Effects associated with insertion of *rol* genes on morphogenic potential in explants derived from transgenic *Bacopa monnieri* (L.) Wettst

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Abstract

The present study deals with the establishment of *rolA*-transgenic and *rolB*-transgenic plants for the first time through *Agrobacterium tumefaciens* mediated transformation, exploiting the inherent morphogenic potential of an important medicinal plant, *Bacopa monnieri* (L.) Wettst. The *rolA*-transgenic and *rolB*-transgenic plants showed integration and expression of *rolA* and *rolB* genes respectively, whereas Ri-transformed plants showed integration and expression of *rolA*, *rolB*, *rolC* and *rolD* genes. Morphogenic potential of different types of explants derived from *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants on basal medium was evaluated. Shoot organogenesis was enhanced significantly in leaf (1.6-fold) and internode (1.4-fold) explants derived from *rolA*-transgenic plants, *rolB*-transgenic leaf (2.4-fold) and internode (1.6-fold) explants as well as leaf (5.2-fold) and internode (3.3-fold) explants derived from Ri-transformed plants compared to explants from non-transformed plants. Substantial increase in root organogenesis was also noticed in *rolA*-transgenic leaf (1.7-fold) explants, *rolB*-transgenic leaf (3.6-fold) and internode (1.4-fold) explants as well as leaf (4.1-fold) and internode (1.9-fold) explants derived from Ri-transformed plants compared to non-transformed ones. In addition to this, growth of root tip and shoot regeneration was also noticed from Ri-transformed root explants, but not in *rolA*-transgenic, *rolB*-transgenic and non-transformed roots. Clones of all three transgenic plants differed morphologically from non-transformed plants; however, *rolB* gene alone has a pronounced effect in alteration of plant phenotype in *B. monnieri*. Clones of *rolB*-transgenic plants were similar in shoot length, internode length, number of nodes/plant and number of leaves/plant when compared with Ri-transformed plant clones

Key Message

Insertion of *rol* genes, individual or in combination, of *Agrobacterium rhizogenes* resulted in enhanced morphogenic potential of excised explants and alteration in phenotype of transgenic plants of *Bacopa monnieri* maintained *in vitro*.

Introduction

Plant cells are highly totipotent to undergo morphogenesis *in vitro* through adventitious shoot bud differentiation and/or somatic embryogenesis (Bhojwani and Razdan 1983). Since decades, this morphogenic competence of the plant cell has been exploited in plant tissue culture for conservation and sustainable production of highly-valued plants throughout the year. Although both the morphogenetic pathways are under the control of several endogenous and exogenous factors, the classical finding of Skoog and Miller (1957) on quantitative auxin/cytokinin ratio plays a pivotal role in determining the morphogenetic pathways. Since then, several pioneer works have been conducted which concluded that the fate of regenerating organs during morphogenesis can be altered either exogenously by the application of hormones in the culture media (Thorpe 1980; Tran Than Van 1980; Hicks 1994) or endogenously within the plant body by introducing T-DNA genes of *Agrobacterium rhizogenes* synthesizing auxins and cytokinins (Owens et al. 1988; Wabiko and Minemura 1996).

For centuries, extensive studies have been conducted to decipher the complex plant-*A. rhizogenes* pathogenic interaction and functions of Ri T-DNA oncogenes (White et al. 1985; Nilsson and Olsson 1997; Mauro et al. 2017). This pathogenic bacterium is known to induce neoplastic root growth in host plant at infection sites by transferring its T-DNA to produce opines as source of food and energy (Tepfer and Tempé 1981; Chilton et al. 1982). Molecular analysis has revealed that four *ro*oting *l*oci (*rolA*, *rolB*, *rolC*, *rolD*) of T-DNA are involved in rhizogenesis by manipulating endogenous hormone metabolism or sensitivity in transformed plant cells although exact mechanism of action is still unclear (White et al. 1985; Mauro et al. 2017). Due to hormonal disequilibrium, developmental programmes controlled by hormones are altered, as a side-effect, in transformed plants affecting morphogenesis *in vitro*. The Ri-transformed rhizoclones can regenerate spontaneously or induced in hormone-supplemented media into whole fertile Ri-transformed plants with altered phenotypes through direct or indirect morphogenesis (Roychowdhury et al. 2013; Makhzoum et al. 2013). Although regeneration of Ri-transformed plants expressing T-DNA genes has been studied in many plant species, few reports are available on transgenic plant production harbouring individual *rol* genes which have explained the contribution of each of *rol* genes in changing plant phenotype (Roychowdhury et al. 2013; Sarkar et al. 2018). The inter-relationship between *rol* genes and morphogenesis is further confirmed when *de novo* morphogenesis in explants of Ri-transformed plants or *rol*-transgenic plants has been investigated although the number of such reports is very limiting. Among them, *rolB* gene has been extensively studied and was considered as a meristem-inducing gene. Altamura (2004) hypothesized that increased auxin sensitivity caused by *rolB* gene product could trigger the formation of meristemoids which in turn can regenerate into a wide range of organs depending upon cell's inherent competence and/or existing hormonal conditions during organogenesis. Casanova et al. (2003), on the other hand, demonstrated the dual effect of *rolC* gene having both cytokinin-like and auxin-like activities due to improved shoot and root regeneration in *rolC*-transgenic *Dianthus caryophyllus* explants in presence of cytokinin and auxin respectively. However, the effect of *rolA* and *rolD* genes on organogenesis has been very less explored. van Altvorst et al. (1992) reported root induction in *rolA* or *rolB* or *rolABC*-expressing tomato leaf explants in presence of auxin but not in controls due to increased auxin sensitivity in *rol*-transformed explants. However, unlike other *rol* genes, *rolD* gene showed enhanced flower induction both in flowering and rooting medium in transgenic tobacco thin cell layer explants indicating the commitment of this gene towards floral organogenesis (Mauro et al. 1996).

Bacopa monnieri (L.) Wettst. of the Plantaginaceae family is mainly recognized as a memory-enhancer herb in the traditional systems of medicine although it has several other pharmacological uses (Majumdar et al. 2013; Ramasamy et al. 2015; Jeyasri et al. 2020). Being the only herbal source of triterpenoid saponins (bacopa saponins or bacosides), tissue culture approaches including micropropagation from pre-existing meristems and *de novo* organogenesis from different explants were undertaken to meet the commercial demand of this medicinal plant species (Tiwari et al. 1998, 2001; Mohapatra and Rath 2005; Sharma et al. 2015; Sarkar and Jha 2017). Some of these studies revealed high morphogenic potency of excised leaf and internode explants even in absence of exogenous hormones suggesting this species to be an ideal system to study the factors underlying morphogenesis

in vitro, circumventing the effects of exogenous phytohormones (Mohapatra and Rath 2005; Sarkar and Jha 2017).

Establishment of hairy root culture and Ri-transformed plant regeneration from Ri-transformed roots following *A. rhizogenes* mediated genetic transformation have been reported in *B. monnieri* which highlighted the stimulatory role of T-DNA genes on secondary metabolite production (Majumdar et al. 2011; Bansal et al. 2014; Paul et al. 2015). Spontaneous shoot regeneration was noted either directly or from dedifferentiated Ri-transformed calli in growth-hormone free basal medium in Ri-transformed root explants but not in control non-transformed root explants (Majumdar et al. 2011; Paul et al. 2015; Largia et al. 2016). This suggests that Ri-transformed roots have higher competence to undergo shoot organogenesis due to the regulation of morphogenic pathways in transformed tissues by T-DNA oncogenes specifying auxin and cytokinin synthesis. As excised *B. monnieri* explants can inherently undergo morphogenesis *in vitro* even in absence of exogenous hormone, the interaction of *rol* genes with endogenous hormones during *in vitro* organogenesis can easily be investigated in this species. Several researches have been conducted on *A. tumefaciens* mediated transformation in *B. monnieri* in order to develop an efficient transformation protocol in this species (Ramesh et al. 2011; Mahender et al. 2012; Aggarwal et al. 2013; Yadav et al. 2014). The *crypt*-transformed plants were also developed in this medicinal herb through *A. tumefaciens* mediated transformation to examine the role of *crypt* gene on plant morphology and bacoside accumulation (Majumdar et. 2013; Paul et al. 2015). To our knowledge, there are no reports till date on the establishment of transgenic *B. monnieri* plants harbouring individual *rol* genes as well as on the role of *rol* genes on morphogenesis. Therefore, in the present research work, *B. monnieri* was transformed by individual *rolA* and *rolB* genes and also by Ri T-DNA through *Agrobaterium*-mediated transformation. The objectives of the study were to evaluate the effects associated with insertion of *rol* genes, either individual or in combination, on plant phenotype and totipotency expression potential of differentiated transgenic explants during adventitious shoot and root organogenesis.

Materials And Methods

Tissue culture

Axenic cultures of *B. monnieri* were established from shoot tip explants obtained from plants growing at the Experimental Garden, Department of Botany, University of Calcutta as described earlier (Sarkar and Jha 2017). After surface-sterilization, shoot tips with nodes (~2–2.5 cm) were placed on MS (Murashige and Skoog 1962) basal medium supplemented with 3 % (w/v) sucrose and 0.75 % (w/v) agar (Sigma) and cultured at 25 ± 1°C and 50–60 % relative humidity under 16-/8-h (light/dark) photoperiod with light supplied by cool white fluorescent tubes (Philips, India) at an intensity of 48 µmol m^{-2} s^{-1}.

Bacterial strains and cultures

A. tumefaciens strains GV3101 harbouring *rolA* and *nptII* genes, LBA4404 harbouring *rolB* and *hptII* genes and wild type agropine *A. rhizogenes* strain A4 harbouring pRiA4 were used for transformation. *A.*

tumefaciens strains GV3101-*rolA*, LBA4404-*rolB* and *A. rhizogenes* strain A4 were cultured on solid AP medium (Petit et al. 1983; pH 6.6) supplemented with neomycin (200 mg l^{-1}) and rifampicin (50 mg l^{-1}), LB medium (Miller 1972; pH 7.0) supplemented with kanamycin (50 mg l^{-1}) and rifampicin (50 mg l^{-1}) and YMB medium (Hooykass et al. 1977; pH 7.0) respectively for 2-3 days in dark at 28 ºC and the petriplates were, thereafter, maintained at 4º C. Bacterial suspensions were prepared by inoculating a loop-full of bacterium from a single colony in corresponding liquid bacterial media (10 ml) from respective bacterial stock cultures and incubated on a gyratory shaker (Certomat) at 28 ºC in dark at 180-200 rpm for 24-48 h. Acetosyringone (200 µM) was added to overnight-grown bacterial suspension (~10^{10} cells ml^{-1}) 2 h prior to infection to improve virulence.

Sensitivity test of kanamycin and hygromycin on shoot organogenesis from explants

Prior to transformation, the amount of kanamycin and hygromycin required to inhibit shoot organogenesis in non-transformed leaf and internode explants was tested. The explants were cultured on MS medium containing different concentrations of kanamycin (25, 50, 75 and 100 mg l^{-1}) or hygromycin (5, 10, 15, 20, 25 and 30 mg l^{-1}). Data on frequency of shoot organogenesis was taken after four weeks of culture. The minimum concentrations of kanamycin and hygromycin that totally inhibit shoot organogenesis was determined and used to select transgenic shoots. For each experiment, 30 excised leaves and 30 excised internodes were used per treatment and the experiment was repeated thrice.

Transformation procedure

Leaves (1-1.5 cm) and internodes (~0.9 cm) excised from 15-day-old *in vitro*-grown plants cultured on MS basal medium were used as explants. Transformation was done following the protocol as reported earlier (Paul et al. 2015). For each experiment, 50 leaves and 50 internode explants excised from 15 different *in vitro* propagated plants were infected and the experiment was repeated thrice.

Transformation with A. tumefaciens strains GV3101-rolA and LBA4404-rolB

After surface-sterilization, control and infected explants were cultured on MS medium supplemented with cefotaxime (500 mg l^{-1}) and kanamycin (100 mg l^{-1}) for GV3101-*rolA* transformation and MS medium supplemented with cefotaxime (500 mg l^{-1}) and hygromycin (15 mg l^{-1}) for LBA4404-*rolB* transformation.
Transformation with A. rhizogenes strain A4

After surface-sterilization, control and infected explants were cultured on MS medium supplemented with cefotaxime (500 mg l^{-1}). Roots induced at wound sites of infected explants were excised and cultured on MS medium containing cefotaxime (500 mg l^{-1}) under 16/8 h (light/dark) photoperiod or under complete darkness. Each excised root was propagated as a separate root line and sub-cultured at 4-weeks interval on the same medium. Fast growing axenic root lines were maintained on cefotaxime-free MS medium after six months of culture initiation with regular subculture at 4-weeks interval. Roots induced from

explants not infected with *A. rhizogenes* (control) were similarly excised and cultured on MS medium supplemented with cefotaxime (500 mg l^{-1}).

Establishment of transgenic plants

Shoot organogenesis and establishment of rolA-transgenic and rolB-transgenic plants

After two months of shoot bud induction from wound sites of infected explants on selection media, infected explants with developing micro shoots (< 1.5 cm) were transferred to selection media supplemented with 1.0 mg l^{-1} 6 benzylaminopurine (BA). Micro shoots regenerated from shoot buds induced from wound sites of different explants derived from different plants were propagated as separate clones. After four weeks, elongated shoots were excised and cultured on selection media devoid of BA (sub culturing was done at an interval of four weeks and cefotaxime concentration was gradually reduced to zero). Each of the micro shoots was rooted spontaneously to develop complete rooted plantlets. After six months of culture initiation, putatively *rolA*-transgenic and *rolB*-transgenic plants were cultured in respective bacterial media on a gyratory shaker at 28 ºC in dark at 180 rpm for 24-48 h to determine their axenic nature. Finally, clones of axenic putatively *rolA*-transgenic and *rolB*-transgenic plants were established on MS medium containing kanamycin (100 mg l^{-1}) and MS medium containing hygromycin (15 mg l^{-1}) respectively.

Shoots regenerated from leaf and internode explants of non-transformed plants via organogenesis cultured on MS medium and MS medium containing cefotaxime (500 mg l^{-1}) were maintained separately as control.

Spontaneous regeneration from Ri-transformed roots and establishment of Ri-transformed plants

Shoot buds (~1 cm) spontaneously regenerated from Ri-transformed roots were excised and cultured on MS medium supplemented with cefotaxime (500 mg l^{-1}) under 16/8 h (light/dark) photoperiod. Micro shoots regenerated from shoot buds induced from different Ri-transformed root lines were multiplied as separate clones. Each of the micro shoots was rooted spontaneously on this medium to develop complete rooted plantlets. Cefotaxime was withdrawn after six months of regular subculture at 4-weeks interval and the axenic cultures were finally established on MS medium. Excised non-transformed roots were similarly cultured on MS supplemented with BA (0.01 mg l^{-1}) for induction of shoots (control).

Confirmation of integration of transgenes

Genomic DNA was extracted from different transgenic and non-transformed plants after one year of their establishment according to the procedure published by Dellaporta et al. (1983). Isolated DNA was analyzed for transgene integration by PCR of *rolA*, *rolB*, *rolC*, *rolD*, *nptII* and *hptII* genes using gene-specific primers (Diouf et al. 1995; Wang et al. 2001; Sevón et al. 1997; Christensen et al. 2008; Beck et al. 1982). Plasmid DNA isolated from the corresponding bacteria following the standard alkali lysis protocol (Sambrook and Russel 2001) was used as positive control, whereas genomic DNA from non-transformed

plants was used as negative control. To eliminate the false positive result due to bacterial contamination, the *virD1* gene specific primers (Alpizar et al. 2008) were used. PCR amplicons were resolved by 1.2 % (w/v) agarose gel electrophoresis with a 100 bp plus DNA ladder (Thermo Scientific, USA) and visualized by ethidium bromide staining under UV light. Documentation was done using BioRad Gel DocTM EZ Imager. For each transgenic plant, ten replicates per clone were used and the experiment was repeated thrice.

Gene expression analysis by RT-PCR

The expression of transgenes at the transcription level was analyzed by reverse transcriptase polymerase chain reaction. Total RNA was extracted from fresh tissues of 4-weeks-old transgenic plant clones and non-transformed plants using HiPurA™ Plant and Fungal RNA Miniprep Purification Kit (HIMEDIA) following the manufacturer's protocol. RT-PCR reactions were performed as described earlier (Paul et al. 2015).

Maintenance of regenerated transgenic plants

Five axenic *rolA*-transgenic plant clones (Bm-At-A-L#5, Bm-At-A-L#7, Bm-At-A-1N#1, Bm-At-A-IN#2, Bm-At-A-IN#4), seven axenic *rolB*-transgenic plant clones (Bm-At-B-L#1, Bm-At-B-L#5, Bm-At-B-L#6, Bm-At-B-L#9, Bm-At-B-L#11, Bm-At-B-L#12 and Bm-At-B-L#15) and five Ri-transformed plant clones (Bm-R-8, Bm-R-10, Bm-R-15, Bm-R-22, Bm-R-23) were randomly selected and maintained for over three years with regular subculture at 8-weeks interval on MS medium containing kanamycin (100 mg l^{-1}), MS medium containing hygromycin (15 mg l^{-1}) and MS basal medium respectively.

Study of *in vitro* morphogenic potential in explants derived from transgenic plants on basal medium

Excised leaves (~0.95 cm), internodes (~0.90 cm) and root (1.5-2.0 cm) segments derived from *in vitro* grown 4-weeks-old different transgenic and non-transformed plants were cultured on MS basal medium as reported earlier (Sarkar and Jha 2017). The excised leaf explants were placed with their abaxial face down in contact with the medium while the internode and root explants were placed horizontally. After four weeks, the morphogenic responses of different explants were evaluated. For leaf and internode explants, the number of shoot buds/ micro shoots induced per explant and number of roots induced per explant were recorded whereas the development of shoot buds and/or growth of root tips were examined for root explants. For each experiment, 30 leaf, 30 internode and 30 root explants per *rolA*-transgenic, *rolB*-transgenic, Ri-transformed plant clone and non-transformed plant were used and the experiment was repeated thrice.

Analysis of morphology of transgenic plants maintained *in vitro*

Morphological characterization of *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants was done based on morphological descriptors reported earlier for Ri-transformed plants (Majumdar et al. 2011). Approximately 2 cm long shoot tips were cultured in culture tubes (15 x 2.5 cm) containing 20 ml of solid

MS supplemented with kanamycin (100 mg l^{-1}) for *rolA*-transgenic, MS with hygromycin (15 mg l^{-1}) for *rolB*-transgenic and MS basal medium for Ri-transformed plant clones under 16/8 h photoperiod. One shoot tip was cultured per culture tube. Shoot tips excised from axenic non-transformed plants were similarly cultured on MS basal medium and used as control. After six weeks, the plants were harvested, washed, blotted dry and morphological data were taken. For each experiment, 30 shoot tip explants were used per *rolA*-transgenic, *rolB*-transgenic, Ri-transformed plant clone and non-transformed plant and the experiment was repeated thrice.

Study of auxin sensitization response and effect of exogenous cytokinins on *in vitro* morphogenesis in explants derived from transgenic plants

Leaves, internodes and root segments excised from 4-weeks-old *rolA*-transgenic, *rolB*-transgenic, Ri-transformed and non-transformed plants were cultured on MS media fortified with 0.01, 0.05 and 1.0 mg l^{-1} of filter-sterilized auxin i.e. IAA (indole-3-acetic acid) and cytokinins i.e. 6 benzylaminopurine (BA) and kinetin (KN). After four weeks, the number of roots induced per leaf and internode explant in auxin-supplemented media and number of shoot buds/ micro shoots induced per leaf and internode explant in cytokinin-supplemented media were recorded whereas the development of shoot buds and/or growth of root tips were examined for root explants in all hormone-treated media. For each hormone concentration, 30 explants of each type per transgenic clone and non-transformed plant were used and the experiment was repeated thrice.

Statistical analysis

All experiments were randomized and were repeated at least thrice. 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 Tukey B test at the same 5 % probability level using SPSS software (version 22.0). Variability in the data was expressed as the mean ± standard deviation (S.D.).

Results

Shoot organogenesis and establishment of putatively *rolA*-transgenic and *rolB*-transgenic plants on selection media following transformation with *A. tumefaciens* strains

Shoot induction was observed in excised explants, cultured on MS medium supplemented with 25 mg l^{-1} (70-75 %) and 50 mg l^{-1} (40-42 %) kanamycin or 5 mg l^{-1} (60-90 %) and 10 mg l^{-1} (0-50 %) hygromycin. Total inhibition of shoot induction from explants was observed on MS medium supplemented with 75-100 mg l^{-1} kanamycin or 10-15 mg l^{-1} hygromycin. Therefore, MS medium containing 100 mg l^{-1} kanamycin or 15 mg l^{-1} hygromycin was used in the transformation experiments for the selection of transgenic shoots.

Direct shoot organogenesis was observed from the wound sites of infected explants, whereas control explants showed no organogenesis and necrosed within 6-8 weeks of culture on selection media (Fig. 1). The frequency of shoot bud induction from leaf and internode explants infected with *A. tumefaciens* strain GV3101-*rolA* was 7 % and 4 % respectively within four weeks of culture on MS medium containing cefotaxime (500 mg l^{-1}) and kanamycin (100 mg l^{-1}). In case of leaf explants infected with *A.tumefaciens* strain LBA4404-*rolB*, the frequency of shoot bud induction was 5 % within four weeks of culture on MS medium containing cefotaxime (500 mg l^{-1}) and hygromycin (15 mg l^{-1}). None of the LBA4404-*rolB* infected internode explants showed shoot organogenesis and eventually necrosed in the same selection medium (Fig. 1k). The induced shoot buds developed into micro shoots within two months but these micro shoots were very slow growing. Improved growth of these micro shoots was achieved when excised and cultured on respective selection media fortified with BA (1.0 mg l^{-1}) for four weeks. Micro shoots regenerated from shoot buds induced from wound sites of different explants derived from different plants were maintained as separate clones. Once growth was achieved, excised shoots were again cultured on respective selection media devoid of BA for next six months with regular sub-culturing. Spontaneous rooting from these micro shoots was observed on these media forming complete rooted plantlets within 1-2 weeks (Fig. 1g and n). Finally, clones of putatively *rolA*-transgenic and *rolB*-transgenic plants were established on MS medium containing 100 mg l^{-1} kanamycin and MS medium containing 15 mg l^{-1} hygromycin respectively. No growth of bacteria was observed in liquid bacterial media after 48 hrs of culture of putatively *rolA*-transgenic and *rolB*-transgenic plants suggesting their axenic nature.

Establishment of Ri-transformed root culture following infection with *A. rhizogenes* strain A4

Roots were induced from wound sites of leaf (30 %) and internode explants infected with *A. rhizogenes* strain A4 within four weeks of culture on MS medium containing cefotaxime (500 mg l^{-1}). The roots induced were thin, light green and fast growing with lateral branches when maintained under complete darkness as well as under 16-/8-h (light/dark) photoperiod. Each putatively transformed primary root induced at each wound site was maintained as root line.

The frequency of root induction from wound sites of non-transformed (uninfected) leaf and internode explants was very low and was 2-4 % after four weeks on MS medium containing cefotaxime (500 mg l^{-1}). The roots were very thin, white or green, slow growing with no branches and necrosed within four weeks when excised and cultured on this selection medium.

Spontaneous regeneration of shoot buds from Ri-transformed roots

Shoot buds (5 per explant) were regenerated spontaneously in 60 % of Ri-transformed root lines within 10 days of culture on MS medium supplemented with cefotaxime (500 mg l^{-1}). Micro shoots were developed from these shoot buds which rooted spontaneously on same medium forming complete Ri-transformed plantlets. Micro shoots regenerated from shoot buds induced from different Ri-transformed root lines were maintained as separate clones.

Shoots were induced from excised non-transformed roots on MS medium supplemented with BA (0.01 mg l^{-1}) within four weeks and were used as control.

Confirmation of transformation

PCR and RT-PCR analysis showed integration and expression of *rolA* and *nptII* genes in *rolA*-transgenic plant clones, *rolB* and *hptII* genes in *rolB*-transgenic plant clones and *rolA*, *rolB*, *rolC*, *rolD* genes in Ri-transformed plant clones after one year of their establishment (Fig. 2). The amplified products were of expected size and identical to their positive controls, confirming integration, retention and expression of respective genes in transgenic plant clones. No amplification of *virD1* gene was noticed during PCR analysis confirming absence of bacterial contamination in the transgenic plants. No amplification was observed in the genomic DNA of non-transformed plants.

Direct shoot and root organogenesis from different explants derived from *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants on basal medium

Shoot organogenesis from leaf, internode and root explants

In the present study, excised leaf and internode explants of non-transformed plants showed inherent shoot organogenic potential and produced adventitious shoot buds/micro shoots on MS basal medium (Fig. 3). However, after four weeks, internode explants showed higher rate of shoot organogenesis near proximal and distal cut ends in contrast to leaf explants near proximal cut ends (Fig. 4a).

The shoot organogenic competence was increased significantly ($p \leq 0.05$) in excised leaf and internode explants derived from *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants after four weeks on MS basal medium (Fig. 4a). Among the three transgenic explants, the rate of shoot organogenesis was highest in explants derived from Ri-transformed plants containing T-DNA. Similar to non-transformed explants, shoot organogenic response was higher in internode explants of Ri-transformed plants (38.6 ± 0.71 shoot buds/micro shoots per explant) near proximal and distal cut ends compared to leaf explants (32.6 ± 0.92 shoot buds/micro shoots per explant) near proximal cut ends (Fig. 4a).

Compared to *rolA*-expressing explants, *rolB*-transgenic explants yielded greater number of shoot buds/micro shoots per explant. Similar to non-transformed and Ri-transformed explants, shoot organogenesis was induced at a higher rate in internode explants than the leaf explants of both *rolA*-transgenic and *rolB*-transgenic plants. Internode explants of *rolB*-transgenic and *rolA*-transgenic plants produced 19.4 ± 0.96 and 16.0 ± 0.63 shoot buds/micro shoots per explant respectively near proximal and distal cut ends. On the other hand, leaf explants of *rolB*-expressing and *rolA*-expressing plants were recorded 15.2 ± 0.44 and 10.2 ± 0.83 shoot buds/micro shoots per explant respectively near proximal cut ends (Fig. 4a).

Thus, in growth-regulator free medium, both leaf and internode explants of Ri-transformed plants showed maximum shoot organogenic potential followed by *rolB*-transgenic and *rolA*-transgenic plants, with

internode explants being the most responsive. The shoot regeneration potential of Ri-transformed, *rolB*-transgenic and *rolA*-transgenic internode explants was almost 3.3-fold, 1.6-fold and 1.4-fold higher whereas in leaf explants it was 5.2-fold, 2.4-fold and 1.6-fold higher, respectively, than in non-transformed explants (Fig. 4a).

In the present study, root explants of Ri-transformed plants showed shoot regeneration on MS basal medium (Fig. 3). Initially, explants swelled followed by the formation of green nodules at several points on their surfaces within 15-20 days of culture. Within four weeks, numerous adventitious shoot buds were differentiated throughout the surfaces of Ri-transformed root explants with the emergence of primary leaves. However, root explants derived from *rolA*-transgenic, *rolB*-transgenic and non-transformed plants showed no shoot bud induction and the explants eventually died within six-eight weeks on MS basal medium (Fig. 3).

Root organogenesis from leaf, internode and root explants

In the present study, roots were induced *de novo* from excised leaf and internode explants of non-transformed plants on MS basal medium. But, unlike shoot organogenesis, the tendency of these explants to undergo root organogenesis was comparatively low. Internode explants showed higher root regeneration (4.7 ± 0.48 roots per explant) near proximal and distal cut ends than leaf explants (2.7 ± 0.51) near proximal cut ends after four weeks of culture (Fig. 4b).

Improved root organogenic potential was observed in excised leaf and internode explants of *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants on MS basal medium (Fig. 4b). But the explants of Ri-transformed plants regenerated maximum roots than *rolA*-transgenic and *rolB*-transgenic explants after four weeks. However, Ri-transformed leaf explants produced greatest number of roots (11.0 ± 0.63 roots per explant) near proximal cut ends than internode explants (9.0 ± 0.57 roots per explant) near proximal and distal cut ends.

Among *rolA*-transgenic and *rolB*-transgenic explants, higher rooting ability was obtained in excised *rolB*-expressing leaf and internode explants than *rolA*-transgenic explants. Like Ri-transformed explants, leaf explants were more competent to form roots than internode explants of *rolB*-transgenic and *rolA*-transgenic plants (Fig. 4b). Leaf explants of *rolB*-transgenic and *rolA*-transgenic plants induced 9.8 ± 0.40 and 4.7 ± 0.51 roots per explant near proximal cut ends respectively. On the other hand, internode explants of *rolB*-transgenic plants yielded 6.6 ± 0.53 roots per explant near proximal and distal cut ends whereas the difference in root regeneration was not notable between *rolA*-transgenic and non-transformed internode explants (Fig. 4b).

Therefore, the rate of root organogenesis was enhanced maximum in excised explants derived from Ri-transformed plants followed by *rolB*-transgenic and *rolA*-transgenic plants compared to non-transformed ones after four weeks of culture on MS basal medium. Unlike in non-transformed explants, excised leaf explants of all transgenic plants showed increased rooting ability than internode explants. The number of roots was 4.1-fold, 3.6-fold and 1.7-fold greater in leaf explants of Ri-transformed, *rolB*-transgenic and

rolA-transgenic plants respectively, whereas it was 1.9-fold and 1.4-fold greater in Ri-transformed and *rolB*-transgenic internode explants, respectively, than in non-transformed ones (Fig. 4b).

On the other hand, root explants of Ri-transformed plants showed extensive growth of root tips (4-6 cm) along with the formation of primary laterals within four weeks on MS basal medium. However, root explants derived from *rolA*-transgenic, *rolB*-transgenic and non-transformed plants showed no growth of root tips or lateral branching formation on MS basal medium until necrosis (Fig. 3).

Comparative morphology of transgenic plants harbouring *rolA*, *rolB* and Ri T-DNA genes

In the present study, comparative morphological characterization was performed between the clones of *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants to evaluate the effects of *rol* genes, either individual or in combination, on plant morphology. Ri-transformed plants of *B. monnieri* expressing four *rol* genes exhibited characteristic morphological changes associated with *A. rhizogenes* mediated transformation as expected (Fig. 5). Clones of both *rolA*-transgenic and *rolB*-transgenic plants also displayed significant differences ($p \leq 0.05$) in phenotypic characters from non-transformed plants such as decrease in shoot and internode lengths, increase in number of nodes/plant, leaves/plant and roots/plant (Fig. 6). However, among the two, the morphology of *rolB*-transgenic plant clones was found to be more or less similar to Ri-transformed plant clones (Fig. 5), suggesting that *rolB* gene alone has a pronounced effect in alteration of plant phenotype in *B. monnieri*. Clones of *rolB*-transgenic plants were similar in shoot length, internode length, number of nodes/plant and number of leaves/plant when compared with Ri-transformed plant clones (Fig. 6). The leaves were smaller in *rolB*-transgenic plants due to alteration in leaf shape (roundish) as in Ri-transformed plants (Fig. 5b). Presence of axillary shoots was also noted in both *rolB*-transgenic and Ri-transformed plant clones. But these *rolB*-transgenic plant clones differed significantly in their rooting ability with lesser number of roots/plant and shorter roots than Ri-transformed plant clones (Fig. 6).

On the other hand, the degree of phenotypic abnormalities in *rolA*-transgenic plant clones was not as intense as in clones of *rolB*-transgenic and Ri-transformed plants and some of the morphological characters were even similar to non-transformed plants. The *rolA*-transgenic plant clones differed significantly in shoot length and were longer, with less number of nodes/plant and leaves/plant than *rolB*-transgenic and Ri-transformed plants (Fig. 6). The rooting ability also differed significantly in *rolA*-transgenic plant clones which produced lesser number of roots/plant with shorter roots than *rolB*-transgenic and Ri-transformed plants. However, unlike *rolB*-transgenic and Ri-transformed plants, *rolA*-transgenic plants were found to be similar to non-transformed ones in terms of leaf length, leaf shape and root length (Fig. 5). Furthermore, no formation of axillary shoots was noticed in any of the *rolA*-transgenic plant clones as in non-transformed plants. However, leaf wrinkling was not observed in any of the transgenic plants in the present study.

Auxin sensitization response of different explants derived from *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants

Root organogenesis was enhanced significantly in both leaf and internode explants derived from *rolA*-transgenic and *rolB*-transgenic plants than non-transformed explants in presence of IAA (Table 1). Leaf explants derived from Ri-transformed plants showed significantly higher root organogenesis while internode explants showed variable response. Moreover, in presence of IAA, root explants derived from *rolA*-transgenic and *rolB*-transgenic plants produced callus near cut ends and lateral branching, thereby showing the increased sensitivity to auxin (Fig. 7). Root explants from Ri-transformed plants showed root tip elongation extensively (6-7 cm) along with shoot organogenesis (4-6 shoot buds/explant) and regeneration of whole plants (Fig. 7). On the other hand, root explants derived from non-transformed plants when cultured on the same medium showed slow growth without any other distinct change.

Shoot organogenesis in different explants derived from *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants on cytokinin supplemented media

In the present study, enhanced rate of shoot organogenesis was noticed in leaf explants of *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants than non-transformed explants in presence of BA and KN (Table 2). The interaction between transgenic explants and media showed that BA was found to be superior to KN. On the other hand, internode explants of Ri-transformed plants induced uncountable shoot buds/micro shoots whereas internode explants derived from *rolA*-transgenic and *rolB*-transgenic plants showed variable response in presence of exogenous cytokinins used (Table 2). Root explants of Ri-transformed plants showed shoot regeneration on BA and KN supplemented media at all concentrations whereas excised *rolA*-transgenic root segments induced shoot buds only in presence of 1.0 mg l^{-1} BA and KN (Fig. 8). However, root explants from *rolB*-transgenic plants did not induce shoot buds in presence of BA or KN and necrosed within eight weeks of culture. On the other hand, the non-transformed root explants induced shoot buds followed by shoot regeneration on medium containing BA only (Fig. 8).

Discussion

Several researchers have established Ri-transformed roots and plants following *A. rhizogenes* infection in *B. monnieri* and demonstrated the effect of T-DNA genes on growth, biomass accumulation and bacosides production in transformed tissues (Majumdar et al. 2011; Bansal et al. 2014; Paul et al. 2015; Largia et al. 2016). However, few attempts were made on *A. tumefacien*s mediated transformation where hygromycin/ kanamycin resistant transgenic plants expressing GUS gene were produced to develop an efficient protocol in this species (Ramesh et al. 2011; Mahender et al. 2012; Aggarwal et al. 2013; Yadav et al. 2014). But, to our knowledge, regeneration of transgenic plants harbouring individual *rolA* or *rolB* or *rolC* or *rolD* gene has not yet been performed. Therefore, the present study reports for the first time on successful establishment of *rolA*-transgenic and *rolB*-transgenic plants in *B. monnieri* through *A. tumefaciens* mediated genetic transformation. The Ri-transformed plants harbouring Ri T-DNA were also developed from Ri-transformed roots through *A. rhizogenes* infection to investigate the effects associated with integration and expression of *rol* genes, individually or synergistically, on plant morphology and morphogenesis *in vitro*.

The *rolA*-transgenic and *rolB*-transgenic plants of *B. monnieri* displayed reduced shoot and internode length, increased number of nodes and leaves per plant with enhanced rooting ability as compared to non-transformed plants, suggesting that *rolA* and *rolB* genes individually could alter plant phenotype. Similar observations were previously made by others in different plant species showing each of these genes is capable to modify plant morphology due to hormonal disequilibrium in transgenic plants (Arshad et al. 2014; Dilshad et al. 2015; Amanullah et al. 2016; Bettini et al. 2016a, b; Kodahl et al. 2016). Moreover, wrinkling of leaves was not observed in *rolB*-transgenic plant clones of *B. monnieri* in the present study which is in agreement with studies on *rolB*-transgenic tomato plants (van Altvorst et al. 1992). However, Kodahl et al. (2016) reported that the leaves of *rolB* of *Arabidopsis thaliana* were somewhat wrinkled in nature upon transformation with *rolB* gene. This suggests that the characteristics of individual *rol* gene also depend on the plant species used.

The abnormal phenotypic traits as observed in *rolA*-transgenic and *rolB*-transgenic plant clones were found to be more exaggerated in Ri-transformed *B. monnieri* plant clones expressing *rolABCD* genes. These alterations in plant morphology collectively called “hairy root syndrome” were reported earlier in *B. monnieri* (Majumdar et al. 2011; Paul et al. 2015) as well as in several other plant species (Roychowdhury et al. 2013). Such exaggeration is probably due to the combined expression of *rol* genes of T_L-DNA of *A. rhizogenes* Ri plasmid since each of the *rol* genes is associated with specific phenotypic alterations (Nilsson and Olsson 1997; Sarkar et al. 2018).

Furthermore, the degree of alterations inferred by *rolB* gene was found to be more intense as compared to transgenic plant clones expressing *rolA* gene indicating that *rolB* gene is more potent in causing developmental abnormalities in transformed *B. monnieri* plants. Additionally, *rolB*-transgenic plant clones exhibited certain transgenic characters due to which its morphology closely resembled Ri-transformed plant clones. This result indicates that *rolB* gene alone has a very pronounced effect in the alteration of plant morphology in *B. monnieri*. It has previously been established that *rolB* gene confers majority of morphological anomalies as noticed in Ri-transformed plants expressing all four *rol* genes thereby ensuring the potentiality of this gene in exerting hairy root syndrome (van Altvorst et al. 1992; Zia et al. 2010; Arshad et al. 2014; Dilshad et al. 2015; Bettini et al. 2016a; Kodahl et al. 2016).

The inherent morphogenic ability of excised leaf and internode explants of *B. monnieri* on hormone-free MS basal medium was demonstrated earlier (Mohapatra and Rath 2005; Majumdar et al. 2012; Koul et al. 2014; Sarkar and Jha 2017) and was again confirmed in the present study. Enhanced shoot and root formation in explants of *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants containing T-DNA therefore indicate the positive correlation between *rol* genes and organogenic competence of this plant species. According to Skoog and Miller (1957), plant organogenesis can be controlled by auxin to cytokinin ratios. Decades-long study of *A. rhizogenes*-plant pathogenic interaction has revealed that the *rol* genes of Ri T-DNA perturb metabolism/sensitivity of endogenous hormones in transformed cells, primarily auxin and cytokinin and their interactions (Delbarre et al. 1994; Faiss et al. 1996) which could explain enhanced morphogenic potential in transgenic explants. However, maximum morphogenic responses in Ri-transformed explants containing T-DNA were probably due to the combined effects of *rol*

genes in the alteration of endogenous auxin/cytokinin ratio in Ri-transformed explants. In addition to this, growth of root tips and shoot regeneration were also noticed from Ri-transformed root explants, but not in *rolA* transgenic, *rolB*-transgenic and non-transformed roots suggesting that *rolA* or *rolB* gene alone is not sufficient for shoot or root organogenesis in excised roots of *B. monnieri*.

The fact that *rol* genes affect hormone-controlled morphogenesis by modulating hormonal metabolism/sensitivity directly or indirectly in transformed cells became more prominent when exogenous auxin and cytokinin were added to the culture medium in the present study. Enhanced rhizogenesis in explants derived from *rolA*-transgenic, *rolB*-transgenic and Ri-transformed plants of *B. monnieri* compared to the control in presence of auxin is supposed to be the consequence of increased auxin sensitivity in *rol*-transgenic cells. This also explains the formation of lateral branching and callus induction in *rolA*-transgenic and *rolB*-transgenic root explants and root tip growth in Ri-transformed root explants in medium supplemented with IAA. van Altvorst et al. (1992) speculated that root formation in leaf explants of RolA, RolB and RolABC tomato plants in medium supplemented with NAA but not in control was due to enhanced sensitivity of *rol*-expressing leaf tissues for auxin. The enhancement of flowering and rooting in *rolB*-transformed tobacco thin cell layers (TCLs) along with altered *in vitro* development of transformed flowers and roots are also thought to be the manifestation of *rolB*-induced increased auxin sensitivity (Altamura et al. 1994). Furthermore, inhibition of root formation by oligogalacturonides in *rolB*-transgenic tobacco leaf explants and protoplasts by inhibiting auxin-dependent *rolB* gene expression makes the idea of involvement of *rolB* gene in auxin signaling pathway more apparent (Bellincampi et al. 1996).

In addition to this, enhanced shoot organogenesis in explants derived from *rolA*-transgenic and *rolB*-transgenic plants of *B. monnieri* in presence of cytokinin describes the possibility of increased cytokinin sensitivity rendered by *rolA* and *rolB* genes in the present study. Altamura et al. (1998) also predicted the presence of a positive interaction between *rolB* gene and exogenous cytokinin due to the promotion of shoot formation in *rolB*-transgenic tobacco TCLs and leaf explants in presence of very low cytokinin concentrations although the mechanism is unclear. Later on, *rolB* is hypothesized to cause meristem formation triggered by increased auxin sensitivity in transformed cells which can develop into different types of organs depending on local hormone level and cell's competency for organogenesis (Altamura 2004). A recent study has shown promising results in the modification of both auxin/cytokinin signaling pathways rendered by *rolB*-transformed cells in *Arabidopsis thaliana* Columbia (Col-0) (Bulgakov et al. 2018).

Apart from *rolA* and *rolB* genes, the effect of *rolC* gene on organogenesis was also discussed earlier. Improved shoot and root regeneration in presence of cytokinin and auxin respectively in *rolC*-transgenic leaf explants of *Dianthus caryophyllus* compared to the control indicates that *rolC* gene probably has both cytokinin-like and auxin-like activities (Casanova et al. 2003). Therefore, each of *rol* genes seems to play a pivotal role in regulating developmental program in plants during organogenesis. Therefore interaction of *rol* genes in combination with hormonal metabolism/sensitivity offers a plausible explanation of enhanced morphogenesis in hormone-supplemented media in Ri-transformed explants

compared to *rolA*-transgenic and *rolB*-transgenic explants of *B. monnieri*. Enhanced differentiation of root and shoot was also reported by Koperdáková et al. (2009) when shoot cuttings of two *Hypercum perforatum* clones transformed with wild agropine strain of *A. rhizogenes* were cultured in presence of exogenous auxin and cytokinin respectively. However, the exact mode of action of these genes on endogenous level of phytohormones is unclear till date and needs further investigations in order to clarify the specific functions of *rol* genes and their biological effects in plants.

Conclusions

In conclusion, the present study demonstrated successful establishment of *rolA*-transgenic and *rolB*-transgenic plants of *B. monnieri* for the first time through direct shoot organogenesis following *A. tumefaciens* mediated genetic transformation. Ri-transformed plants harbouring Ri T-DNA were spontaneously regenerated from Ri-transformed roots induced by *A. rhizogenes* infection. Respective transgenes were integrated and expressed in all three transgenic plants, confirming their transgenic nature. The *rol* genes, either singly or in combination, played significant role in substantial increase in shoot and root organogenic potential on hormone unsupplemented medium by interacting positively with inherent morphogenic potential of this species. Each of the *rol* genes is found to be associated with the alteration of plant morphology in *B. monnieri*, although their degree varied. Furthermore, enhanced morphogenic response in transgenic explants containing *rolA* or *rolB* or Ri T-DNA genes even in presence of low concentrations of exogenous auxin and cytokinin might therefore be a manifestation of increased auxin and cytokinin sensitivity respectively although the exact mode of action of these genes needs further investigations.

Declarations

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Author contributions

SS and SJ conceived and designed research. SS conducted this research, analyzed the results and wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare no conflict of interests.

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Tables

Table 1 Adventitious root regeneration in leaf and internode explants derived from *rolA*-transgenic, *rolB*-transgenic, Ri-transformed and non-transformed plants of *B. monnieri* after four weeks of culture on MS medium in presence of exogenous auxin

Concentration of IAA (mg l^{-1})	Plant Lines			
	Non-transformed	*rolA*-transgenic	*rolB*-transgenic	Ri-transformed
Leaf explants				
0.0	2.6 ± 0.74^{a}	4.8 ± 0.83^{b}	10.0 ± 0.70^{c}	11.4 ± 0.54^{d}
0.01	2.5 ± 0.53^{a}	4.6 ± 0.51^{b}	7.4 ± 0.51^{d}	5.7 ± 0.71^{c}
0.05	2.7 ± 0.71^{a}	6.6 ± 0.91^{b}	8.1 ± 0.83^{c}	6.5 ± 0.92^{b}
1.0	7.6 ± 1.30^{a}	13.4 ± 1.06^{c}	9.1 ± 1.24^{b}	7.7 ± 0.71^{a}
Internode explants				
0.0	4.5 ± 0.54^{a}	3.8 ± 0.83^{a}	6.8 ± 0.44^{b}	8.7 ± 0.50^{c}
0.01	4.2 ± 0.75^{a}	6.4 ± 0.54^{b}	9.2 ± 0.83^{c}	5.5 ± 0.57^{b}
0.05	4.7 ± 0.75^{a}	7.0 ± 0.71^{b}	8.2 ± 0.83^{b}	5.2 ± 0.95^{a}
1.0	2.0 ± 0.63^{b}	2.0 ± 0.71^{b}	2.8 ± 0.44^{b}	0.0 ± 0.0^{a}

Values represent mean ± S.D. For each transgenic and non-transformed plant, 30 leaf and 30 internode explants were used per treatment and each experiment was repeated thrice. Means with different letters in a row were significantly different ($p \leq 0.05$) according to ANOVA and Tukey B's multiple comparison test.

Table 2 Adventitious shoot regeneration from leaf and internode explants derived from *rolA*-transgenic, *rolB*-transgenic, Ri-transformed and non-transformed plants of *B. monnieri* after four weeks of culture on MS medium containing exogenous cytokinins

Concentration of cytokinins (mg l^{-1})		Plant Lines			
BA	KN	Non-transformed	*rolA*-transgenic	*rolB*-transgenic	Ri-transformed
Leaf explants					
0.0	0.0	6.6 ± 1.14^{a}	10.4 ± 0.55^{b}	15.0 ± 0.71^{c}	33.0 ± 0.71^{d}
0.01	0.0	15.4 ± 0.91^{a}	16.5 ± 0.92^{b}	18.1 ± 0.64^{c}	54.2 ± 1.16^{d}
0.05	0.0	17.6 ± 0.92^{a}	18.2 ± 0.70^{a}	22.4 ± 0.74^{b}	52.2 ± 1.91^{c}
1.0	0.0	41.2 ± 1.38^{b}	53.9 ± 1.64^{c}	26.6 ± 0.91^{a}	71.6 ± 1.30^{d}
0.0	0.01	10.7 ± 1.16^{a}	13.7 ± 0.88^{b}	16.5 ± 0.53^{c}	24.5 ± 0.92^{d}
0.0	0.05	11.0 ± 0.75^{a}	15.7 ± 1.38^{b}	24.9 ± 1.45^{d}	22.1 ± 1.12^{c}
0.0	1.0	18.5 ± 0.92^{a}	25.5 ± 0.92^{b}	34.6 ± 1.18^{c}	26.4 ± 0.74^{b}
Internode explants					
0.0	0.0	11.6 ± 0.89^{a}	16.6 ± 0.55^{b}	19.6 ± 0.54^{c}	39.2 ± 0.44^{d}
0.01	0.0	41.2 ± 1.50^{c}	15.2 ± 0.95^{b}	12.5 ± 1.0^{a}	> 70
0.05	0.0	43.2 ± 0.50^{b}	17.0 ± 0.81^{a}	17.5 ± 0.57^{a}	> 70
1.0	0.0	> 70	> 70	> 70	> 70
0.0	0.01	27.0 ± 1.82^{c}	18.2 ± 0.50^{b}	8.0 ± 0.81^{a}	> 70
0.0	0.05	26.0 ± 0.81^{b}	28.7 ± 0.50^{c}	16.2 ± 0.50^{a}	> 70
0.0	1.0	39.7 ± 0.95^{a}	40.7 ± 0.95^{a}	54.0 ± 1.15^{b}	> 70

Values represent mean ± S.D. For each transgenic and non-transformed plant, 30 leaf and 30 internode explants were used per treatment and each experiment was repeated three times. Means with different letters in a row were significantly different ($p \leq 0.05$) according to ANOVA and Tukey B's multiple comparison test.

Figures

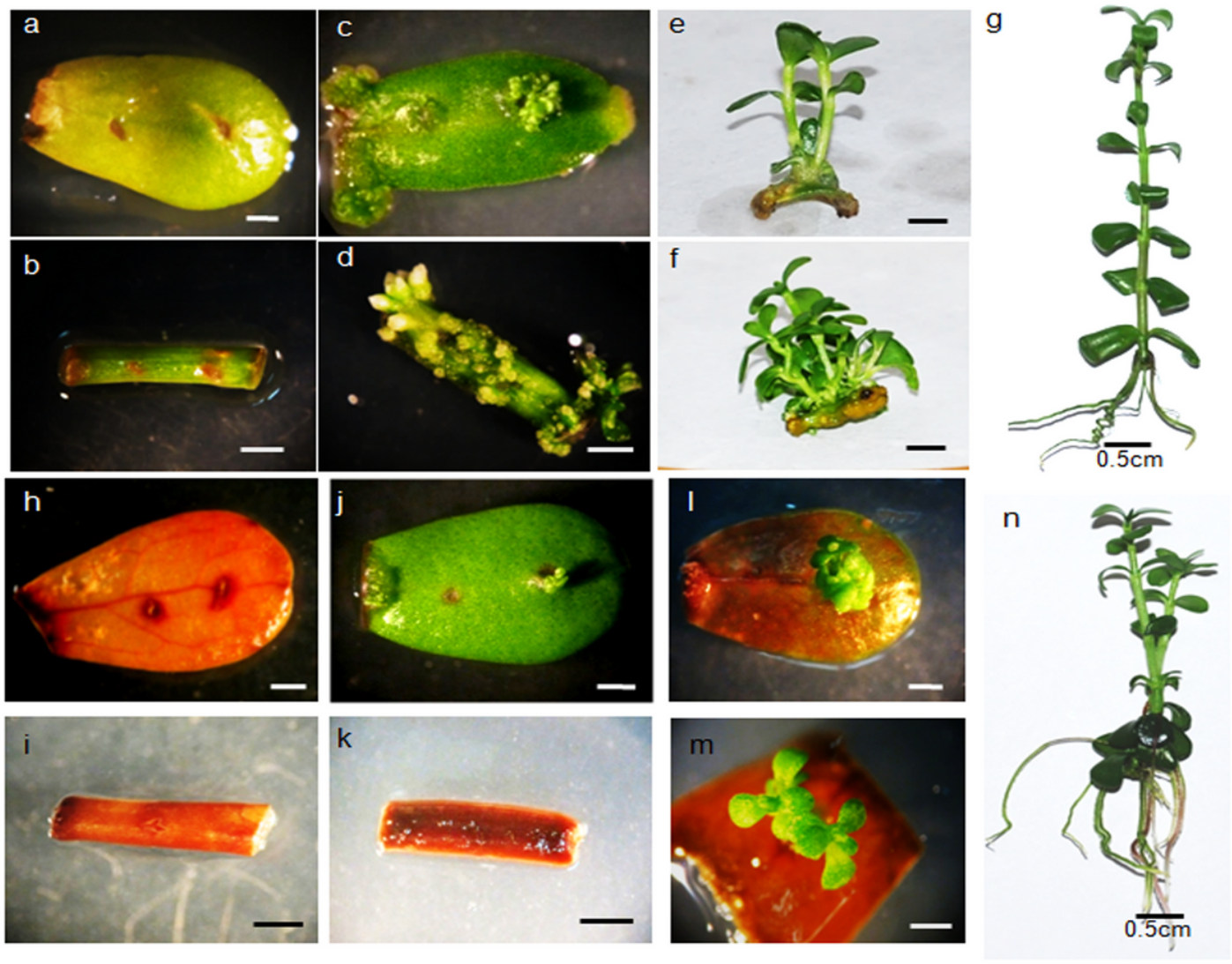

Figure 1

Shoot bud induction following infection of B. monnieri explants with different A. tumefaciens strains and establishment of rolA-transgenic and rolB-transgenic plants on respective selection medium. a, b uninfected (control) leaf and internode explants after four weeks of culture on MS+Cefo500+Kan100, c-f shoot bud induction and micro shoot development from wound sites of leaf and internode explants following transformation with A. tumefaciens strain GV3101 harboring rolA gene on MS+Cefo500+Kan100, g rooted rolA-transgenic micro shoot, h, i uninfected (control) leaf and internode explants after four weeks of culture on MS+Cefo500+Hygro15, j shoot bud induction from wound site of leaf explant following transformation with A. tumefaciens strain LBA4404 harboring rolB gene after two weeks, k no response from infected internode explant after four weeks, l, m developing shoot buds from infected leaf explants after five and eight weeks of culture respectively on MS+Cefo500+Hygro15, n rooted rolB-transgenic micro shoot. Scale bar = 10 mm.

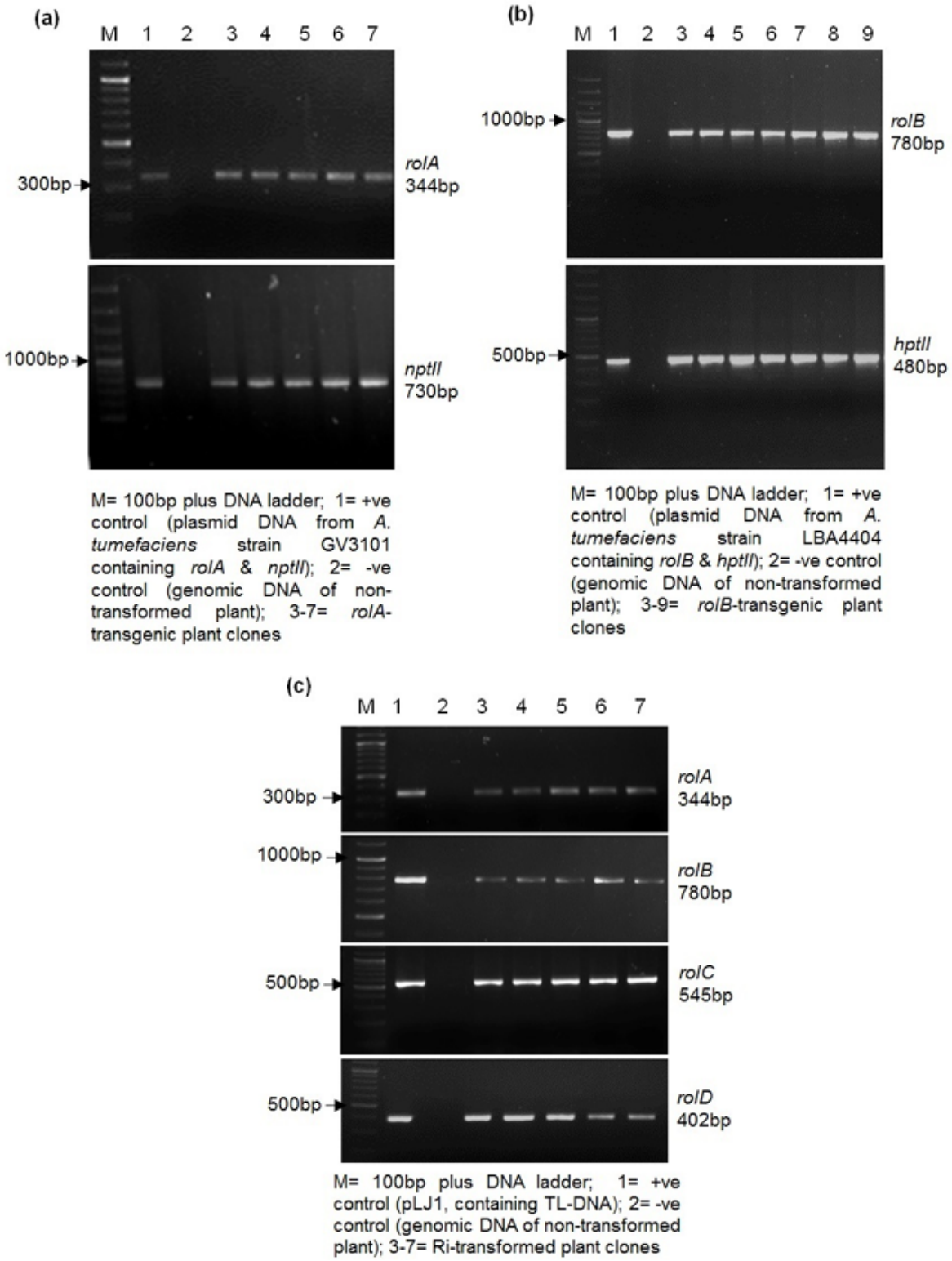

Figure 2

Agarose gel electrophoresis showing expression of a rolA and nptII genes in rolA-transgenic plants, b rolB and hptII genes in rolB-transgenic plants, and c rolA, rolB, rolC, rolD genes in Ri-transformed plants by RT-PCR analysis.

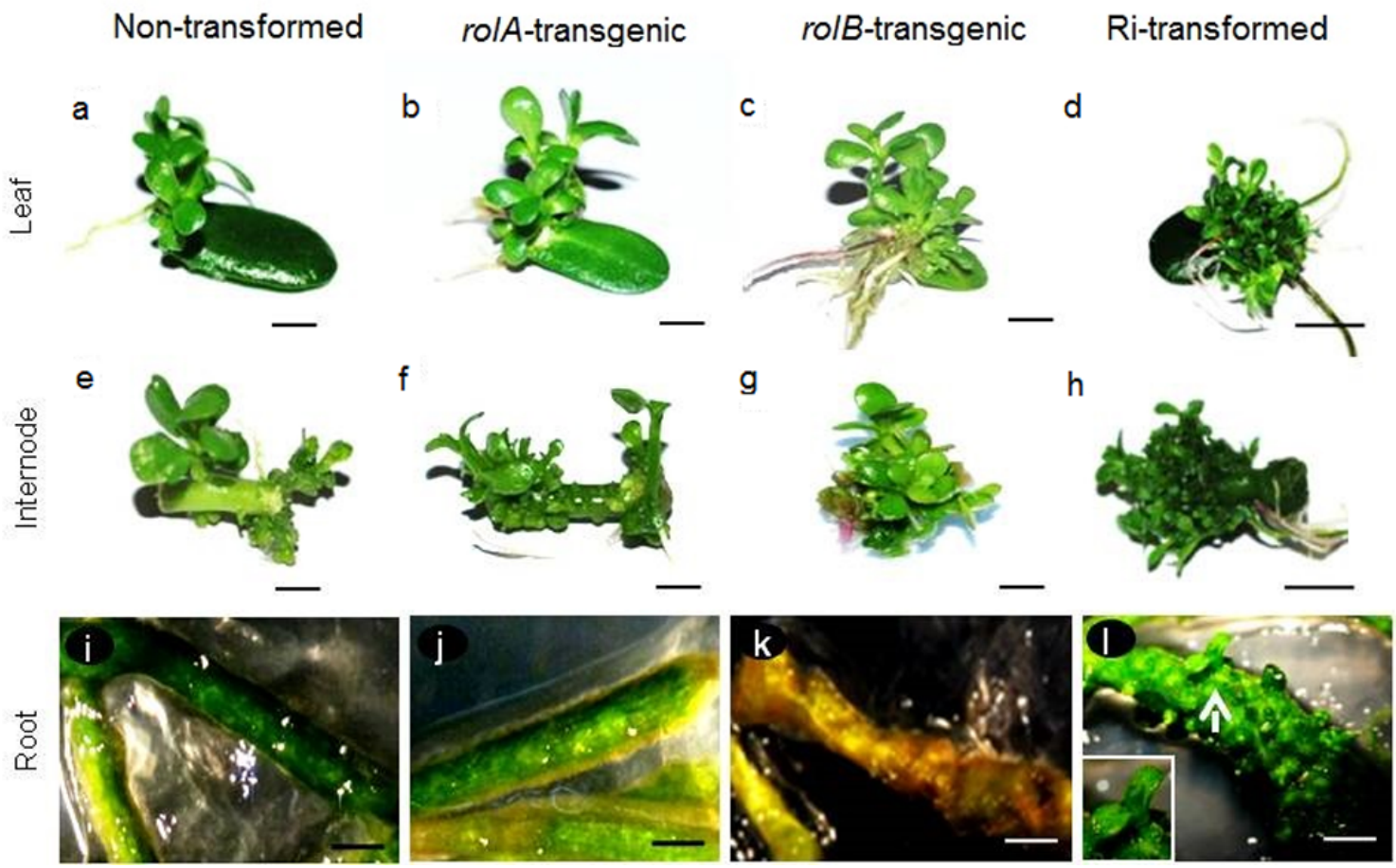

Figure 3

Morphogenic responses showing shoot buds/micro shoots formation from excised a-d leaf and e-h internode explants derived from different transgenic and non-transformed plants, i-k excised root explants from non-transformed, rolA-transgenic and rolB-transgenic plants showing no morphogenic response, l induction of shoot buds from excised Ri-transformed root explants after four weeks of culture on MS basal medium. Scale bar = 2 mm

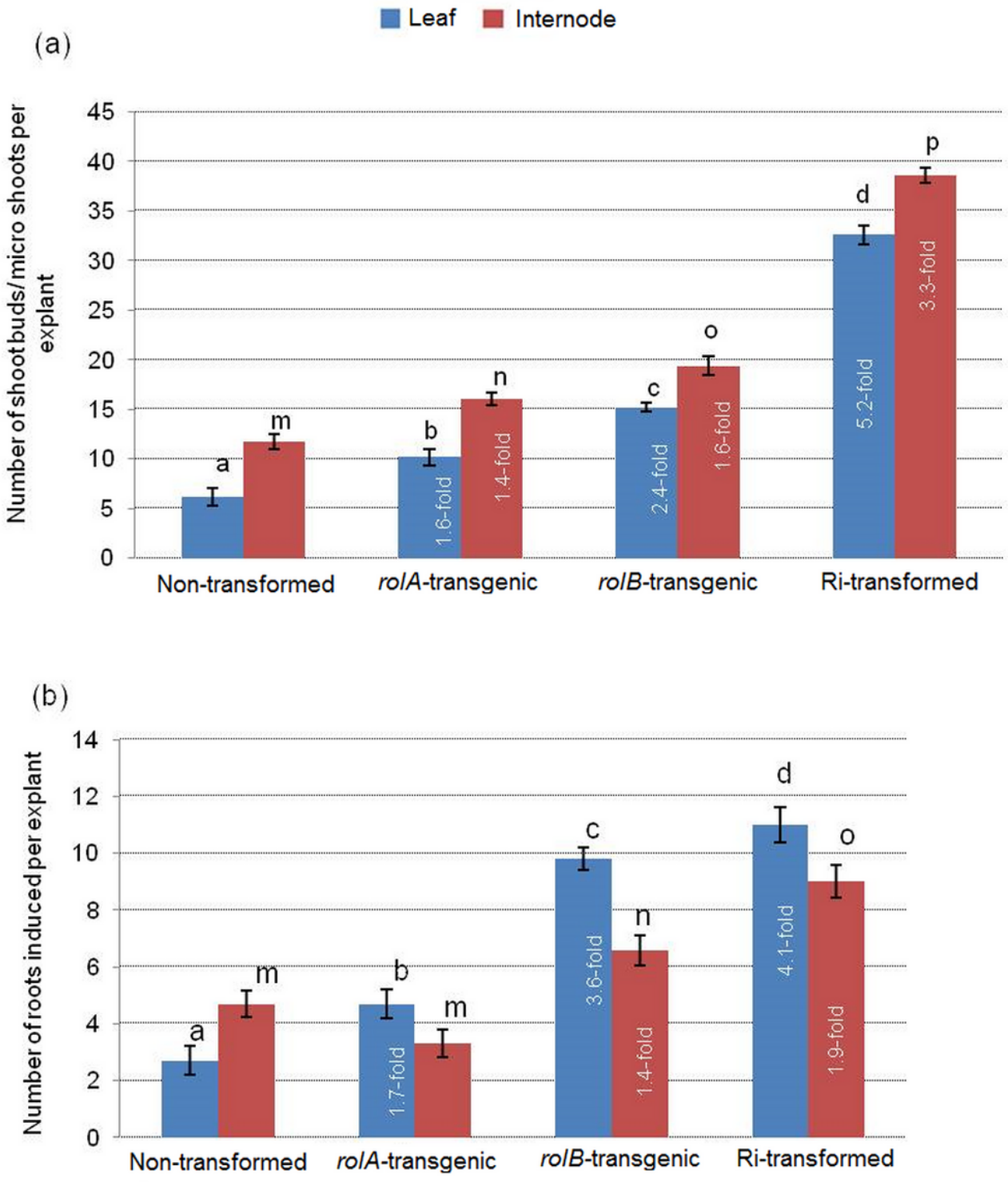

Figure 4

Shoot and root regeneration potential expressed as a number of shoot buds/micro shoots induced per explant, and b number of roots induced per explant excised from different transgenic and non-transformed plants after four weeks of culture on MS basal medium. Values represent mean ± S.D. Means marked with different letters are significantly different at $p \leq 0.05$.

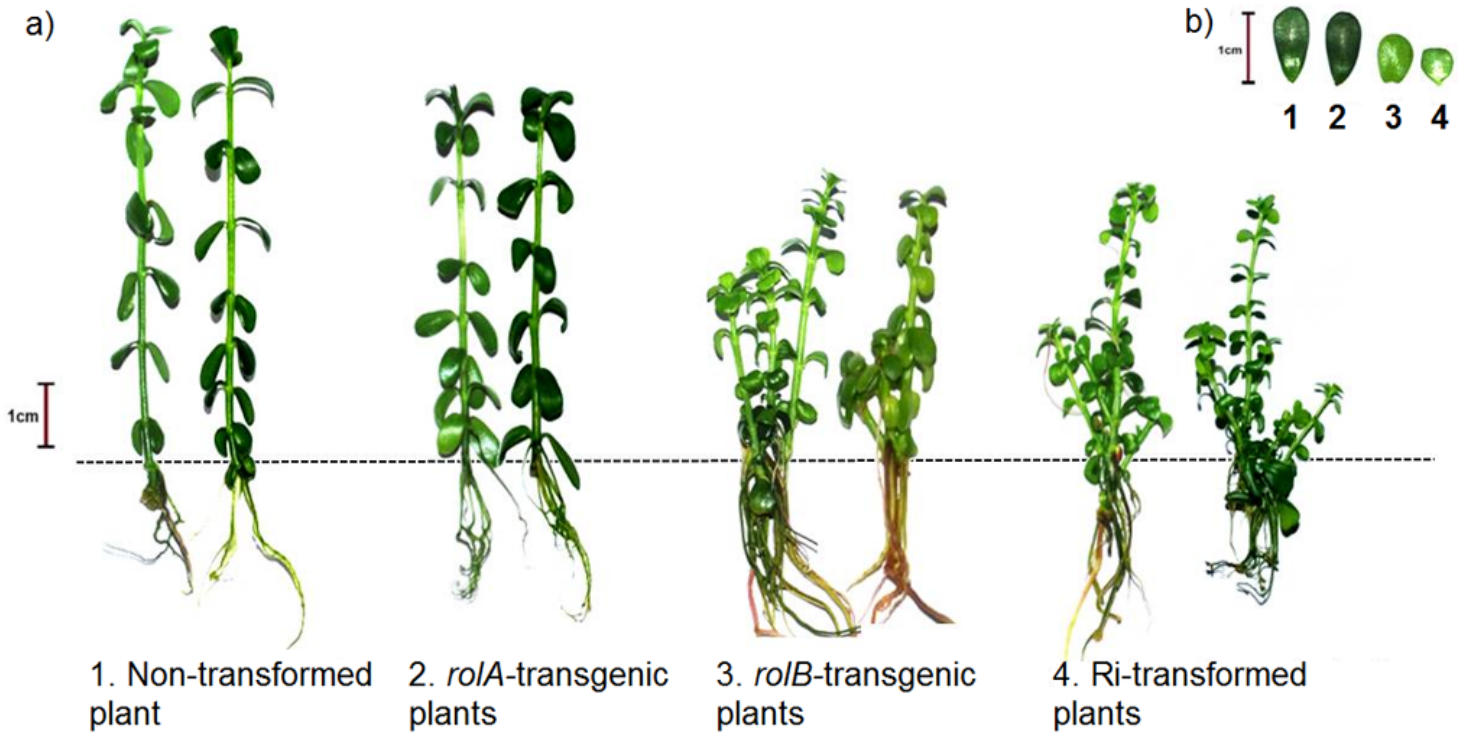

Figure 5

Comparative morphology of six-weeks-old in vitro grown non-transformed, rolA-transgenic, rolB-transgenic and Ri-transformed rooted plants, b individual leaf of transgenic and non-transformed plants.

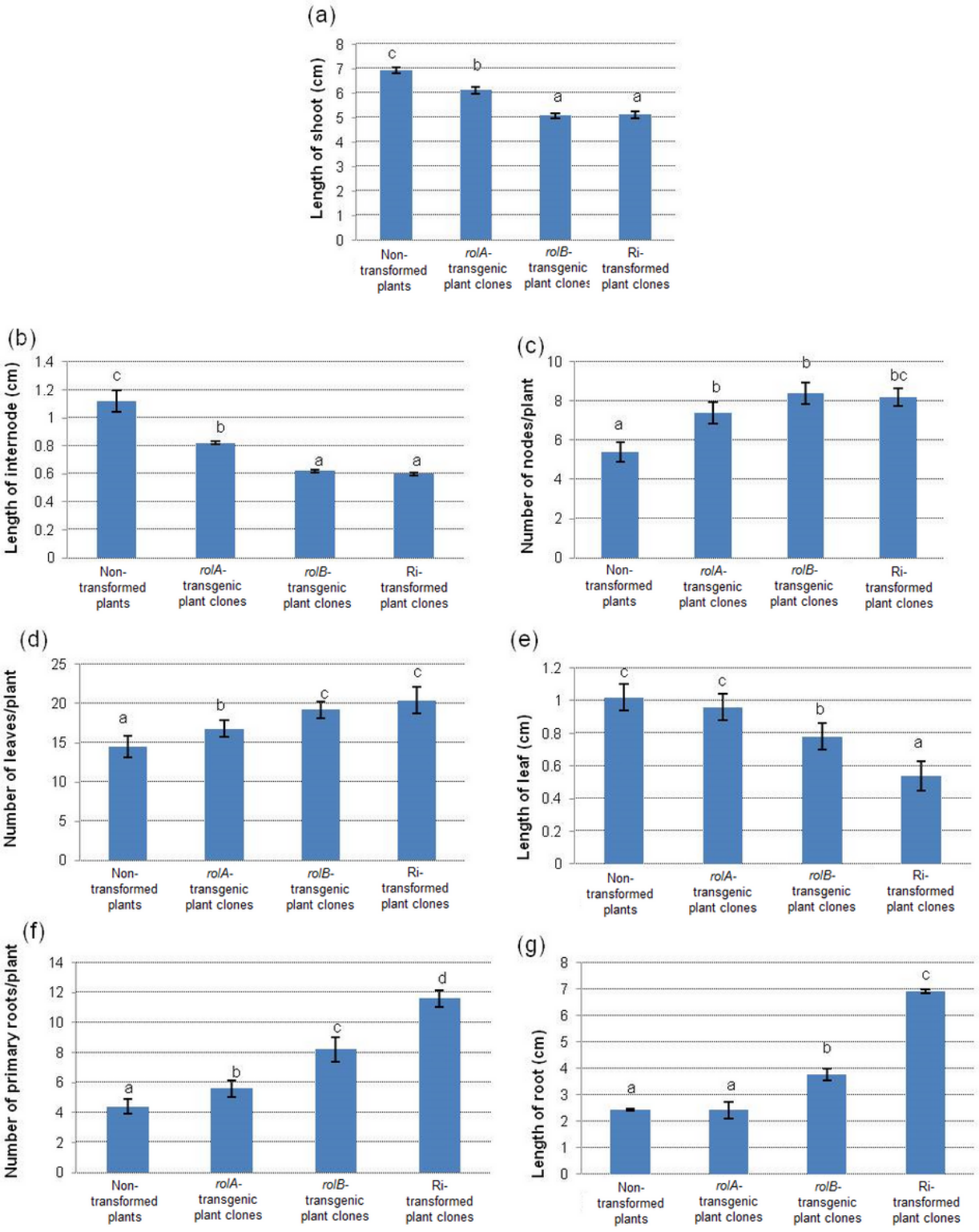

Figure 6

Morphological characterization of six-weeks old in vitro grown rolA-transgenic, rolB-transgenic, Ri-transformed plant clones and non-transformed ones based on morphological descriptors: a length of shoot (cm), b length of internode (cm), c number of nodes/plant, d number of leaves/plant, e length of sub-apical leaf (cm), f number of primary roots/plant, and g length of root (cm). Values represent mean ± S.D. Means marked with different letters are significantly different at $p \leq 0.05$.

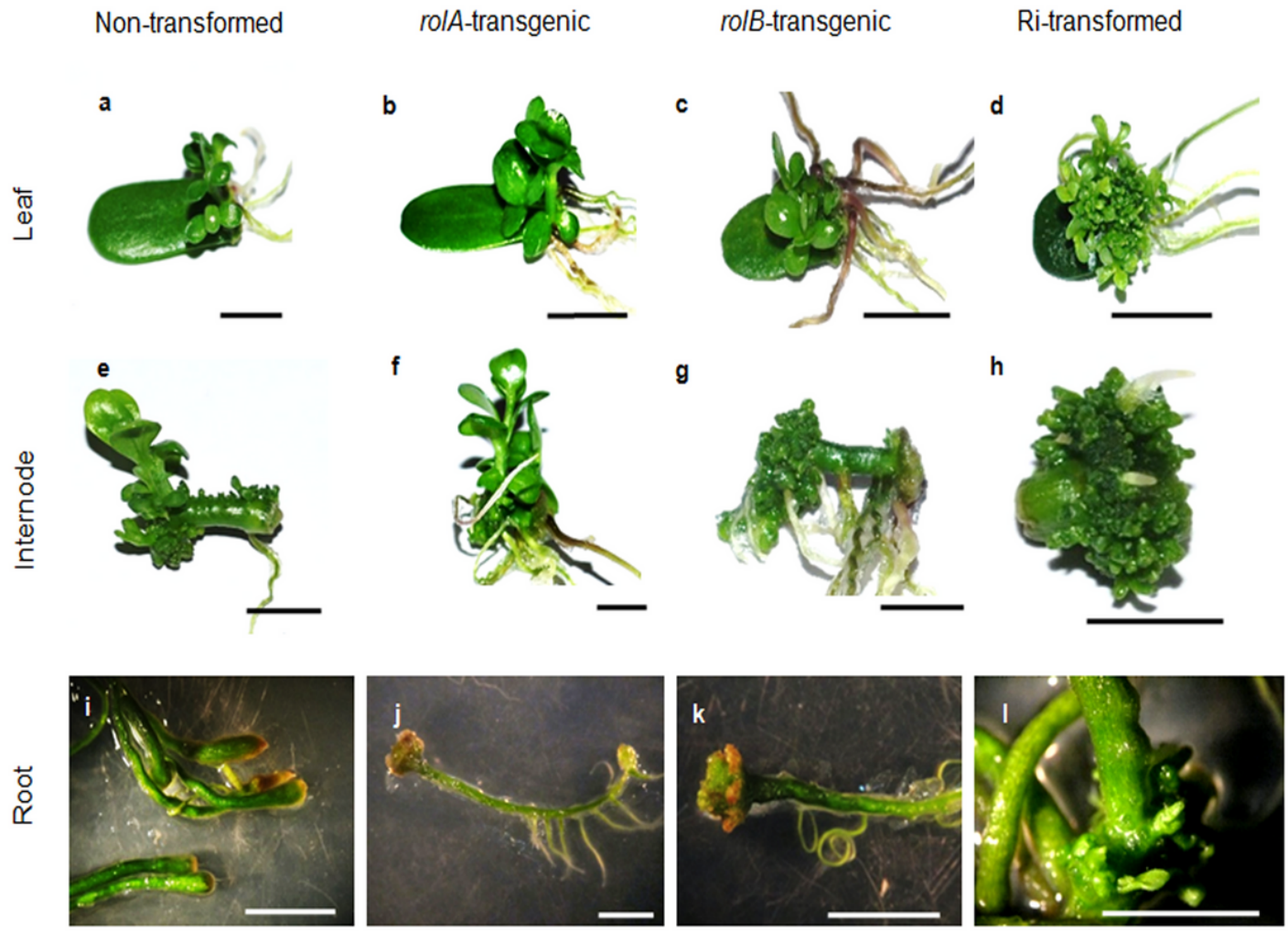

Figure 7

Morphogenic responses showing adventitious shoot and root organogenesis from excised a-d leaf and e-h internode explants derived from different transgenic and non-transformed plants, i portion of excised non-transformed root explants with no organognic response, j and k portion of excised rolA-transgenic and rolB-transgenic root explants inducing callus at cut ends and lateral branching formation, and l portion of excised Ri-transformed root explant showing shoot regeneration, after four weeks of culture on 0.05 mg l-1 IAA supplemented MS medium. Scale bar = 4 mm

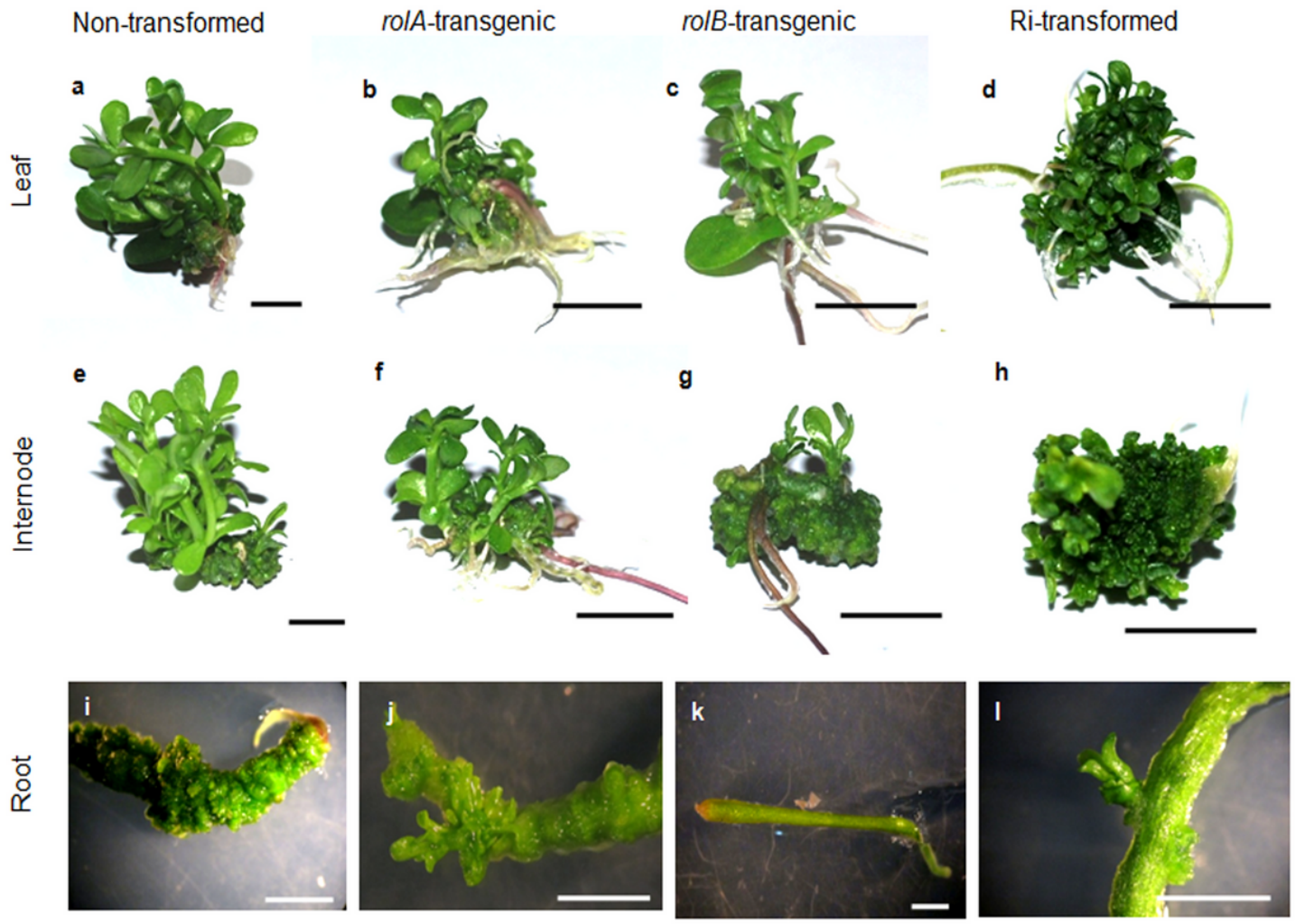

Figure 8

Adventitious shoot formation from excised a-d leaf and e-h internode explants derived from different transgenic and non-transformed plants. Excised i non-transformed, j rolA-transgenic and l Ri-transformed plants-derived root segments induced shoot buds, whereas k excised rolB-transgenic root explant showed no response, after four weeks of culture on 1.0 mg l-1 BA supplemented MS medium. Scale bar = 4 mm