A simple and efficient protocol for hairy root culture of *Arabidopsis thaliana*

Pijush Paul1 · Sukanya Majumdar2 · Sumita Jha2

Received: 11 November 2021 / Accepted: 30 January 2022 / Published online: 22 February 2022
© The Author(s), under exclusive licence to Springer Nature B.V. 2022

Abstract

Hairy root culture (HRC) represent a valuable biotechnological tool for the production of plant secondary metabolites. Secondary metabolome study of *Arabidopsis thaliana* may help to understand the biological roles of various secondary metabolites present in it. The present work deals with the establishment of *Agrobacterium rhizogenes* strains transformed HRC of *A. thaliana* with very high transformation frequency resulting in long term hairy root cultures grown in hormone free media. Optimization of culture medium and standardisation of co-cultivation period are the key role players in obtaining high frequency of hairy roots. Four days of preculture in CIM medium and five min of co-cultivation in the bacterial suspension were found to be optimal conditions for root induction. This protocol could become a powerful tool for transcriptomics, metabolomics and proteomics-based studies for different transgenic root lines of *A. thaliana*.

Key message

Insertion of *rol* genes of *Agrobacterium rhizogenes* enhanced rhizogenic potential of excised explants in *Arabidopsis thaliana*. The optimization process of hairy root culture induction and establishment led to maintenance up to 4 years in hormone free media.

Keywords *Arabidopsis thaliana* · *Agrobacterium rhizogenes* · Hairy root · Transformation efficiency

Introduction

*Agrobacterium rhizogenes* mediated transgenic root system or the hairy roots allow the production of highly diverse molecules (Halder et al. 2018). These hairy roots can express plant natural biosynthesis pathways required to produce specialized metabolites. With the adoption of different binary vector system based on Ri plasmid, hairy roots are excellent system for specialized areas of research as well as innovative applications (Bahramnejad et al. 2019). Industries in the pharmaceuticals, cosmetics and food fields use this expression system in combination with the use of the centralized large-scale bioreactors (Gutierrez-Valdes et al. 2020).

*Arabidopsis thaliana* (Brassicaceae family) is widely considered as a model organism in plant biology mainly because of its small genome size (~ 125 Mb), short generation time (~ 6 weeks), and complete genomic sequence elucidated. This plant has a diverse secondary metabolite arsenal composed of anthocyanins, flavonoids, sinapoyl esters, glucosinolates, terpenoids, camalexin, and other tryptophan derivatives (Chapple et al. 1994; Van Poecke et al. 2001; Chen et al. 2003). Significant progress towards understanding the biological role of different secondary metabolites in *Arabidopsis* has been made. However, many questions remain unanswered. Karimi et al. (1999) provided the first evidence of hairy root production from leaf explants in *A. thaliana* using *Agrobacterium rhizogenes* strains A4, 15834 and other recombinant strains. Mai et al. (2016) performed the *A. rhizogenes* mediated genetic transformation of *A. thaliana* hypocotyls to establish hairy roots capable of producing heterologous green fluorescence protein following
the method of Limpens et al. (2004). The same transformation protocol was followed by Guerineau et al. (2020) using A. rhizogenes ATCC® 15834TM to initiate and culture hairy roots producing human gastric lipase. In the present study A. thaliana was genetically transformed with two A. rhizogenes strains, wild type strain LBA 9402 and LBA 9402 crypt which harbors β cryptogein gene. The cryptogein gene, which encodes a proteinaceous elicitor (Ricci et al. 1993), has been shown to be associated with modulation in second-compounds metabolism in several plant species (Chaudhuri et al. 1962; Amelot et al. 2011; Vuković et al. 2013; Majumdar et al. 2012; Sil et al. 2015). A successful high frequency transformation protocol has been established here to produce fast-growing long-term culture of HRC of A. thaliana which might be studied in future for several important research purposes including effects associated with cryptogein on metabolic profile of A. thaliana hairy root culture.

Materials and methods

Arabidopsis thaliana (ecotype Col 0) seeds were obtained from Dr. David Tepfer, INRA, Versailles, France. A. thaliana seeds were taken in a sterile 2 ml eppendorf tube, washed with 10% Teepol® (Reckitt and Colman, India) for 5 min, followed by 70% ethanol wash for 2 min and finally sterilised with 0.1% aqueous mercuric chloride (HgCl₂) for 5 min. Seeds were then thoroughly washed with sterile distilled water, imbibed for 10 min and incubated for germination in dark on 0.5× MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose and 0.6% agar under a 16/8 h (light/dark) photoperiod at 24 °C. Axenic germinated seeds were maintained on the same media for 4 weeks.

Optimisation of transformation conditions

Rosette shaped leaves from 3-week-old A. thaliana plants were used as the explant for the A. rhizogenes mediated transformation study. The transformation procedures comprised four steps, namely (1) pre-culture of excised leaf explants (cut at both petiolar and apex ends of the leaf) on callus induction medium (CIM; Karimi et al. 1999), (2) cocultivation of leaf explants with A. rhizogenes strain LBA9402 and strain LBA9402-crypt in bacterial suspension containing acetosyringone, (3) incubation on standard medium (SM; Karimi et al. 1999) for 72 h and (4) washing in sterile distilled water, followed by thorough washing in cefotaxime (1000 mg l⁻¹) for 5 min. The explants were then cultured on SM supplemented with cefotaxime (500 mg l⁻¹) with or without kanamycin (50 mg l⁻¹) for 4 weeks. The effect of pre-culture on CIM (0–5 days) and cocultivation period (1–7 min) on transformation efficiency of two A. rhizogenes strains using A. thaliana excised leaf explants was determined. Each treatment was with 30 explants repeated five times (n = 30 × 5 = 150 for each treatment). Infected explants were kept on filter paper soaked with liquid SM in the dark for 72 h. The explants were then washed three times in sterile distilled water followed by thorough washing in cefotaxime (1000 mg l⁻¹ in double distilled water) for 5 min. and cultured on solid SM supplemented with 500 mg l⁻¹ cefotaxime with or without 50 mg l⁻¹ kanamycin for LBA 9402crypt and LBA 9402 infected explants respectively under 16/8-h (light/dark) photoperiod at 24 °C. After 4 weeks on SM medium, the number of explants showing root induction was scored for each treatment for all experiments.

For control, excised leaf explants were similarly pre-cultured on CIM (0–5 days), cocultivated in fresh uninoculated YMB media with acetosyringone (200 µM) for 1–7 min. Then the explants were incubated on filter paper soaked with liquid SM in dark for 72 h. All the explants were washed in sterile distilled water followed by washing in cefotaxime (1000 mg l⁻¹ in double distilled water) for 5 min. Washed explants were blotted dry and cultured on solid SM, supplemented with cefotaxime (500 mg l⁻¹) with or without kanamycin (50 mg l⁻¹). Root induction frequency was scored after 4 weeks on solid SM medium in each treatment for all experiments.

Establishment and maintenance of hairy root lines of A. thaliana

Roots (~2–3 cm) induced from the infected leaf explants on SM medium were excised and cultured on Petri dishes containing 20 ml of solid MS or MS N/5 medium (Amselem and Tepfer 1992; Tepfer 1995) with cefotaxime (500 mg l⁻¹) with or without kanamycin (50 mg l⁻¹) for

Genetic transformation of Arabidopsis thaliana with Agrobacterium rhizogenes strains

Bacterial culture

Agrobacterium rhizogenes wild type strain LBA9402 (pRi 1855) (Petit et al. 1983) and A. rhizogenes strain LBA9402-cryptogein containing pBIN19 vector harboring the synthetic β cryptogein gene under the control of CaMV promoter as previously described (O’Donohue et al. 1995; Sil et al. 2015) obtained from Dr. David Tepfer, INRA, Versailles, France, were grown separately in liquid YMB medium (Hooykass et al. 1977) and liquid YMB medium containing kanamycin (100 mg l⁻¹) respectively, pH 7.0 in a gyratory shaker in dark at 28 °C at 180 rpm for 24–48 h or till the O.D. at 600 nm reached > 0.8. Acetosyringone (200 µM) was added to the bacterial suspensions (approximately 10¹⁰ cells ml⁻¹) 2 h prior to infection to improve virulence.
LBA 9402 crypt and LBA 9402 infected explants respectively under 16-/8-h (light/dark) photoperiod at 24 °C and subcultured after every 4 weeks.

In MS N/5 medium, nitrogen content had been reduced by 80% i.e. MS medium containing 330 mg/l Ammonium nitrate and 380 mg/l Potassium nitrate or one fifth of the total N of MS medium (Amselem and Tefer 1992; Tepfer 1995) and 3% w/v sucrose. Each excised root was propagated and maintained as clones of a separate root line on MS N/5 medium. Fifteen vigorously growing putatively transformed LBA9402 root lines (At-IX 1–15) and LBA9402-crypt- root lines (At-IXcry 1–15) maintained on solid MS N/5 medium ± kanamycin (50 mg l⁻¹) were selected for the confirmation of transgenes integration and expression study.

After 1 year, the LBA9402 and LBA9402-crypt-transformed axenic hairy root cultures were maintained in liquid MS N/5 and liquid MS N/5 with kanamycin (50 mg l⁻¹) respectively (50 ml medium/250 ml Erlenmeyer flask) in a gyrotary shaker at 70 rpm with a regular 8-weekly subculture for over 4 years under 16/8 h (light/dark) photoperiod at 24 °C.

**Confirmation of integration and expression of transgenes by PCR and RT-PCR analysis**

Transgene integration and expression study was conducted 1 year after establishment of root cultures in MS N/5 medium (solid) as well as after 4 years of maintenance in liquid MS N/5 medium.

Integration of transgenes were confirmed by PCR detection of the rol genes (rolA, rolB, rolC and rolD), ags gene of TR-DNA, npt II and crypt genes using genomic DNA extracted from the 15 selected root lines each of At-IX and At-IXcry and primers spanning the corresponding genes (Table S1) following the method described earlier (Majumdar et al. 2012; Paul et al. 2015). The gene-specific primers used as polymerase chain reactions are shown in Table S1. A negative result in PCR amplification of virD1 gene ruled out the chance of Agrobacterium contamination. The plasmid pLJ1 (Jouanin 1984), covering the Ri TL-DNA was used as positive control and genomic DNA isolated from non-transformed excised roots from whole plant (At-NT) was used as negative control.

Expression of transgenes were confirmed by Reverse Transcription (RT)-PCR analysis of the rol genes (rolA, rolB, rolC and rolD), ags gene of TR-DNA, npt II and crypt genes in transformed root lines. Total RNA from 15 selected root lines each of At-IX (LBA9402-transformed) and At-IXcry (LBA9402-crypt-transformed) was extracted and the subsequent cDNA preparation and RT-PCR analysis were performed as described earlier (Paul et al. 2015).

**Growth study of hairy root cultures of A. thaliana**

For this study, a five cm square tuft of hairy root from At-IX and At-IXcry root lines was used as inoculum and cultured in 250 ml flasks containing 50 ml liquid MS N/5 ± kanamycin for 8 weeks in a gyrotary shaker at 70 rpm under 16/8 h (light/dark) photoperiod at 24 °C. The initial fresh weight (FW) and dry weight (DW) were recorded at inoculation time. After 8 weeks of culture, roots were harvested, blotted dry and weighed to determine the final FW. Growth was also measured on a dry weight (DW) basis, after desiccation for 8 h at 50 °C. Growth index was calculated as (Final FW or DW/Initial FW or DW). Growth study was conducted with three randomly selected root lines each of At-IX (LBA9402-transformed) and At-IXcry (LBA9402-crypt-transformed). The experiments were with three replicates repeated three times (n = 3 × 3 × 3 = 27) for each At-IX and At-IXcry transformed root lines.

**Statistical analysis**

All of the experiments were randomized and were repeated at least three times. Percentage data were arcsine transformed (Gomez and Gomez 1984) prior to examination by a one-way analysis of variance (ANOVA) to detect significant differences (p ≤ 0.05) in the mean (Sokal and Rohlf 1987). A post hoc mean separation was performed by the Duncan multiple range test (DMRT) at the same 5% probability level using SPSS software (version 16.0). Variability in the data was expressed as the mean ± standard deviation (SD).

**Result and discussion**

**Establishment of hairy root lines of A. thaliana following transformation with A. rhizogenes strains LBA9402 and LBA 9402 crypt**

**Effect of pre-culture and cocultivation period in bacterial suspension on root induction frequency**

As the expression of rol genes gives rise to rhizogenesis, appearance of hairy root was considered as the indication of putative successful transformation. Pre-culture on callus induction medium (CIM) prior to infection as well as cocultivation period in bacterial suspensions were observed to influence root induction frequency in A. rhizogenes mediated transformation of A. thaliana leaf explants. Leaf explants which were not pre-cultured, on cocultivation with A. rhizogenes strain LBA 9402 and LBA 9402 crypt, showed root induction in 2–10% and 0–3% of the explants respectively. At the same time, 5 min of cocultivation in bacterial suspension culture resulted in highest root induction
frequency under optimum preculture conditions (Figs. 1, 2), for explants infected with A. rhizogenes strain LBA 9402 (95%) and LBA 9402 crypt (65%). Root induction frequency was scored after 4 weeks of culture on solid SM medium for all experiments.

So, 4 days of pre-culture prior to infection and 5 min of cocultivation time were found to be optimum for both the A. rhizogenes strains mediated transformation using A. thaliana leaf explants. Pre-culture on CIM increased the root induction frequency by 9 and 20-fold respectively for LBA9402 and LBA9402-crypt strains (Figs. 1, 2).

Although, the effect of preculture and cocultivation period was not published in Karimi et al. (1999), but they used in their protocol the optimized conditions found in this study. Karimi et al. (1999) used other A. rhizogenes strains than LBA 9402. It is interesting that the optimized values of these parameters seemed to be independent of the strain used. Mai et al. (2016) reported transformation rate approximately 1/6 using hypocotyl explants.

Control leaf explants did not show root induction after 4 weeks of culture on SM, supplemented with cefotaxime (500 mg l⁻¹) and kanamycin (50 mg l⁻¹) and eventually turned brown. However, control leaf explants showed root induction in a very low frequency (5%) and in lower number (1–2 roots per explant) in absence of kanamycin, after 4 weeks of culture on standard medium (SM), supplemented with cefotaxime (500 mg l⁻¹) only. The non-transformed roots so obtained when cultured on MS or MS N/5 medium, supplemented with or without cefotaxime (500 mg l⁻¹) did not grow and turned brown within 2 weeks. As the non-transformed roots did not survive on hormone free media, the non-transformed control for the subsequent studies were not be considered from these experiments.

The excised leaf explants infected with LBA9402 (Fig. 3) showed initiation of root induction (10–20 roots per explant) within 4–7 days. However, maximum root induction frequency (95%) was attained within 15 days of culture on SM, supplemented with cefotaxime (500 mg l⁻¹). The excised leaf explants infected with LBA9402-crypt showed (Fig. 3) initiation of root induction (5–10 roots per explant) within 12–15 days. However, maximum root induction frequency (65%) was attained within 25 days of culture on SM, supplemented with cefotaxime (500 mg l⁻¹) and kanamycin (50 mg l⁻¹).

Establishment and maintenance of HRC's

When induced roots (~ 2–3 cm) from the LBA 9402 and LBA9402-crypt infected explants were excised and cultured for 4 weeks initially on solid MS medium or MS-N/5 medium (Tepfer 1995), supplemented with cefotaxime (500 mg l⁻¹) with or without kanamycin (50 mg l⁻¹), the response was different in the two medium. The roots did not grow and turned brown within 2 weeks on MS medium, while they grew well in MS N/5 medium, they were found to be hairy, whitish in colour, highly branched, showing fine thread like appearances (Fig. 4).

Root culture media are generally more dilute than cell culture media (Tepfer 1995) although hairy root cultures in many species will grow on MS medium (Halder et al. 2018). Karimi et al. (1999) used H-EM medium (De Greef
and Jacobs 1979) instead of MS medium. In the present study, the LBA9402-transformed hairy root lines (At-IX) and the LBA9402-crypt-transformed hairy root lines (At-IXcrypt) were maintained in MS N/5 medium without or with kanamycin (50 mg l⁻¹) respectively for 1 year on solid medium and subsequently in liquid culture for 4 years (Fig. 5). Induced root lines from the LBA 9402 crypt-infected explants were found to be morphologically similar as the LBA 9402 transformed ones. The hairy roots induced by A. rhizogenes strain A4 and 2659 (Karimi et al. 1999) showed very condensed growth with little branching; whereas, 15834-induced roots showed more branching and the 8196-induced roots were calloused.

**Confirmation of transformation**

Fifteen independent root lines characterised by their rapid, plagiotropic growth were selected for subsequent analysis of transgene integration and expression after 1 year and after 4 years of maintenance in vitro. The T-DNA genes (rol genes and ags) were found to be integrated and expressed in both At-IX and At-IXcrypt root lines (Figs. S1, S2). As expected, the integration and expression of crypt and npt II genes were confirmed only in At-IXcrypt root lines. Absence of virD1 amplicon in the transformed root lines eliminates the possibility of bacterial contamination. As expected, amplification was not observed for any of transgenes in the negative control (non-transformed root excised from axenic 4-week-old non-transformed plants). Integration and expression of transgenes were confirmed even after 4 years of maintenance in liquid MS N/5 medium. For use in production of commercially important pharmaceuticals etc. hairy root transformants need to be stable in long term culture ideally in absence of selective pressure (Lipp Joao and Brown 1994). In the present study we have used continual selection and not performed experiments for comparison with maintenance in non-selection media for the LBA9402-crypt-transformed hairy root lines. Hence at present, we are unable to infer on the stability of transgene (retention and expression of crypt and nptII) in hairy root cultures maintained in non-selection medium.

**Growth of HRCs**

Growth index of three selected root lines each of LBA9402-transformed (At-IX) and LBA9402-crypt-transformed (At-IXcrypt) hairy root cultures were compared. Despite similar morphology and growth index (GI) on the basis of FW, the GI on the basis of DW was significantly higher (1.4-fold) in At-IXcrypt root lines than that of At-IX after 8 weeks of culture on liquid MS N/5 medium (Fig. S3). It suggests the higher accumulation of dry matter in Ri-crypt-transformed root lines than Ri-transformed root lines of A. thaliana as has been reported in Convolvulus sepium, Withania somnifera (Chaudhuri et al. 2009). Basu et al. (2017) reported significantly higher biomass accumulation in crypt-cotransformed hairy root lines of Tylophora indica than Ri-transformed hairy root lines. Interestingly, inducers of plant defences have been reported to stimulate growth (Dörnenburg and Knorr 1995; Walker et al. 2002; Wu et al. 2007), which might be a generalised response to stress (Chaudhuri et al. 2009). Thus, cryptogein as a known inducer of plant defences, may stimulate growth as well as
Fig. 4 Selected LBA9402-transformed (At-IX) (a–c) and LBA9402 crypt-transformed (At-IX crypt) (d–f) root lines of A. thaliana maintained for 1 year on solid MS N/5 medium with or without kanamycin (50 mg l⁻¹) under 16-/8-h (light/dark) photoperiod at 24 °C. Bar = 3 cm

Fig. 5 Maintenance of LBA9402-transformed hairy root culture of A. thaliana in liquid MS N/5 medium after a 1 week, b 4 weeks, c 8 weeks of culture under 16-/8-h (light/dark) photoperiod at 24 °C. Bar = 3 cm
induce antioxidant enzyme activity in as reported in tobacco by Kumar et al. (2016).

Thus, in the present study a successful protocol for the establishment and long-term maintenance of HRC of *A. thaliana* was standardised. The transformation procedure was performed following four different steps. The effect of CIM on the transformation efficiency was determined, revealing a gradual increase in root induction till 4th day of incubation. Co-cultivation for 5 min with the bacterial strains gave the highest root induction frequency. LBA 9402 *crypt* transformed root lines and LBA9402-transformed were maintained for 4 years in liquid MS N/5 media with or without kanamycin (50 mg l⁻¹). In whole plants of *A. thaliana*, transformation (via *A. tumefaciens*) with *cryptogoein* led to enhancement in flavonoids (Chaudhuri et al. 2009). In tobacco, transgenic plantlets obtained from hairy roots expressing *cryptogoein* showed enhanced accumulation of total phenolics and total flavonoids (Kumar et al. 2016). Recently, Kumar et al. (2020) correlated changes in metabolites including nicotine biosynthesis with expression analysis of selected genes of phenylpropanoid/benzenoid pathway in tobacco hairy roots expressing a β-cryptogein gene. As a model plant, *A. thaliana* provides an excellent scaffold for the metabolomics and proteomic studies. The LBA 9402 *crypt* transformed HR of *A. thaliana* will be utilized in our future studies on antioxidative responses in addition to effects associated with insertion of *cryptogein* on metabolic profile of the host plant. Such HRCs of different ecological niches might also in the long term be displaying different metabolomics signatures which can be compared using seeds of different ecotype in *A. thaliana*.

Southern-blot of the same HRC lines with probes at different times/years to check for the absence of somaclonal variations will be required for in depth studies (transcriptomics, metabolomics and proteomics) on selected HRC lines. Moreover, as for other GMOs, one should surely select HRC lines with few or single insertions. Multiple copy insertions have been mentioned in HRC by Karimi et al. (1999) and may cause recombination or somaclonal variations overtime. Such variations might interfere with the results of in-depth studies proposed for HRC (omics) and hence should be resolved before undertaking such in depth studies in future.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11240-022-02248-x.

**Acknowledgements** Pijush Paul gratefully acknowledges the University Grant Commission (UGC), Govt. of India for award of Senior Research Fellowship in the Department of Genetics, University of Calcutta. SJ is thankful to the National Academy of Sciences (NASI, Allahabad, India), for award of “Senior Scientist, NASI” and providing the financial support to continue the research. SM acknowledges NASI, Allahabad for Research Associateship. The authors thank the Head, Department of Botany, University of Calcutta for the facilities provided and Dr. Mihir Halder for technical help.

**Author contributions** PP and SJ conceived and designed research. PP conducted this research, analyzed the results. SM wrote the manuscript. All authors read and approved the manuscript.

**Funding** Authors acknowledge UGC (GOI) and NASI (India) for financial support.

**Data availability** All data generated or analysed during this study are included in this published article [and its supplementary information files].

**Code availability** Not applicable.

**Declarations**

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** Not applicable.

**Consent for publication** Authors declare consent for publication.

**References**

Alpizar E, Dechamp E, Lapreye-Montes F, Guilhaumon C, Bertrand B, Jourdan C et al (2008) *Agrobacterium rhizogenes*-transformed roots of *Coffee (Coffeea arabica)*; conditions for long-term proliferation and morphological and molecular characterization. Ann Bot 101:929–940

Amelot N, Carrouche A, Danoun S, Bourque S, Haich J et al (2011) Cryptogein, a fungal elicitor, remodels the phenylpropanoid metabolism of tobacco cell suspension cultures in a calcium-dependent manner. Plant Cell Environ 34:149–161

Amsellem J, Teperf M (1992) Molecular basis for novel root phenotypes induced by *Agrobacterium rhizogenes* A4 on cucumber. Plant Mol Biol 19:421–432

Bahramnejad B, Naji M, Bose R, Jha S (2019) A critical review on use of *Agrobacterium rhizogenes* and their associated binary vectors for plant transformation. Biotechnol Adv 37:107405. https://doi.org/10.1016/j.biotechadv.2019.06.004

Basu A, Roychowdhury D, Joshi RK, Jha S (2017) Effects of *cryptogein* gene on growth, phenotype and secondary metabolite accumulation in co-transformed roots and plants of *Tylophora indica*. Acta Physiol Plant. https://doi.org/10.1007/s11738-016-2306-4

Beck E, Ludwig G, Averswald EA, Reiss B, Schaller H (1982) Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene 19(327–336):161

Chappell CCS, Shirley BW, Zook M, Hammerschmidt R, Somerville SC (1994) Secondary metabolism in Arabidopsis. In: Meyerowitz EM, Somerville CR (eds) Arabidopsis. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 989–1030

Chaudhuri KN, Das S, Bandyopadhyay M, Zalar A, Kollmann A, Jha S, Teperf D (2009) Transgenic mimcry of pathogen attack stimulates growth and secondary metabolite accumulation. Transgenic Res 18:121–134. https://doi.org/10.1007/s11248-008-9201-8

Chen F, Tholl D, D’Auria JC, Farooq A, Pichersky E, Gershenzon J (2003) Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. Plant Cell 15:481–494. https://doi.org/10.1105/tpc.007989

Christensen B, Sriskandarajah S, Serek M, Müller R (2008) Transformation of *Kalanchoe blossfeldiana* with rol-genes is useful
in molecular breeding towards compact growth. Plant Cell Rep 27:1485–1495
De Greef W, Jacobs M (1979) In vitro culture of the sugar beet: description of a cell line with high regeneration capacity. Plant 
Sci Lett 17:55–61
Diof D, Gherbi H, Prin Y, Franche C, Duhoux E, Bogusz D (1995) 
Hairy root nodulation of Casuarina glauca: a system for studying 
symbiotic gene expression in an actinorhizal tree. Mol Plant 
Microbe Interact 8:532–537
Dörnenburg H, Knorr D (1995) Strategies for the improvement of 
secondary metabolite production in plant cell cultures. Enzym 
Microb Technol 17:674–684
Gomez KA, Gomez AA (1984) Statistical procedures for agricultural 
research. 2nd edn. Wiley, New York, pp 306–308
Guerrineau F, Mai NTP, Boitel-Conti M (2020) Arabidopsis hairy roots 
producing high level of active human gastric lipase. Mol Bio-
technol 62:168–176. https://doi.org/10.1007/s12033-019-00233-y
Gutierrez-Valdes N, Häkkinen ST, Lemasson C, Guillet M, Oksman-
Caldentey KM, Ritaia A, Cardon F (2020) Hairy root cultures—a 
versatile tool with multiple applications. Front Plant Sci 11:33(1–11. https://doi.org/10.3389/fpls.2020.00033
Halder M, Roychowdhury D, Jha, S (2018) A critical review on bio-
technological interventions for production and yield enhancement 
of secondary metabolites in hairy root cultures. In: Srivastava et al. (eds) Hairy roots. Springer Nature, Singapore. pp 21–44. 
https://doi.org/10.1007/978-981-13-2562-5_2
Hooikass PJJ, Klapwjik PM, Nuti MP, Schilperoort RA, Rorsch 
A (1977) Transfer of the Ti plasmid to aviru-
lent Agrobacteria and Rhizobium ex planta. J Gen Microbiol 98:477–484
Jouanin L (1984) Restriction map of an agropine Ri plasmid and its 
homologies with Ti plasmids. Plasmid 12:91–102
Karimi M, Van Montagu M, Gheygens G (1999) Hairy root production 
in Arabidopsis thaliana: cotransformation with a promoter-trap vector results in complex T-DNA integration patterns. Plant Cell Rep 19:133–142
Kumar M, Basu A, Kumari P, Jha S, Mitra A (2016) Tobacco plantlets 
ameliorate oxidative stress upon expression of a cryptogein gene. 
Plant Cell Tissue Organ Cult 125(3):553–570
Kumar M, Jha S, Mitra A (2020) Targeted profiling reveals metabolic 
perturbations in cryptogein-cotransformed hairy root cultures of 
Nicotiana tabacum. Acta Physiol Plant 42:166. https://doi.org/10.
1007/s11738-020-00355
Limpens E, Ramos J, Franken C, Raz V, Compaan B, Fransen H, Bisseling T, Geurts R (2004) RNA interference in Agrobacterium 
 rhizogenes transformed roots of Arabidopsis and Medicago tran-
catula. J Exp Bot 55:983–992. https://doi.org/10.1093/jxb/erh122
Lipp Joao KH, Brown TA (1994) Long-term stability of root cultures 
of tomato transformed with Agrobotecrahizogenes R1601. J Exp 
Bot 27:464–467
Mai NTP, Boitel-Conti M, Guerrineau F (2016) Arabidopsis thaliana 
hairy roots for the production of heterologous proteins. Plant 
Cell Tissue Organ Cult 127:489–496. https://doi.org/10.1007/
s11240-016-1073-7
Majumdar S, Garai S, Jha S (2012) Use of the cryptogein gene to stim-
ulate the accumulation of Bacoside saponins in transgenic Bacopa 
monnieri plants. Plant Cell Rep 31:1899–1909. https://doi.org/10. 
1007/s00299-012-1303-3
Murashige T, Skoog F (1962) Revised medium for rapid growth and 
bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
O’Donohue M, Gousseau H, Huet JC, Tepfer D, Pernollet JC (1995) 
Chemical synthesis, expression and mutagenesis of a gene encoding 
β-cryptogein, an elicitor produced by Phytophthora cryptogea. 
Plant Mol Biol 27:577–586
Paul P, Sarkar S, Jha S (2015) Effects associated with insertion of 
cryptogein gene utilizing Ri and Ti plasmids on morphology and 
secondary metabolites are stable in Bacopa monnieri transformed 
plants grown in vitro and ex vitro. Plant Biotechnol Rep 9:231– 
245. https://doi.org/10.1007/s11816-015-0360-9
Petit A, David C, Dahl GA, Ellis JG, Guyon P, Casse-Delbart F, Tempé 
J (1983) Further extension of the opine concept: plasmids in Agro-
bacterium rhizogenes cooperate for opine degradation. Mol Gen 
Genet 190:204–214
Ricci P, Panabière F, Bonnet P, Maia N, Ponchet M, Devergne JC, 
Marais A, Cardin L, Milat ML, Blein JP (1993) Proteinaceous 
elicitors of plant defense responses. In: Fritig B, Legrand M (eds) 
Mechanisms of plant defense responses. Kluwer, Dordrecht, pp 
121–135
Sevón N, Dräger B, Hiltunen R, Oksman-Cal dentey K-M (1997) Char-
acterization of transgenic plants derived from hairy roots of 
Hyoscyamus muticus. Plant Cell Rep 16:605–611
Sil B, Mukherjee C, Jha S, Mitra A (2015) Metabolic shift from with 
asteroid formation to phenylpropanoid accumulation in cryp-
togein cotransformed hairy roots of Withania somnifera (L.) 
Dunal. Protoplasma 252:1097–1110. https://doi.org/10.1007/ 
s00709-014-0743-8
Sokal RR, Rohlf FJ (1987) Introduction to biostatistics. WH Freeman, 
New York
Taneja I, Jaggi M, Wankhede DP, Sinha AK (2010) Effect of loss of 
T-DNA genes on MIA biosynthetic pathway gene regulation and 
alcaloid accumulation in Catharanthus roseus hairy roots. Plant 
Cell Rep 29:1119–1129
Tepfer D (1995) Agrobotecrahizogenes-mediated transformation: 
transformed roots to transformed plants. In: Potrykus I, Spangen-
berg G (eds) Gene transfer to plants. Springer, Berlin, pp 45–52
Van Poecke RMP, Posthumus MA, Dicke M (2001) Herbivore-induced 
volatile production by Arabidopsis thaliana leads to attraction of 
the parasitid Cotesia rubecula: chemical, behavioral and gene-
expression analysis. J Chem Ecol 27:1911–1928. https://doi.org/ 
10.1023/A:1012213116515
Vukovic R, Bauer N, Perica MC (2013) Genetic elicitation by inducible 
expression of β-cryptogein stimulates secretion of phenolics from 
Coelus blumei hairy roots. Plant Sci 199–200:18–28
Walker T, Bais HP, Vivanco JM (2002) Jasmonic acid-induced hypericin production in cell suspension culture of Hypericum 
perforatum L. (St. John’s Wort). Phytochemistry 60:289–293. 
https://doi.org/10.1016/S0031-9422(02)00074-2
Wang YM, Wang JB, Luo D, Jia JF (2001) Regeneration of plants from 
callus cultures of roots induced by Agrobotecrahizogenes on 
Alhagi pseudoalhagi. Cell Res 11:279–284
Wu CH, Tewari RK, Hahn EJ, Paek KY (2007) Nitric oxide elicitation 
induces the accumulation of secondary metabolites and antioxi-
dant defense in adventitious roots of Echinacea purpurea. J Plant 
Biol 50:636–643
Publisher’s Note Springer Nature remains neutral with regard to 
jurisdictional claims in published maps and institutional affiliations.