A Simple and Efficient Protocol for Hairy Root Culture of *Arabidopsis Thaliana*

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Abstract

Hairy root culture (HRC) is a valuable biotechnological tool for the production of plant secondary metabolites. Secondary metabolome study of *Arabidopsis thaliana* can be helpful in understanding the biological roles of various secondary metabolites present in it and HRC constructs the base of such study. 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 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 upto 4 years in hormone free media.

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 such as pharmaceuticals, cosmetics and food sectors use this expression system in combination with the use of the centralized large-scale bioreactors (Gutierrez-Valdes et al. 2020).

Arabidopsis thaliana (family Brassicaceae) is widely considered as a model organism in plant biology mainly because of its small genome size (~125 Mb) and short generation time (~6 weeks). This plant is with a complete genomic sequence elucidated and a diverse secondary metabolite arsenal. This arsenal is composed of anthocyanins, flavonoids, sinapoyl esters, glucosinolates, terpenoids, camalexin, and other tryptophan derivatives (Chapple et al. 1994; Van Poecke, Posthumus & Dicke 2001; Chen et al. 2003). Significant progress towards understanding the biological role of different secondary metabolites in *Arabidopsis* has been made. However many unanswered questions are still left. A total picture of *Arabidopsis* secondary metabolites can be presented only after identifying the complete *Arabidopsis* secondary metabolome. In such work HRC of *Arabidopsis* can play a significant role as a ground work. 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® 15,834™ to initiate and culture hairy roots producing human gastric lipase. In the present study *A. thaliana* is 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 secondary metabolism in several plant species (Chaudhuri et al. 2009; Amelot et al. 2011; Vukovic 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.

Material 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 minutes, followed by 70 % ethanol wash for 2 minutes and finally sterilised with 0.1 % aqueous mercuric chloride ($HgCl_2$) for 5 minutes. Seeds were then thoroughly washed with sterile distilled water, imbibed for 10 minutes and incubated for germination in dark on 0.5 x 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 plants were maintained on the same media for 4weeks.

Genetic transformation of *Arabidopsis thaliana* with *Agrobacterium rhizogenes* strains

Bacterial culture

A. 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 10^{10} cells ml^{-1}) 2 h prior to infection to improve virulence.

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 (1,000 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 four 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 = 30x5=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 (1,000 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 9402*crypt* 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 (1,000 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 LBA 9402 *crypt* andLBA 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-IXcrypt 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 gyratory shaker at 70 rpm with a regular 8-weekly subculture for over four 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 one year after establishment of root cultures in MS N/5 medium (solid) as well as after four 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-IXcrypt 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-IXcrypt (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-IXcrypt root lines was used as inoculum and cultured in 250 ml flasks containing 50 ml liquid MS N/5 ± kanamycin for eight weeks in a gyratory 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 eight 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 ºC. 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-IXcrypt (LBA9402-*crypt*-transformed). The experiments were with three replicates repeated three times (n=3x3x3=27) for each At-IX and At-IXcrypt transformed root lines.

Statistical analysis

All of the experiments were randomized and were repeated at least three times. Data were examined by a one-way analysis of variance (ANOVA) to detect significant differences ($p \leq 0.05$) 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, five minutes of cocultivation in bacterial suspension culture resulted in highest root induction frequency under optimum preculture conditions (Fig. 1, Fig. 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, four days of pre-culture prior to infection and five minutes 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 (Fig. 1, Fig. 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. Control leaf explants did not show root induction after four 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) after four weeks of culture on standard medium (SM), supplemented with cefotaxime (500 mg l^{-1}). 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 two weeks. As the non-transformed roots did not survive on hormone free media, the non-transformed control for the subsequent studies could 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 mgl^{-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 four 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 two 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 one year on solid medium and subsequently in liquid culture for four 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 callused.

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 (Fig. S1, Fig. 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 four 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 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 4 different steps. The effect of CIM on the transformation efficiency was determined, revealing a gradual increase in root induction till 4^{th} day of incubation. Co-cultivation for 5 mins 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 *cryptogein* led to enhancement in flavonoids (Chaudhuri et al. 2009). In tobacco, transgenic plantlets obtained from hairy roots expressing *cryptogein* 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*.

Declarations

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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.

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Figures

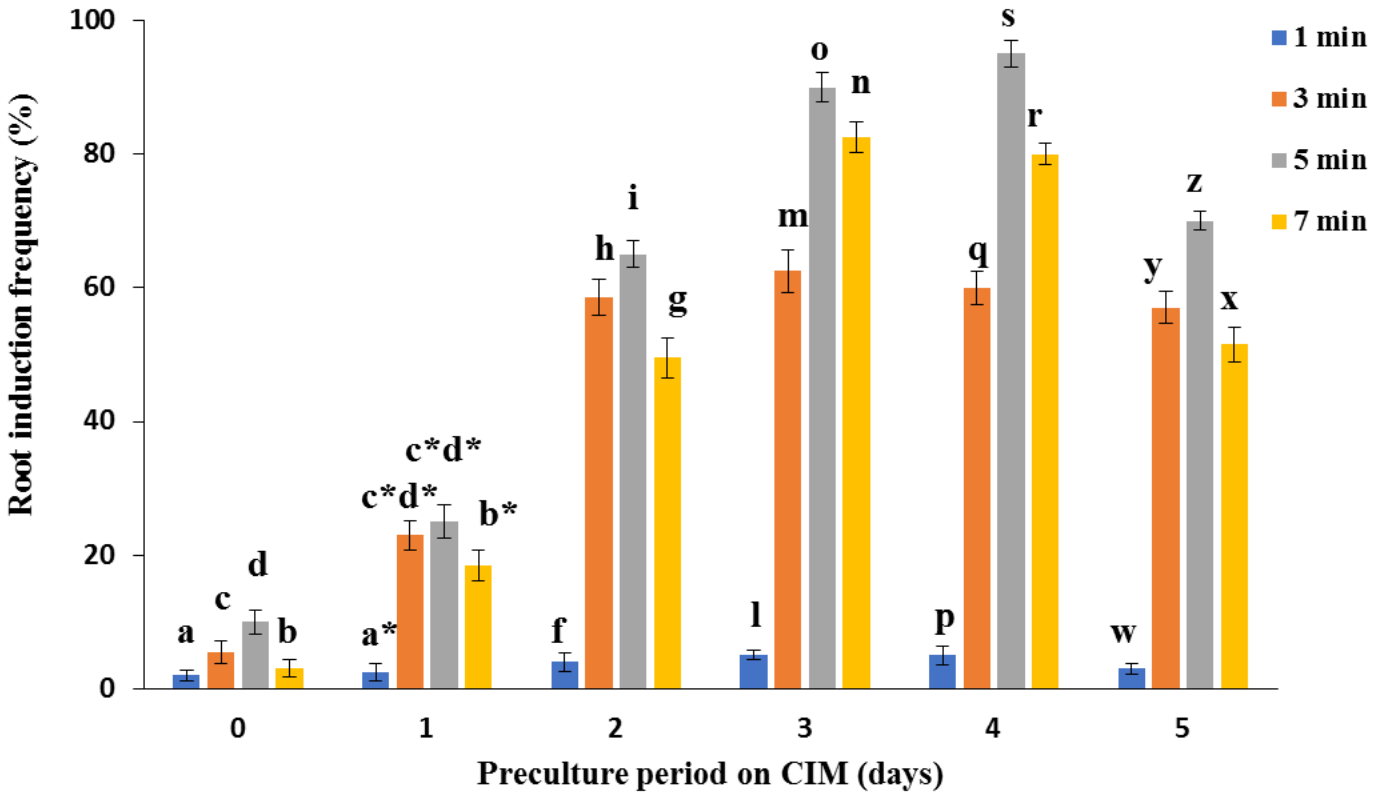

Figure 1

The effect of pre-culture on CIM (0-5 days) and cocultivation period (1-7 min) on root induction frequency in A. thaliana leaf explants infected with A. rhizogenes strain LBA 9402. Values represent mean ± standard deviation (SD) of five independent experiments (n = 150). Bars with the same letter are not significantly different according to ANOVA ($p \leq 0.05$) and DMRT for each data point

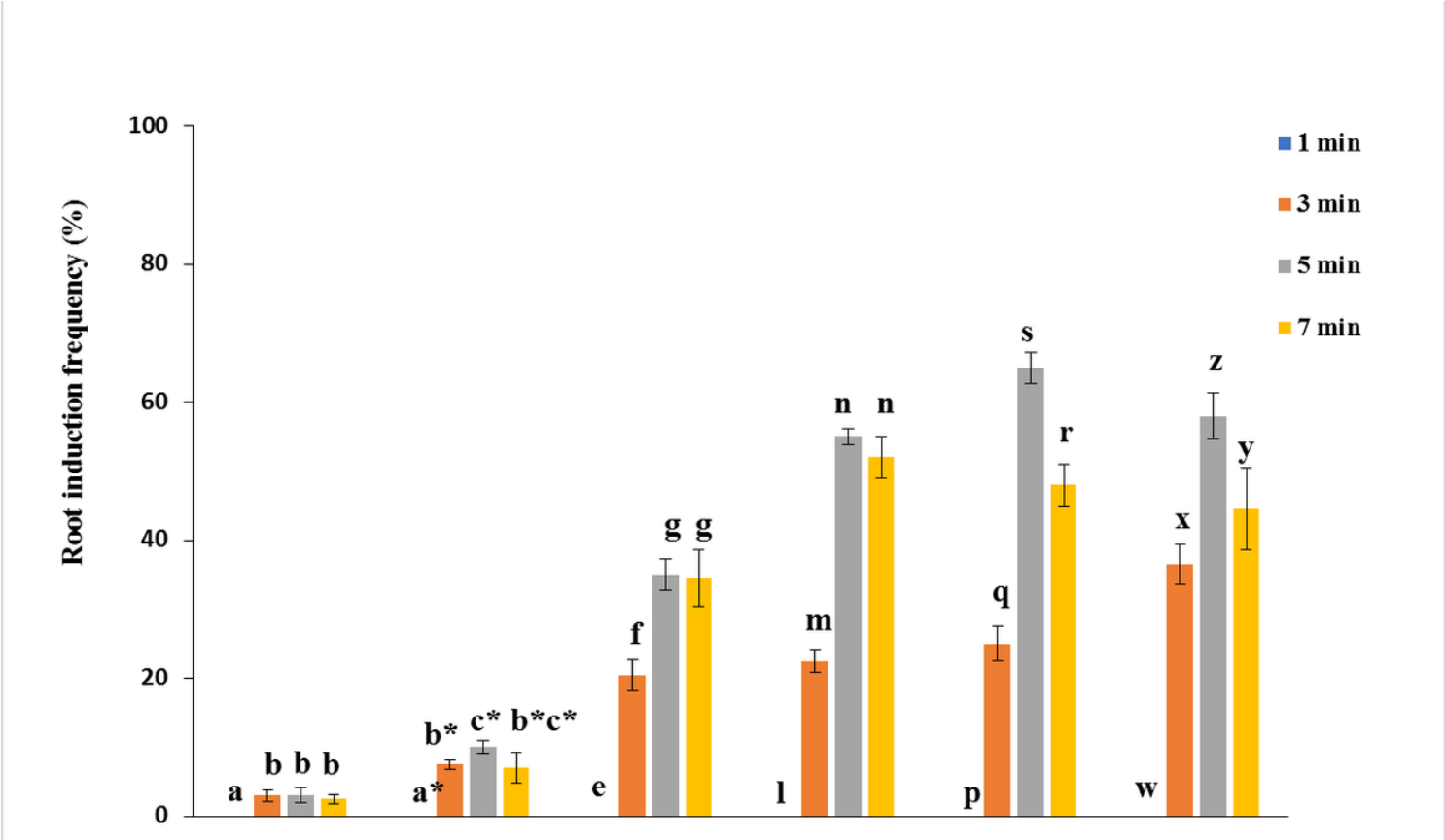

Figure 2

The effect of pre-culture on CIM (0-5 days) and cocultivation period (1-7 min) on root induction frequency in A. thaliana leaf explants infected with A. rhizogenes strain LBA 9402crypt. Values represent mean ± standard deviation (SD) of five independent experiments (n = 150). Bars with the same letter are not significantly different according to ANOVA ($p \leq 0.05$) and DMRT for each data point

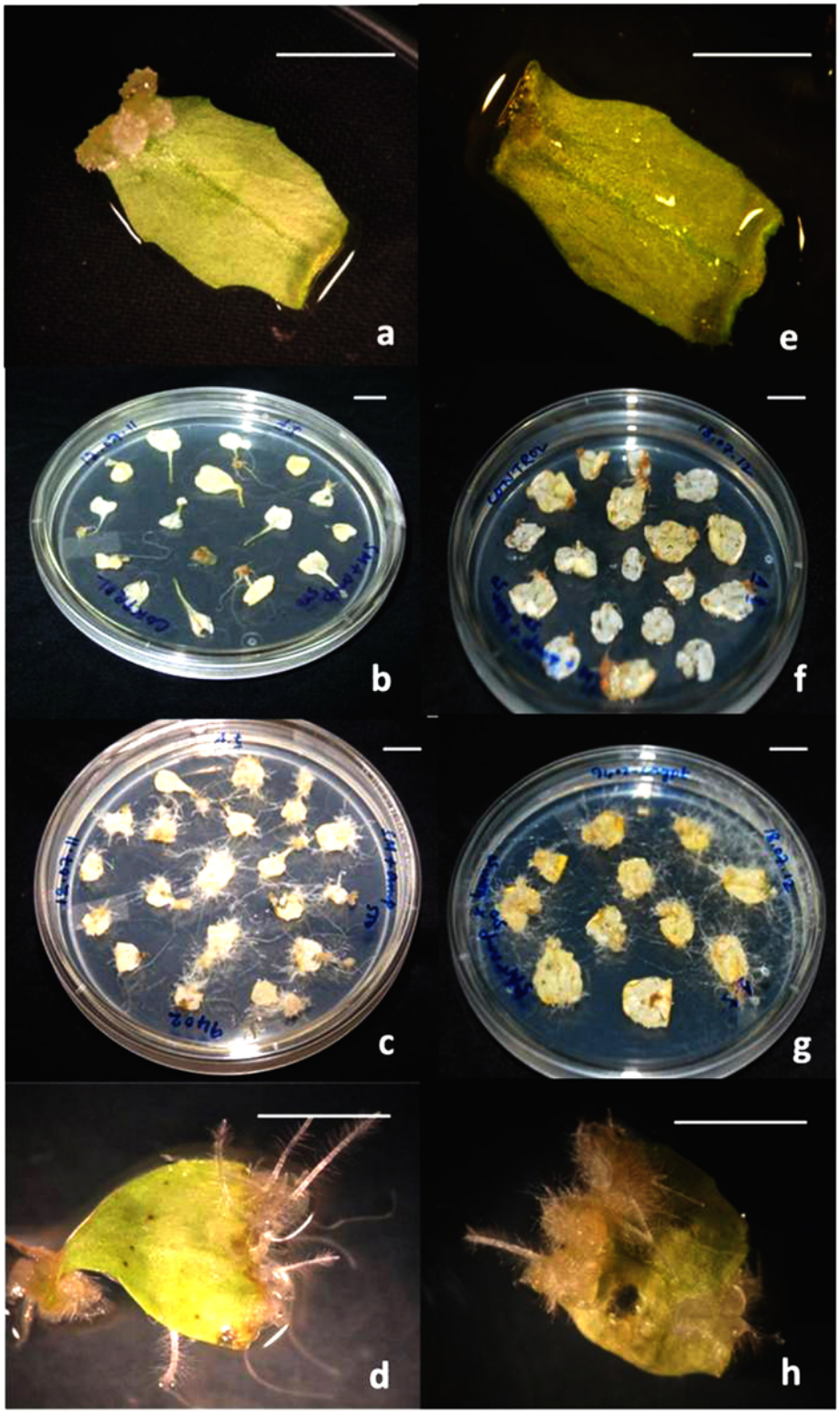

Figure 3

Response of excised leaf explants of A. thaliana following infection with A. rhizogenes strains LBA9402 and LBA9402-crypt. a-b control leaf explants after 15 (a) and 30 (b) days of culture on SM+cefo500; c-d leaf explants infected with LBA9402 after 30 days of culture on SM+cefo500; e-f control leaf explants after 15 (a) and 30 (b) days of culture on SM+cefo500+kan50, g-h leaf explants infected with LBA9402-crypt after 30 days of culture on SM+cefo500+kan50. Bar = 1 cm

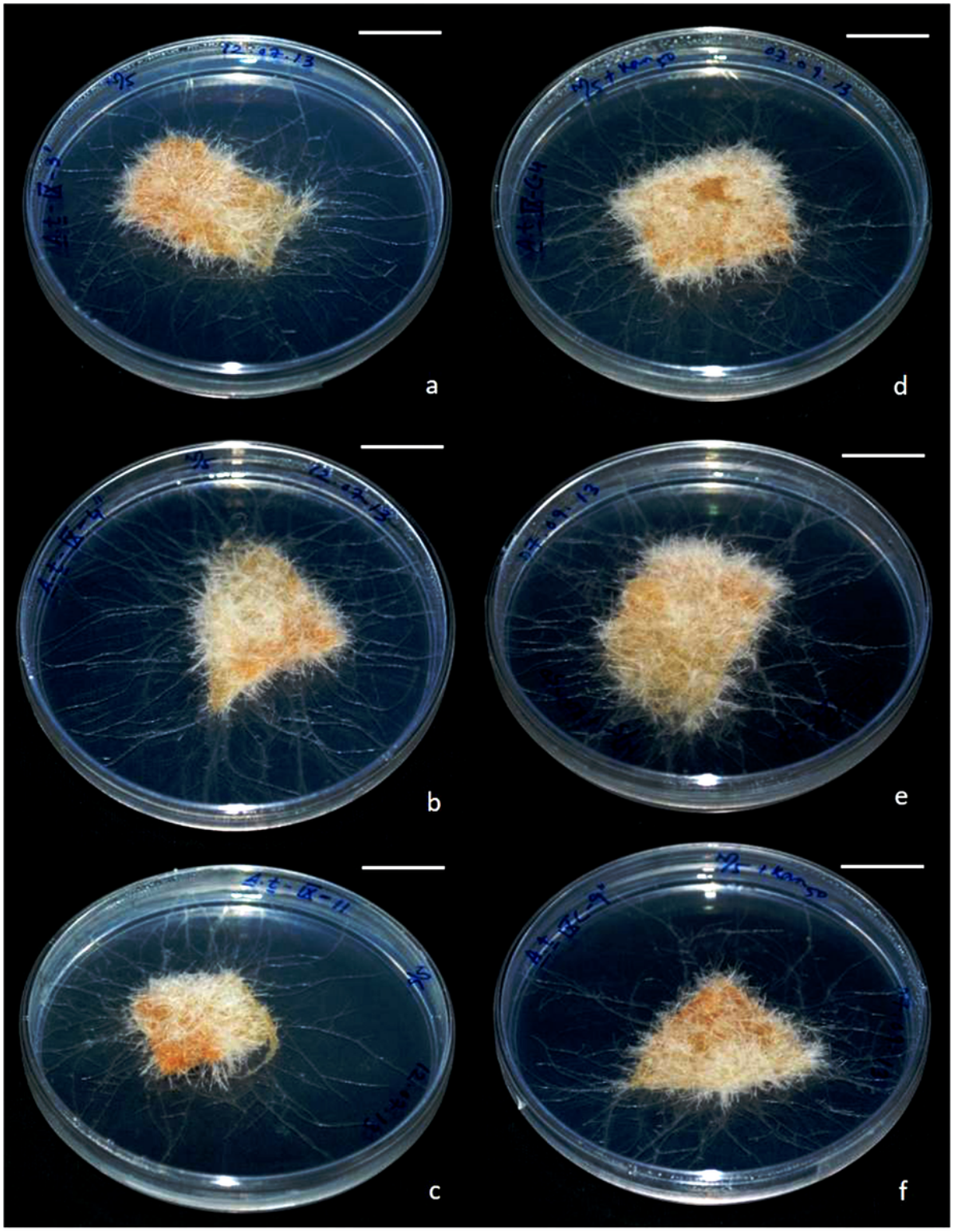

Figure 4

Selected LBA9402-transformed (At-IX) (a-c) and LBA9402 crypt-transformed (At-IX crypt) root lines of A. thaliana maintained for one year on solid MS N/5 medium with or without kanamycin (50 mg l-1) under 16-/8-h (light/dark) photoperiod at 24 ºC. Bar = 3 cm

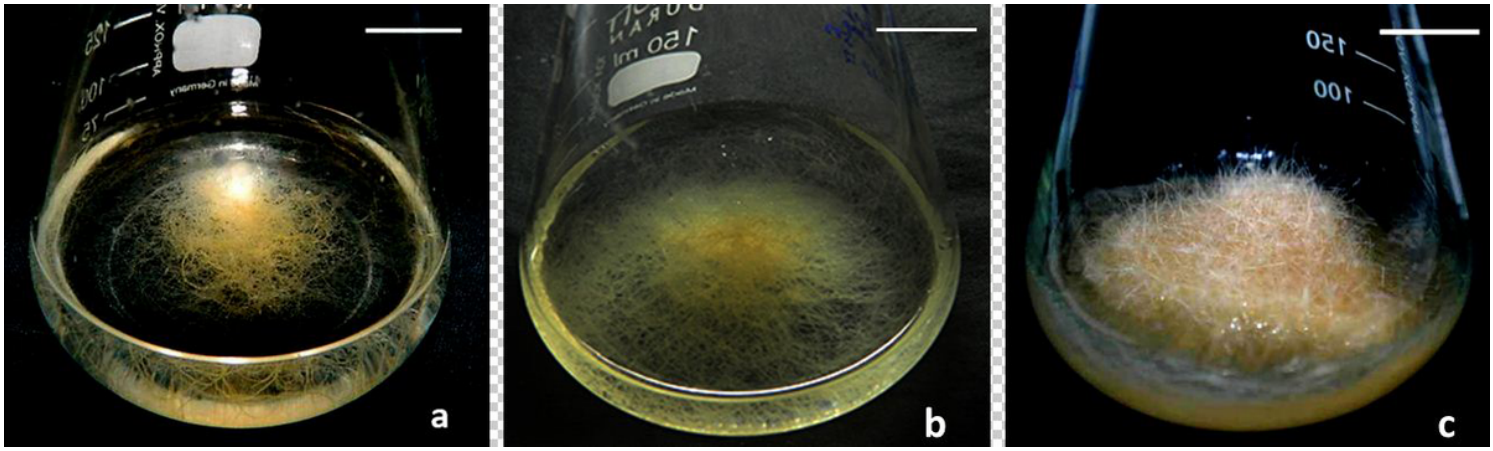

Figure 5

Maintenance of LBA9402-transformed hairy root culture of A. thaliana in liquid MS N/5 medium after (a) one week, (b) 4 weeks (c) 8 weeks of culture under 16-/8-h (light/dark) photoperiod at 24 ºC. Bar = 3 cm

Supplementary Files

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