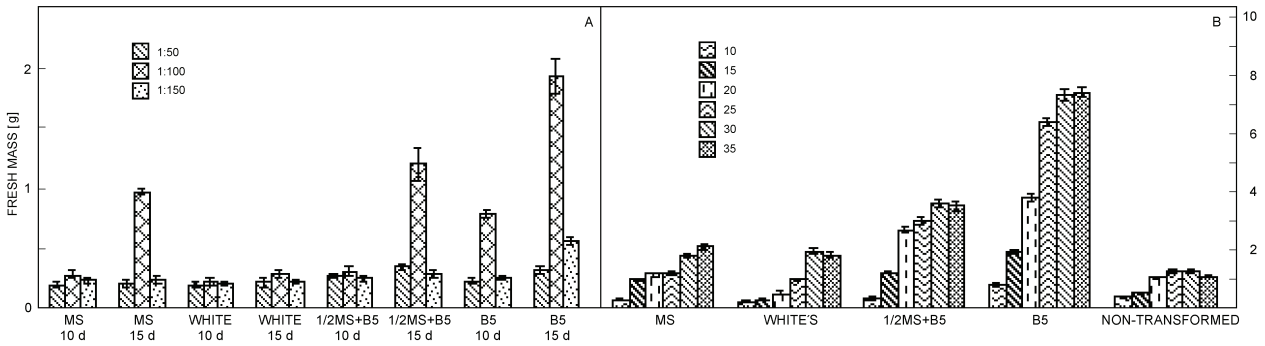

Fig. 4. Establishment of *Scutellaria baiclensis* hairy roots in liquid cultures. *A* - Effect of culture:media ratio and *B* - biomass accumulation in different liquid media at 5, 10, 15, 20, 25, 30, 35 d. Means ± SE, $n = 5$.

regulating the expression of *rol*B thereby triggering meristem formation (Baumann *et al*. 1999).

Further, the determination of a meristem towards the development of a specific organ type depends on the local levels of hormones and on the specific cell competence, which also depends on the physiological stage of the plant. These *rol*B-induced meristem will favour the root formation under higher auxin content. Endogenous auxin content of target cells is regulated by the expression of auxin biosynthesis gene present at T_R region of T-DNA (Huffman *et al*. 1984). In present study hybridization results showed stable integration of T_L DNA and at least single copy integration of T-DNA in plant genome. Evidence collected from GUS assay, PCR amplification, Southern blot and RT-PCR analysis provided sufficient proof of integrative transformation of *S. baicalensis.*

Growth and establishment of root liquid cultures: Initially more than 32 transformed *Scutellaria baicalensis* root clones of A_4GUS were maintained. At the end of the culture period (35 d), transformed root clones varied ($P \leq 0.05$) with respect to total root length, lateral branching density and they accumulated significantly more biomass than control roots (Fig. 3). Total root length

and lateral root density varied significantly also among the clones and was highest in HQ-16 (length 16.3 ± 0.4 cm and density 3.1 ± 0.2 cm^{-1}) (Fig. 3*A*,*B*). No lateral roots were observed on non-transformed roots. Clone HQ-16 was found best for biomass accumulation (3.94 ± 0.58 g; Fig. 3*C*), which was 5.6-fold higher than that of control roots and thus it was selected for further study. Similar difference in growth rate of hairy roots was observed earlier (Mano *et al.* 1986, Yoshikawa and Furuya 1987).

To determine the best medium composition and culture conditions, liquid cultures of clone HQ-16 was initiated in MS, White's, B_5, and 1/2 MS + B_5 vitamins media and growth was monitored every 5 d after inoculation. Approximately 200 mg FM were cultured in different amount of medium to obtain root:media ratios 1:50, 1:100, and 1:150 (m/v). Maximum growth rate was recorded at 1:100 root:medium ratio (1.92 ± 0.152 g) after 15 d of culture (Fig. 4*A*). Growth rate was 3.4-fold higher than at 1:50 and 2.9-fold higher than at 1:150 after 10 d. Results indicate that the root media ratio is extremely important for initial establishment. At lower or higher ratios the increase in fresh mass was significantly reduced and culture did not established at all. Therefore, further experiments were performed only on 1:100 root:medium ratio.

In an independent experiment, the maximum biomass increase (37-fold)was found on B_5 vitamin medium after 30 - 35 d followed by ½ MS+ B_5 vitamin (18-fold), while non-transformed root showed only 6-fold increase in FM after 25 d and turned to watery and eventually died (Fig 4*B*). The maximum biomass increase was from 15 to 30 d. In the present work we found root: medium ratio was very important for establishment of liquid culture, however, after initial establishment the ratio was not found important. High growth rate in B_5 medium showed that low inorganic and high organic nitrogen source is suitable for hairy root survival and growth. As compare to biomass (FM 3.94 ± 0.58 g) on solid media, biomass accumulation was 1.8-fold higher on liquid media after 35 d of culture.

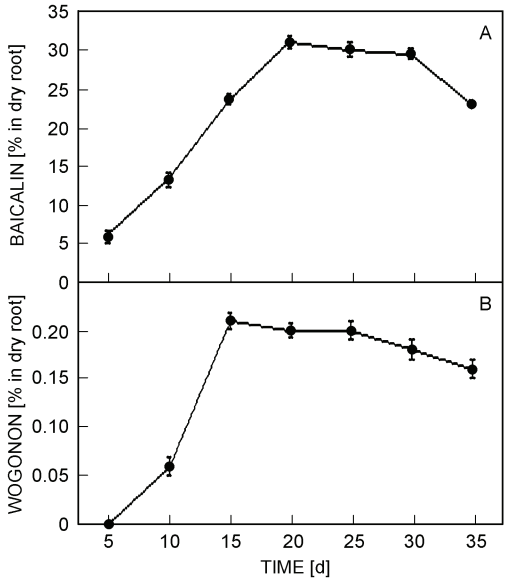

Fig. 5. Time course for baicalin (*A*) and wogonin (*B*) accumulation in hairy root clone HQ-16 in B_5 liquid medium at 1:50 inoculum:medium ratio. Means ± SE, $n = 3$.

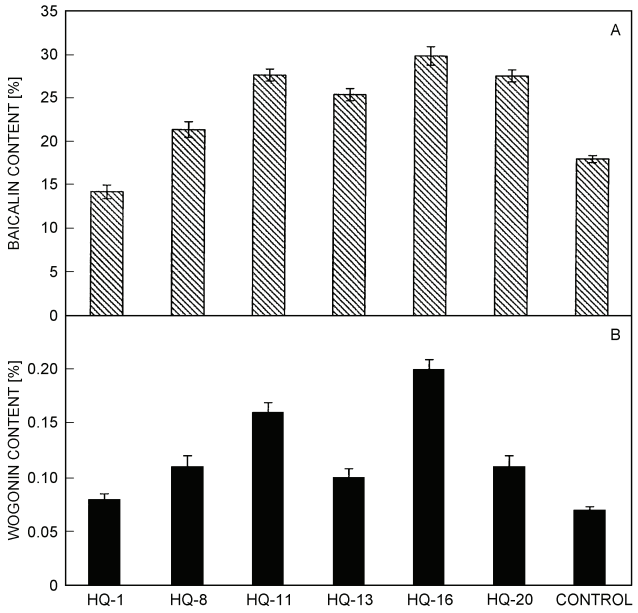

Fig. 6. Baicalin (*A*) and wogonin (*B*) contents after 25 d in liquid culture in transformed root clones on B_5 medim at inoculum:medium ratio 1:50. Means ± SE, $n = 3$.

HPLC analysis of hairy root clones: In the present study, we used HPLC to analyze independent hairy root clones of *Scutellaria baicalensis* for the production of flavonoids baicalin and wogonin in hairy roots and non-transormed roots as a control. In clone HQ-16 showed the baicalin content stabilized at 25 - 30 d, however, maximum content

(31.1 % of dry mass) was recorded at 20 d of culture. Synthesis of wogonin started after 10 d, rapidly increased to maximum (0.21 %) at 15 d, became stable at 20 - 25 d followed by slight decrease (Fig. 5*A*,*B*). These flavonoids accumulated in all hairy root lines but in varying amount (Fig. 6*A*,*B*). The lowest baicalin content was recorded in clone HQ-1 (14.1 %), which was lower than baicalin content of the roots of non-transformed plants (18 %). The highest baicalin content was found in clones HQ-16 and HQ-20. The highest wogonin content was found in clone HQ-16 and HQ-11 and the lowest in clone HQ-1 (0.08 %), which was only slightly higher than in control roots (0.07 %). Clone HQ-16 was found most promising as it accumulated highest content of both the compounds and showed best growth. In principle, alkaloid productivity and accumulation by hairy root cultures is directly related to biomass yield and cellular alkaloid content (Jung and Tepfer 1987).

Hairy roots of *S. baicalensis* grew faster (37 times) than non-transformed root in standardized liquid culture and accumulate the higher amount of baicalin and wogonin. This might offer exciting possibility for large-scale production of these flavonoids in bioreactors for pharmaceutical use.

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