Plant Cell Rep (2012) 31:1899–1909
DOI 10.1007/s00299-012-1303-3

ORIGINAL PAPER

Use of the cryptogein gene to stimulate the accumulation of bacopa saponins in transgenic *Bacopa monnieri* plants

Sukanya Majumdar · Saraswati Garai · Sumita Jha

Received: 17 May 2012 / Revised: 5 June 2012 / Accepted: 8 June 2012 / Published online: 26 June 2012

Abstract Genetic transformation of the Indian medicinal plant, *Bacopa monnieri*, using a gene encoding cryptogein, a proteinaceous elicitor, via Ri and Ti plasmids, were established and induced bioproduction of bacopa saponins in *crypt*-transgenic plants were obtained. Transformed roots obtained with *A. rhizogenes* strain LBA 9402 *crypt* on selection medium containing kanamycin (100 mg l^{-1}) de-differentiated forming callus and redifferentiated to roots which, spontaneously showed shoot bud induction. Ri *crypt*-transformed plants thus obtained showed integration and expression of *rol* genes as well as *crypt* gene. Ti *crypt*-transformed *B. monnieri* plants were established following transformation with disarmed *A. tumefaciens* strain harboring *crypt*. Transgenic plants showed significant enhancement in growth and bacopa saponin content. Bacopasaponin D (1.4–1.69 %) was maximally enhanced in transgenic plants containing *crypt*. In comparison to Ri-transformed plants, Ri *crypt*-transformed plants showed significantly ($p \leq 0.05$) enhanced accumulation of bacoside A_3, bacopasaponin D, bacopaside II, bacopaside III and bacopaside V. Produced transgenic lines can be used for further research on elicitation in *crypt*-transgenic plants as well as for large scale production of saponins.

Key message The cryptogein gene, which encodes a proteinaceous elicitor is associated with increase in secondary metabolite accumulation—either alone or in addition to the increases associated with transformation by *A. rhizogenes.*

Keywords Cryptogein · Genetic transformation · *Bacopa monnieri* · Jujubogenin glycosides

Communicated by J. Register.

S. Majumdar · S. Jha (✉)
Department of Botany, Centre of Advanced Study, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India
e-mail: sumitajha.cu@gmail.com

S. Garai
Indian Institute of Chemical Biology, 4 Raja SC Mullick Road, Jadavpur, Kolkata 700032, India

Introduction

Plant secondary metabolites are chemically diverse and abundant, and they have numerous and complex functions, serving in defense mechanisms, general resistance to stress, signaling, etc. *Bacopa monnieri* (L.) Wettst. (Scrophulariaceae) is used in Indian Ayurvedic medicine to enhance cognition. The saponins in polar extracts of *B. monnieri* include three jujubogenin glycosides, bacoside A1 (Jain and Kulshreshtha 1993), bacoside A_3 (Rastogi et al. 1994) and bacoside A2 (Rastogi and Kulshreshtha 1998), six dammarane-type saponins, bacopasaponin A, B, C, D, E and F (Garai et al. 1996a, b; Mahato et al. 2000). Of these six saponins, bacopasaponin A, E and F are jujubogenin glycosides and the other three are pseudojujubogenin glycosides. In addition, bacopasides I, II, III, IV and V (Chakravarty et al. 2001, 2003) are also dammarane-type saponins.

Application of elicitors to plant cell and organ cultures has been useful for enhancing the productivity of secondary metabolites (Singh 1999) and a wide variety of elicitors have been employed to modify cell metabolism. These modifications are designed to enhance the productivity of useful metabolites in plant cell/tissue cultures (Pitta-Alvarez et al. 2000; Broeckling et al. 2005; Ghosh et al. 2006). As little is known regarding the biosynthetic pathways of most plant secondary metabolites, the effects of elicitation in plant cell or tissue cultures are difficult to

predict. Fungal elicitors have been most widely studied for enhancement of synthesis of commercially important compounds from plant cell cultures (Baldi et al. 2009).

β-Cryptogein, a proteinaceous elicitor from the phytopathogenic Oomycete *Phytophthora cryptogea* (Huet and Pernollet 1989), is known to induce non-specific resistance against a wide range of tobacco pathogens and cause the accumulation of pathogenesis related proteins (Bonnet et al. 1986). This protein belongs to a novel family of 10-kDa holoproteins (98-amino acid; Mr of 10,329) called elicitins (Ricci et al. 1993) and has recently been shown to induce accumulation of phenylpropanoids (Amleot et al. 2011). β-Cryptogein is a sterol carrier protein which elicits a defense response in tobacco through interaction with receptor, followed by signal transduction which leads to the expression of a transcription factor (Guo et al. 2004) and a MAPK (Ren et al. 2006), which in turn is thought to be responsible for a generalized stress response. Genetic transformation of tobacco with *crypt* gene, under the control of the 35S CaMV promoter, rendered two independent lines of tobacco plants resistant to *P. parasitica* var. *nicotianae* (Tepfer et al. 1998).

The production of plant secondary metabolites can be improved by manipulating specific genes (Hashimoto et al. 1993; Canel et al. 1998) or through indirect approaches, e.g. by treating with substances (elicitors) that simulate pathogen attack (Zhao et al. 2004; Menke et al. 1999) or by relying on natural transformation by *Agrobacterium rhizogenes* (Ray et al. 1996; Chaudhuri et al. 2005). These strategies can be combined, using for instance, the cryptogein gene, which encodes a fungal elicitor and is associated with resistance to fungal pathogens in tobacco and increases in secondary metabolite accumulation—either alone (*Arabidopsis*) or in addition to the increases associated with transformation by *A. rhizogenes* (morning glory) (Chaudhuri et al. 2009). Such indirect approaches obviate the need for detailed knowledge of the underlying biochemistry and genetics, but they do not work in every species (Chaudhuri et al. 2009).

Agrobacterium tumefaciens mediated genetic transformation in *B. monnieri* with strain EHA105 was reported by Nisha et al. (2003). *A. rhizogenes* (wild-type strains) mediated transformation in this species were reported recently (Majumdar et al. 2011) showing the effect of Ri T-DNA on morphology, growth and bacopa saponins accumulation of the transformed cultures. For testing the hypothesis that the gene encoding cryptogein (*crypt*) could also increase metabolite accumulation via production of the cryptogein protein in planta, the synthetic gene was introduced by Chaudhuri et al. (2009) into the roots of *Withania somnifera*, *Convolvulus sepium* and *Tylophora tanakae*, via *A. rhizogenes* mediated transformation. In all cases, increases in growth and secondary metabolite accumulation were found to be associated with the presence of the *crypt* gene.

We transformed *B. monnieri* with the cryptogein gene (*crypt*) to study effects associated with insertion of *crypt* gene on secondary metabolites in transgenic plants utilizing both Ri and Ti plasmids for the first time.

Materials and methods

Agrobacterium strains and culture

Agrobacterium rhizogenes LBA 9402 *crypt* (pRi1855+pBin19++*crypt*) (O'Donohue et al. 1995) and a disarmed *A. tumefaciens* strain LBA 4404 *crypt* (pTi 4404+pBin19++*crypt*) under the control of 35S CaMV viral promoter and the nos terminator (Tepfer et al. 1998) were used in the present study. For plant transformation, single colony was grown overnight in 10 ml of liquid YMB medium (Hooykaas et al. 1977), pH 7.0, on a gyratory shaker at 28 °C at 180 rpm.

Effects of ampicillin, cefotaxime and kanamycin on organogenesis: sensitivity test

Prior to transformation, the amount of kanamycin required to inhibit direct organogenesis in leaf explants was tested. The wild-type leaf explants were placed on MS media supplemented with different concentrations of kanamycin (0–100 mg l^{-1}) and ampicillin or cefotaxime (0–500 mg l^{-1}) and subcultured 2 times at 4 week intervals. Survival rates of explants were evaluated. The concentration of kanamycin that inhibited root induction and shoot induction was used in transformation experiments.

Transformation procedure

Subapical leaves (1–1.5 cm) of in vitro grown 15-day-old plants of *B. monnieri* cultured on MS medium, were used as explants for *A. rhizogenes and A. tumefaciens* inoculation. Leaf explants were infected on the leaf lamina with a hypodermic needle loaded with 10 ml (for 25 explants) of bacterial suspension. Infected and control explants were kept on filter paper soaked with liquid MS medium (Murashige and Skoog 1962) for co-cultivation in the dark for 72 h. All the explants were washed in sterile distilled water. They were then cultured on solid MS medium supplemented with 500 mg l^{-1} ampicillin or cefotaxime and 100 mg l^{-1} kanamycin for 8 weeks.

Control leaf explants were inoculated with bacterial growth medium without bacteria. Control leaves were cultured on MS medium supplemented with 500 mg l^{-1} ampicillin or cefotaxime with and without kanamycin

(100 mg l^{-1}). Fifty leaf explants were used for each experiment. Each experiment was repeated twice.

Transformed root culture

Roots that were induced on the LBA 9402 *crypt*-infected explants were excised and cultured on Petri dishes containing 20 ml MS supplemented with 500 mg l^{-1} ampicillin and 100 mg l^{-1} kanamycin under 16/8-h (light/dark) photoperiod to establish axenic kanamycin resistant transformed root cultures. Each excised primary root was propagated as a separate clone and subcultured at 4-week intervals. Six months after initiation of the culture, fast-growing axenic root clones were transferred to ampicillin-free media and further subcultured at 4-week intervals. Each clone was screened for the presence of bacteria by culturing the root segments on YMB medium. Roots induced from WT leaves (control) were similarly excised and grown on MS with or without ampicillin (500 mg l^{-1}). WT roots (control) on medium with kanamycin did not grow at all.

Establishment and culture of spontaneously induced transformed callus

Calli spontaneously induced on the putatively transformed LBA 9402 *crypt* root cultures growing on MS + ampicillin 500 mg l^{-1} + kanamycin (100 mg l^{-1}) were excised and cultured on solid MS medium + ampicillin 500 mg l^{-1} kanamycin (100 mg l^{-1}) under a 16/8-h (light/dark) photoperiod at 25 °C.

Excised wild-type roots did not show callus induction on MS with or without ampicillin 500 (mg l^{-1}).

Establishment of redifferentiated root cultures

Roots redifferentiating from calli transformed by Ri T-DNA plus *crypt* were excised and cultured on MS medium for 4 weeks. Clumps of roots were then cultured on Petri dishes containing 20 ml of modified MS medium (BM) with total nitrogen reduced to 1/5th of the normal strength, macro nutrients, micro nutrients, myoinositol, ammonium iron citrate, filter sterilized Gamborg's vitamin (Gamborg et al. 1968), 3 % w/v sucrose supplemented with kanamycin (100 mg l^{-1}) and subcultured at 6–8 week interval.

Shoot organogenesis and establishment of transformed plants

Shoot buds (1 cm) developing from the Ri *crypt* root line, cc5r1, were excised after 1 week of induction and cultured on MS medium supplemented with kanamycin (100 mg l^{-1}) to establish plants transformed by Ri T-DNA and the *crypt* gene. Transformed plants were kept under 16/8-h (light/dark) photoperiod with light provided by cool-white fluorescent tubes at an intensity of 48 mol m^{-2} s^{-1} at 25 °C.

Shoot bud regeneration and transformant selection from Ti *crypt*-infected leaf explants

Shoot buds induced from leaf explants transformed with Ti T-DNA plus *crypt* on MS medium, supplemented with cefotaxime (500 mg l^{-1}) and kanamycin (100 mg l^{-1}) were considered to be putatively transformed. Regenerated microshoots (1 cm in length) were cultured on MS medium supplemented with cefotaxime (500 mg l^{-1}) and kanamycin (100 mg l^{-1}) under a 16/8-h (light/dark) photoperiod at 25 °C.

Control shoots regenerated via organogenesis from leaf explants cultured on MS and MS + cefotaxime (500 mg l^{-1}), were maintained separately.

DNA isolation and PCR analysis

Transformation was confirmed by PCR detection of the TL and TR-DNAs on genomic DNA extracted from different putative transformants. Primers spanned the *rolA* and *rolAB* genes (Chaudhuri et al. 2005, 2006). Primers for the TR-DNA genes, *ags* gene and virD1. (Bandyopadhyay et al. 2007) were also used. The pLJ1 and pLJ85 plasmids (Jouanin 1984), covering the Ri TL-DNA and TR-DNA were used as positive controls. Putatively Ri- and Ti-transformed culture lines harboring *crypt* was analyzed for the presence of the *crypt* gene. The sequences used for *crypt* gene primers were 5′-CCATGTCGACCTACAAGG AAGAGCACTTGT-3′ and 5′-TCCGGTCGACATGGCTT GCACTGCTAC-3′ (O'Donohue et al. 1995). PCR for detection of *crypt* gene was performed following the published method of O'Donohue et al. (1995). The amplified product had the expected size of 329 bp. DNA samples were analyzed by 1.5 % agarose gel electrophoresis and visualized under UV light after ethidium bromide staining.

Negative results obtained with PCR analysis using primers for the virD1 gene were taken as evidence against contamination by *Agrobacterium*.

Gene expression analysis by RT-PCR

Expression of transgenes at the transcription level was analyzed using the reverse transcriptase polymerase chain reaction. Total RNA from transformed plants/calli/roots was extracted with the TRIzol® Reagents (Invitrogen, USA) (Chomczynski and Sacchi 1987). The amount of RNA was determined spectrophotometrically. The RT reaction

was performed by incubating 5 µg of total RNA in a 11 µl reaction volume containing 15–20 pmol of reverse primers for the *rolA* and *rolAB* genes. The same reaction procedure was used for RT-PCR of all genes tested (*rolA rolB*, TR T-DNA genes, *ags* and *crypt*). The reaction mix was kept at 70 °C for 5 min. To the above reaction mixture 5× reaction buffer, 10 mM dNTP mix and 20 U ribonuclease inhibitor were added to make a final volume of 19 µl. This reaction mix was incubated at 37 °C for 5 min. 200 U RevertAid^{TM} M-MuLV Reverse Transcriptase was added and then incubated at 42° C for 60 min. The reaction was stopped by heating at 70 °C for 10 min. The cDNA samples were stored at 4 °C until PCR analysis.

Growth of the Ri/Ti-transformed plants

To study the growth of transgenic in vitro plants, 2 cm long (0.5 g) shoots rooted on solid MSO were cultured in culture flasks (250 ml, with 100 ml medium) for 12 weeks. Shoots regenerated from wild-type root segments and Ri-transformed root segments (Majumdar et al. 2011) were maintained on MSO and used as the control. The experiment was repeated three times and ten replicates were used for each clone. After 12 weeks of culture, the plants were washed with deionized water blotted dry and their fresh weight was determined. The tissues were oven dried (60 °C) for 48 h. and their dry weight measured.

Extraction, identification and quantitative analysis of bacopa saponins

Quantitative and qualitative analysis of eight different bacopa saponins viz., bacoside A3, bacopasaponins (C, D, E and F) and bacopasides (II, III and V) were done following the published method of Mahato et al. (2000) in 12-week-old Ri- and Ti-transformed plants harboring the *crypt* gene and compared with 12-week-old wild-type and Ri-transformed whole plants (Majumdar et al. 2011). Approximately 1 g of dried (60 °C for 8 h) tissues was used for the extraction of bacopa saponins and each experiment was repeated three times. Dried materials were powdered and defatted in a soxhlet apparatus with petroleum ether (40–60 °C) for 24 h. The residue was then extracted with methanol at room temperature for 24 h. The filtered extract was evaporated to dryness and the residue was dissolved in HPLC grade methanol. HPLC analysis (Shimadzu Liquid Chromatograph LC 10 AD, Japan) was performed following method described by Mahato et al. (2000) on a C-18 column (Supelco Hypersil ODS 5 µm id 150 mm × 4.6 mm) using methanol and water [65:35 (v/v)] as the mobile phase at the rate of 1 ml min^{-1} under isocratic condition. Bacopa saponins were detected at 210 nm using a UV–Vis detector (SPD 10 AD) and comparison of retention times with standard samples was as described earlier (Majumdar et al. 2011).

Statistical analysis

All transformation experiments were set up in a randomized design. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means (Sokal and Rohlf 1987). Variability around the mean was represented as the standard deviation.

Means differing significantly were compared using Duncan's Multiple Range Test (DMRT) at the 5 % probability level. Variability in data has been expressed otherwise as mean ± standard deviation.

Results and discussion

Kanamycin sensitivity of leaf explants

Bacopa monnieri leaves have a high organogenic potential. Roots and shoot buds developed spontaneously on unsupplemented basal medium. Hence, it was important to optimize kanamycin selection of transformed roots and shoot buds. Thus, leaf explants (50 explants per treatment repeated twice) were cultured on a range of kanamycin (0–100 mg l^{-1}) concentrations. Shoot and root regeneration was 100 % on medium without kanamycin, but no shoot or root regeneration was observed on medium supplemented with 75–100 mg l^{-1} kanamycin, even after 1 month. Ampicillin or cefotaxime have no inhibitory effect on direct root or shoot organogenesis in *B. monnieri* leaf explants.

Induction and selection of roots following infection with *A. rhizogenes* LBA 9402 *crypt*

After 30 days, uninoculated leaf explants (control experiments, $n = 150$) did not show any root induction on selection medium: MS + ampicillin (500 mg l^{-1}) + kanamycin (100 mg l^{-1}). When non-transformed roots (NT) induced on MS medium with or without ampicillin (500 mg l^{-1}) were cultured on MS medium with or without ampicillin (500 mg l^{-1}), they did not grow after 2 weeks and necrosed.

Leaf explants infected with *A. rhizogenes* strain LBA 9402 *crypt* showed root induction from laminar wound sites in 34.84 ± 3.24 % explants within 30 days of infection on selection medium: MS + ampicillin (500 mg l^{-1}) + kanamycin (100 mg l^{-1}) (Fig. 1a). Induced roots were thin, light green in color, fast growing with lateral branches.

Dedifferentiation in transformed roots

Thirty-five percent of the root lines transformed with LBA 9402 *crypt* (14 out of 40 root lines) showed continued growth on MS + ampicillin (500 mg l^{-1}) + kanamycin (100 mg l^{-1}). Spontaneous dedifferentiation was noticed in root lines after 1 month of initiation of root cultures (Fig. 1b). Out of 14 transformed callus clones, seven light greenish, fast growing, friable callus lines were selected for further study.

Establishment of redifferentiated root cultures

The transformed calli lines showed redifferentiation and rhizogenesis after 9 months of establishment of callus

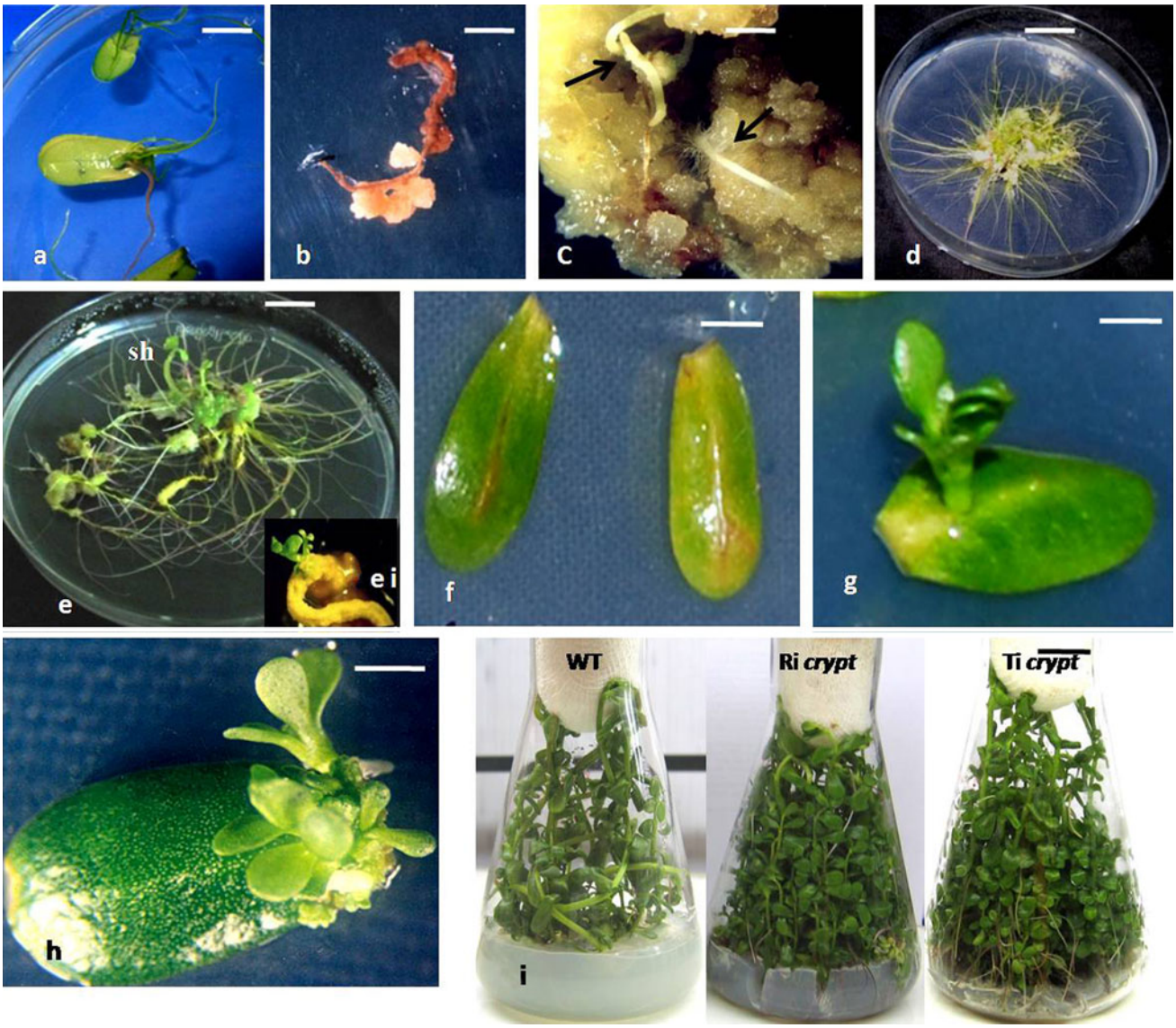

Fig. 1 **a** Induction of transformed roots from laminar wound sites of *B. monnieri* leaf explants after 4 weeks on MS + kan (100 mg l^{-1}) following infection with *A. rhizogenes* strain LBA 9402 *crypt* (*bar* 5 mm). **b** Spontaneous dedifferentiation of Ri *crypt*-transformed root lines on MS + kan (100 mg l^{-1}) within 1 month of establishment of root culture (*bar* 3.3 mm). **c** Redifferentiation of Ri *crypt*-transformed roots from callus cultures on MS + kan (100 mg l^{-1}) after 9 months of callus cultures establishment (*bar* 0.9 mm). **d** Establishment of redifferentiated root cultures on BM + kan (100 mg l^{-1}) (*bar* 1.4 cm). **e** Spontaneous regeneration of shoot buds (*arrow*, sh) from Ri *crypt* root line cc5r1 on BM + kan (100 mg l^{-1}) (*bar* 1.2 cm). **f** Inhibition of shoot bud induction from the leaves of *B. monnieri* on selection medium containing MS + kanamycin (100 mg l^{-1}) (*bar* 3.3 mm). **g** Induction of putative transformed shoot buds from leaf explants of *B. monnieri* following infection with *A. tumefaciens* strain LBA 4404 *crypt* on selection medium containing MS + kanamycin (100 mg l^{-1}) after 4 weeks of culture (*bar* 2.5 mm). **h** Shoot bud development on selection medium after 6 weeks of culture (*bar* 1.7 mm). **i** Ten-week-old Ri *crypt*- and Ti *crypt*-transformed plants growing on MS + kan (100 mg l^{-1}) and wild-type (WT) plant of similar age growing on MS (*bar* 1.75 cm)

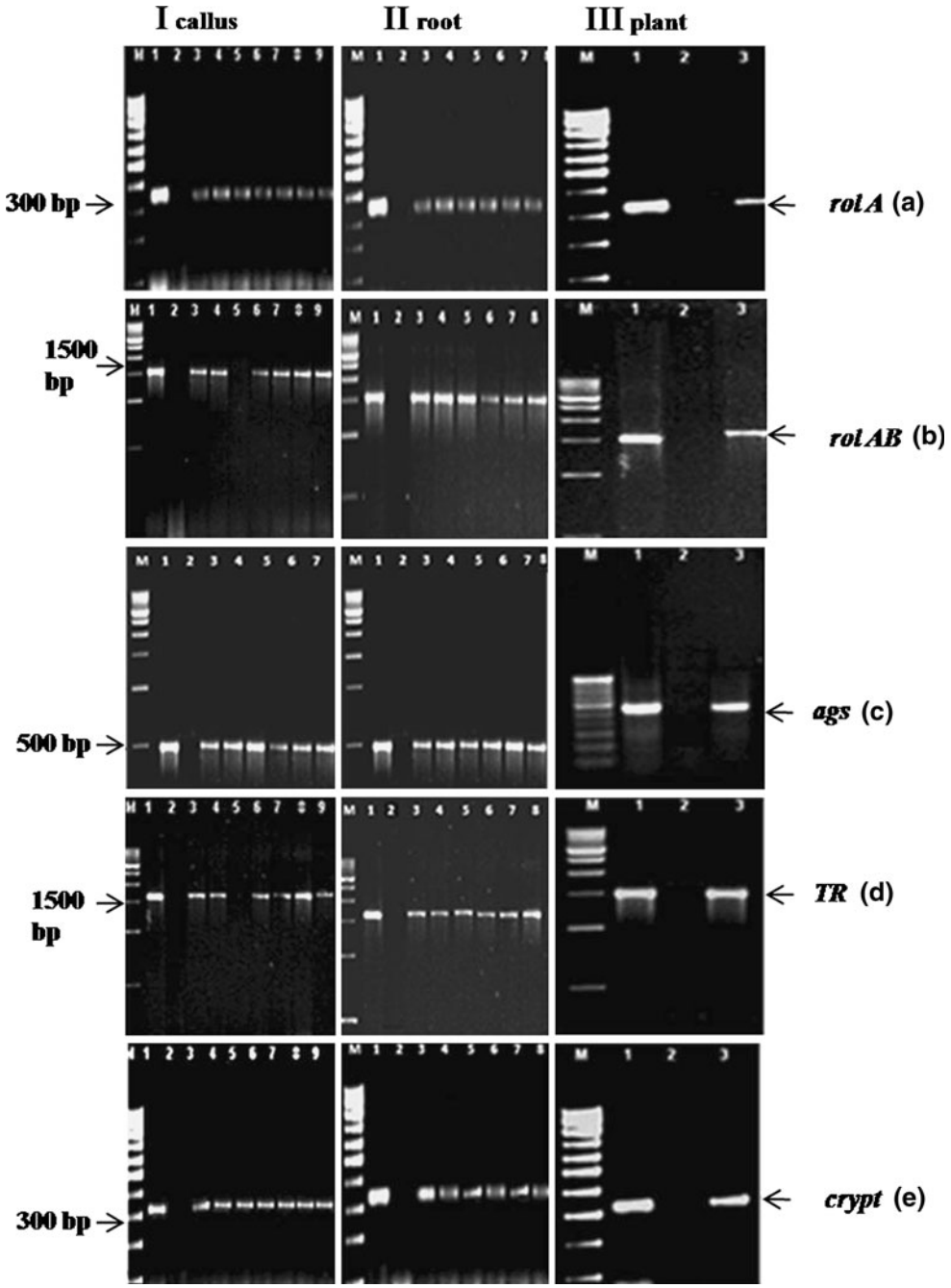

Fig. 2 Expression of Ri T-DNA and *crypt* genes at the transcription level in Ri *crypt*-transformed *I* callus, *II* redifferentiated root lines and *III* regenerated plants by RT-PCR using *rolA, rolAB,* agropine synthase (*ags*), TR genes and *crypt* primers. *I–III*: *Lane 1* positive control (bacterial plasmid), *lane 2* negative control (genomic DNA of non-transformed plants). *I*: *Lanes 3–9* amplified cDNAs of Ri *crypt*-transformed callus lines. **a** *Lane M* molecular marker (100 bp DNA ladder), *lane 1* positive control (pLJ1). **b** *Lane M* molecular marker (500 bp DNA ladder), *lane 1* positive control (pLJ1). **c** *Lane M* molecular marker (500 bp DNA ladder), *lane 1* positive control (pLJ85). **d** *Lane M* molecular marker (500 bp DNA ladder), *lane 1* positive control (pLJ85). **e** *Lane M* molecular marker (100 bp DNA ladder), *lane 1* positive control (plasmid of LBA 9402 *crypt*). *II*: *Lane 3–8* amplified cDNAs of Ri *crypt*-transformed root lines. **a** *Lane M* molecular marker (100 bp DNA ladder), *lane 1* positive control (pLJ1). **b** *Lane M* molecular marker (500 bp DNA ladder), *lane 1* positive control (pLJ1). **c** *Lane M* molecular marker (500 bp DNA ladder), *lane 1* positive control (pLJ85). **d** *Lane M* molecular marker (500 bp DNA ladder), *lane 1* positive control (pLJ85). **e** *Lane M* molecular marker (100 bp DNA ladder), *lane 1* positive control (plasmid of 9402 *crypt*). *III*: *Lane 3* amplified cDNAs of Ri *crypt*-transformed plant. **a** *Lane M* molecular marker (100 bp DNA ladder), *lane 1* positive control (pLJ1). **b** *Lane M* molecular marker (500 bp DNA ladder), *lane 1* positive control (pLJ1). **c** *Lane M* molecular marker (100 bp DNA ladder), *lane 1* positive control (pLJ85). **d** *Lane M* molecular marker (500 bp DNA ladder), *lane 1* positive control (pLJ85). **e** *Lane M* molecular marker (100 bp DNA ladder), *lane 1* positive control (plasmid of LBA 9402 *crypt*)

culture (Fig. 1c). Regenerated roots were excised to initiate transformed root cultures (Fig. 1d).

Shoot organogenesis and establishment of plants transformed by Ri T-DNA and *crypt*

Root clones derived from Ri *crypt*-transformed calli showed spontaneous, light independent shoot bud induction (15 %; 3 per explant) after 15 weeks of culture on MS medium supplemented with kanamycin (100 mg l^{-1}) (Fig. 1e). Shoot buds appeared predominantly on the older, basal regions of the transformed root. Microshoots developed from shoot buds within 2 weeks after induction. The microshoots were excised and multiple shoot cultures were maintained on MS + kanamycin (100 mg l^{-1}). Transformed microshoots rooted spontaneously in presence of kanamycin.

Thus, while LBA 9402-transformed roots have been reported to dedifferentiate to callus that do not show any redifferentiation or organogenesis (Majumdar et al. 2011), LBA 9402 *crypt*-transformed roots showed ability to redifferentiate from callus and regenerate Ri *crypt*-transformed plants.

Shoot bud regeneration and selection following transformation with *A. tumefaciens* strain LBA 4404 *crypt*

Shoot bud induction (100 %) was observed within 20 days of culture from excised *B. monnieri* leaf explants on MS medium with or without cefotaxime (500 mg l^{-1}). On an average 10 buds were induced per leaf explants in uninoculated control explants on MS + cefotaxime (500 mg l^{-1}). Thus cefotaxime does not inhibit the regenerative potential of *B. monnieri* leaf explants. No shoot organogenesis was obtained in leaf explants on selection medium (Fig. 1f).

Leaves infected with *A. tumefaciens* strain LBA 4404 (*crypt*) showed direct shoot bud organogenesis in 4 % of the explants (2 buds per explants) on MS supplemented with cefotaxime (500 mg l^{-1}) and kanamycin (100 mg l^{-1}) (Fig. 1g) within 20–30 days of culture. Developing shoot buds (Fig. 1h) were excised and cultured on MS + cefotaxime (500 mg l^{-1}) + kanamycin (100 mg l^{-1}). Microshoots (1 cm long) were maintained as clones, each derived from a single shoot bud developing following infection with *A. tumefaciens* strain LBA 4404 *crypt* on selection medium.

Transformed microshoots (3–4 cm long) multiplied and rooted spontaneously on MS + cefotaxime (500 mg l^{-1}) + kanamycin (100 mg l^{-1}).

Molecular analysis of dedifferentiating calli lines, redifferentiated root lines and Ri *crypt*-transformed plants

PCR and RT PCR analysis revealed the presence and expression of the *rolA* and *rolAB* genes at the transcription level in all Ri *crypt* calli lines (Fig. 2Ia, Ib) that dedifferentiated from hairy roots, indicating that the TL-DNA was integrated, retained and expressed. The presence and expression of *ags* and TR-DNA (Fig. 2Ic, Id) genes in such calli lines confirmed the integration and expression of TR-DNA in those lines. The presence and expression of *crypt* in Ri *crypt*-transformed calli lines were also confirmed by the present molecular study (Fig. 2Ie). The PCR products were of the expected size and identical with those of the corresponding positive controls (pLJ1, pLJ85 and LBA 9402 *crypt* plasmid).

All putative transformed root lines (cc1r1, cc2r1, cc4r1, cc5r1, cc6r1 and cc7r1) and regenerated shoots showed integration and expression of the *rolA, rolAB* and the *ags* genes from the TL and TR-TDNAs, and the *crypt* gene (Fig. 2IIa–e, IIIa–e). The PCR products were of the expected size and identical with those of the corresponding positive controls (pLJ1, pLJ85 and LBA 9402 *crypt* plasmid).

Molecular analysis of Ti *crypt*-transformed plants

PCR and RT-PCR analysis of putatively Ti *crypt* plants showed the presence and expression of the *crypt* gene (Fig. 3). The PCR products were of the expected size and identical with those of the corresponding positive control (plasmid of LBA 4404 crypt).

Growth of transgenic plants in vitro

After 12 weeks of growth, biomass accumulation (Fig. 4) was significantly ($p \leq 0.05$) higher in the Ri *crypt*-transformed shoots (3.3-fold) and the roots (5.3-fold) than in the wild-type tissue cultured plants. Ri *crypt*-transformed plants showed more than twofold enhancement of shoot biomass but there was not a significant increase in root biomass compared to Ri-transformed plant of similar age.

Accumulation of bacopa saponins in Ri *crypt*- and Ti *crypt*-transformed plants

Qualitative analysis of 12-week-old Ti *crypt*-transformed plants Ri *crypt*-transformed plants, Ri-transformed plants and WT plants revealed presence of bacoside A_3, bacopasaponin C, bacopasaponin D, bacopaside II, bacopaside III, bacopasaponin F and bacopaside V. Bacopasaponin E

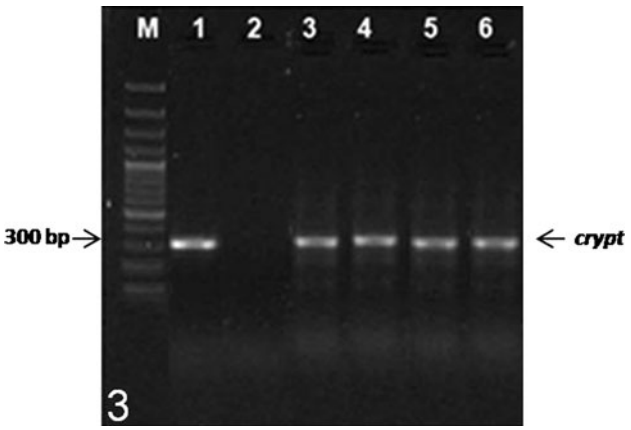

Fig. 3 Expression of *crypt* gene in Ti *crypt*-transformed plants at the transcription level by RT-PCR. *Lane 1* positive control (plasmid of LBA 4404 *crypt*), *lane 2* negative control (genomic DNA of WT plant), *lanes 3–6* amplified DNAs and cDNAs of Ti *crypt*-transformed plants

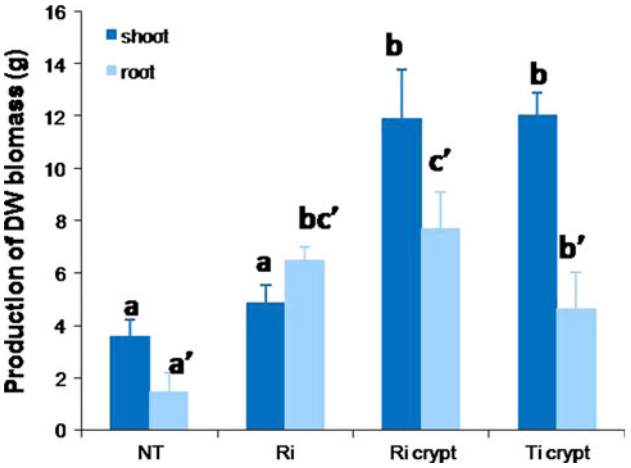

Fig. 4 Biomass accumulation (g) in shoots and roots of wild-type non-transformed (NT), Ri-transformed, Ri *crypt*- and Ti *crypt*-transformed plants of *B. monnieri* after 12 weeks of growth. Values represent mean ± standard deviation of three independent experiments ($n = 30$). *Bars with the same letters* are not significantly different at $p \leq 0.05$ according to ANOVA and DMRT for each data point

could not be detected in Ri, Ri *crypt*- and Ti *crypt*-transformed plants (Table 1).

Of the seven bacopa saponins analyzed in Ti *crypt*-transformed plants, the content of six bacopa saponins (bacoside A_3, bacopasaponin C, bacopasaponin D, bacopasaponin F, bacopaside II and bacopaside III) were enhanced by up to sixfold as compared to WT plants of similar age.

Bacoside A3 in Ti *crypt* plants (0.49 %) increased more than sixfold as compared to WT plants of same age. In Ti *crypt* plants enhancement of bacopasaponin C accumulation (0.58 %) was recorded to be significantly ($p \leq 0.05$) higher than the WT (0.3 %). Bacopasaponin D (1.69 %) was maximally enhanced in transgenic plants containing *crypt*. Ti *crypt* plants accumulated 6 times higher content of bacopaside II compared to the WT plants. Accumulation of bacopaside III in Ti *crypt* plants (0.09 % ± 0.003) was recorded to be nearly twofold more than the WT plants. There was no significant difference in Bacopaside V content in Ti *crypt* plants and WT plants.

Ri *crypt*-transformed plants show significantly ($p \leq 0.05$) enhanced accumulation of seven bacopasaponins as compared to wild type. In comparison to Ri-transformed plants (Majumdar et al. 2011), Ri *crypt*-transformed plants showed significantly ($p \leq 0.05$) enhanced accumulation of bacoside A_3, bacopasaponin D, bacopaside II, bacopaside III and bacopaside V. Accumulation of bacopasaponin C and bacopasaponin F was comparable in two types of transformed plants.

Bacopaside A3 in Ri *crypt* plant (0.39 %) enhanced more than 4 times than the Ri-transformed plant. Bacopasaponin D (1.42 %) content was found to be significantly ($p \leq 0.05$) higher than the Ri-transformed plants. Ri *crypt* plants (0.15 %) accumulated 3 times higher bacopaside II compared to Ri-transformed plants. Accumulation of bacopaside III in Ri *crypt* plants (0.07 %) were recorded to be nearly fourfold increased than Ri-transformed plants. Bacopaside V content in Ri *crypt* plants (0.139 %) were recorded to be twofold increased from the Ri plants.

Genetic transformation of *B. monnieri* with *A. rhizogenes* strain LBA 9402 *crypt* and *A. tumefaciens* strain LBA 4404 *crypt* enabled us to study effects associated with the integration of elicitor protein gene in the transformed cultures, and in regenerated plants.

In *B. monnieri*, although roots were induced on leaf explants following infection with *A. rhizogenes* strains LBA 9402 (Majumdar et al. 2011) and 9402 *crypt,* roots could not be maintained as root cultures (unlike other species transformed with *A. rhizogenes*), as they spontaneously dedifferentiated with induction of callus on MS medium with or without kanamycin (100 mg l^{-1}).

Spontaneous dedifferentiation of the hairy roots induced by the transformation with strains of *A. rhizogenes*, LBA 9402 and 9402 *crypt* suggests that the process of the dedifferentiation of the transformed roots in the hormone free basal media is due to the effects of the expression of the TL and TR DNA genes of *A. rhizogenes* (Batra et al. 2004; Bandyopadhyay et al. 2007) in different copies and insertion site of the host chromosome. The presence and expression of the *crypt* gene in transformed roots does not inhibit dedifferentiation in roots transformed by Ri T-DNA plus *crypt*.

The regeneration of roots occurred spontaneously in all but one callus lines transformed by Ri T-DNA plus *crypt* unlike the Ri-transformed calli lines (Majumdar et al.

Table 1 Accumulation of eight different bacopa saponins in Ri *crypt*- and Ti *crypt*-transformed plants and WT tissue cultured plants of *B. monnieri* after 12 weeks of culture

Name of the bacopa saponins	Plant type		
	Wild type	Ri *crypt* transformed	Ti *crypt* transformed
Bacopasaponin A3	0.069 ± 0.0186a	0.39 ± 0.0404b	0.49 ± 0.0101b
Bacopasaponin C	0.306 ± 0.0327a	0.47 ± 0.0551b	0.58 ± 0.0656b
Bacopasaponin D	0.355 ± 0.04a	1.42 ± 0.1127b	1.69 ± 0.1442b
Bacopasaponin E	0.631 ± 0.0147	Not detected	Not detected
Bacopasaponin F	0.002 ± 0.001a	0.21 ± 0.0404b	0.17 ± 0.0721b
Bacopaside II	0.02 ± 0.0044a	0.15 ± 0.03b	0.12 ± 0.0435b
Bacopaside III	0.041 ± 0.0203a	0.07 ± 0.0208b	0.09 ± 0.0264b
Bacopaside V	0.011 ± 0.0026a	0.139 ± 0.006b	0.009 ± 0.0052a

Values represent mean ± standard deviation of three independent experiments ($n = 30$). Bars with the same letters are not significantly different at $p \leq 0.05$ according to ANOVA and DMRT for each data point

2011). The *crypt* protein facilitates transport of the auxin carrier protein into the cell (Leborgne-Castel et al. 2008), which might lead to an increase in the auxin pool of the host cells. Regeneration of roots from the *crypt* harboring callus lines probably was due to the combined effect of TL-DNA and *crypt*. RolB is mostly responsible for the induction of transformed roots (Nilsson and Olsson 1997).

Regenerated transgenic plants expressing *crypt* at the transcription level produced more biomass and bacopa saponins, as compared to wild-type plants. Increase in growth in *crypt*-transformed cultures was reported in other species (Chaudhuri et al. 2009). Chemical inducers of plant defenses have been reported to stimulate growth (Dörnenburg and Knorr 1995; Walker et al. 2002; Wu et al. 2007); thus, increased rate of growth might be a generalized reaction to stress. In transgenic plants harboring *crypt*, accumulation of seven different bacopa saponins was increased. Similar result was also obtained by Chaudhuri et al. (2009), showing increases in metabolite contents in transformed cultures containing *crypt* in four different species. Elicitation is the induction of secondary metabolite production by biotic or abiotic molecules or treatments (Singh 1999). Elicitation can be applied as a strategy to elucidate metabolic flux redistribution in the metabolic engineering of plant products (Shanks et al. 1998). Several types of products have been successfully elevated by elicitation such as the production of catharanthine in *Catharanthus roseus* (Zhao et al. 2001), cryptotanshinone in *Salvia miltiorrhiza* cultures (Chen and Chen 2000), paclitaxel in *Taxus* cultures (Zhang and Wu 2003), sesquiterpenes in *Hyoscyamus muticus* root cultures (Singh et al. 1998) and saponin in *Panax ginseng* cultures (Lu et al. 2000) etc. Elicitation of *Nicotiana plumbaginifolia* cultures with cryptogein generated specific calcium signals exhibiting different lag and peak times, intensity, and duration (Lecourieux et al. 2000). Like other elicitors (Zhao et al. 2005) the *crypt* protein in plant tissues may initiate a signal transduction cascade that results in the induction of one or more transcription factors, responsible for a generalized defense response which may in turn increase secondary metabolite accumulation in the host cells. Insertion of the *crypt* gene into the *B. monnieri* plant cells is likely a similar signal transduction cascade. Produced transgenic lines can be used for large-scale production of bacopa saponins as well as for further research on elicitation in *crypt* transgenic plants.

Acknowledgments We thank Dr. David Tepfer, INRA, Versailles, France, for providing the *Agrobacterium* strains as well as for his help in preparation of the manuscript. SM gratefully acknowledges financial assistance from the University Grants Commission, New Delhi.

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