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ORIGINAL ARTICLE

Metabolic shift from withasteroid formation to phenylpropanoid accumulation in cryptogein-cotransformed hairy roots of *Withania somnifera* (L.) Dunal

Bipradut Sil · Chiranjit Mukherjee · Sumita Jha · Adinpunya Mitra

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Abstract Cotransformed hairy roots containing a gene that encodes a fungal elicitor protein, β-cryptogein, were established in *Withania somnifera*, a medicinal plant widely used in Indian systems of medicine. To find out whether β-cryptogein protein endogenously elicits the pathway of withasteroid biosynthesis, withaferin A and withanolide A contents along with transcript accumulation of farnesyl pyrophosphate (FPP) synthase, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), and sterol glycosyltransferase (SGT) were analyzed in both cryptogein-cotransformed and normal hairy roots of *W. somnifera*. It was observed that the withaferin A and withanolide A contents were drastically higher in normal hairy roots than cryptogein-cotransformed ones. Similar trends were also observed on the levels of transcript accumulation. Subsequently, the enzyme activity of phenylalanine ammonia lyase (PAL), one of the key enzymes of phenylpropanoid pathway, was measured in both cryptogein-cotransformed and normal hairy roots of *W. somnifera* along with the levels of PAL transcript accumulation. Upliftment of PAL activity was observed in cryptogein-cotransformed hairy roots as compared to the normal ones, and the PAL expression also reflected a similar trend, i.e., enhanced expression in the cryptogein-cotransformed lines. Upliftment of wall-bound ferulic acid accumulation was also observed in the cryptogein-cotransformed lines, as compared to normal hairy root lines. Thus, the outcome of the above studies suggests a metabolic shift from withanolide accumulation to phenylpropanoid biosynthesis in cryptogein-cotransformed hairy roots of *W. somnifera*.

Keywords *Withania somnifera* · Cotransformed hairy roots · β-Cryptogein · Phenylalanine ammonia lyase

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BS and CM contributed equally to this work.

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Introduction

Plants are sessile, and therefore constantly challenged by potentially harmful organisms. Plants evolved a diversity of defense responses to resist these aggressors. Plant defense responses are initiated by recognition of pathogen-derived molecules called elicitors. Cryptogein, a proteinaceous elicitor secreted by the oomycetal pathogen *Phytophthora cryptogea*, was known to induce hypersensitive responses and systemic acquired resistance, when introduced in tobacco, and thus used to elucidate molecular mechanism underlying disease resistance in plants (Tepfer et al. 1998). Cryptogein binds to the plasma membrane and initiates rapid Ca^{2+} influx and activates protein kinases and nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) for reactive oxygen species (ROS) production and nitric oxide synthesis (Lecourieux et al. 2000). Tobacco cells challenged with cryptogein showed upregulation of phenylpropanoid genes leading to enhanced accumulation of cell wall-bound phenolic compounds (Amelot et al. 2011).

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β-Cryptogein is a necrotic elicitin protein produced naturally by *P. cryptogea*. A synthetic β-cryptogein gene was constructed to study the pathogen-induced molecular interactions in plants for initiation of the defense response, mediated by elicitin (O'Donohue et al. 1995). Genetic (endogenous) expression of the β-cryptogein protein was shown to mimic the effects of exogenous cryptogein treatment on growth and secondary metabolite accumulation (Chaudhuri et al. 2009). Through transfer of cryptogein gene in target plants, earlier studies aimed either to improve secondary metabolite production in transformed plants and cotransformed hairy root cultures, such as *Tylophora tanachae*, *Bacopa monnieri*, and *Coleus blumei* (Chaudhuri et al. 2009; Majumdar et al. 2012; Vuković et al. 2013), or to achieve the resistance against plant pathogens in *Arabidopsis thaliana* and *N. tabacum* (Tepfer et al. 1998; O'Donohue et al. 1995). Transgenic tobacco expressing a *P. cryptogea* cryptogein gene under the control of a rice phenylalanine ammonia lyase (PAL) promoter showed enhanced salt tolerance. These transgenic plants when challenged with *P. parasitica* var. *nicotiana* and other pathogens showed induction of PR-1a gene that constitute a part of plant defense responses to pathogen attack and tolerance to salt stress (Donghua et al. 2004). Such study implied that endogenously synthesized oomycetal β-cryptogein elicitor could also trigger the production of secondary metabolites.

Effects of expressing a β-cryptogein gene under the control of cauliflower mosaic virus (CaMV35S) promoter in hairy roots were also studied in several plants such as *T. tanachae* and *Convolvulus sepium* (Chaudhuri et al. 2009). The overall increase in growth of hairy roots was observed in these species. It was recently reported that ethanol-induced expression of β-cryptogein gene in *C. blumei* hairy roots caused enhancement of soluble phenolic accumulation, such as rosmarinic acid and caffeic acid in the culture medium, which suggests that β-cryptogein might act as a potential regulatory factor for phenolic secretion from roots (Vuković et al. 2013).

Role of cryptogein gene in modulating phenylpropanoid pathway raised an obvious question as to whether expression of cryptogein gene could also uplift metabolites of terpenoid origin! In tobacco, it was recently shown that the genes of sesquiterpene synthase were strongly upregulated in cryptogein-treated cells (Amelot et al. 2012). Therefore, it is plausible that expression of a cryptogein gene in a terpene-accumulating species might trigger the synthesis of terpene metabolites through elicitation responses exerted by the endogenously synthesized elicitor protein cryptogein. The well-known medicinal plant *Withania somnifera* (L.) Dunal. offers a novel opportunity to explore the above hypothesis. Roots of *W. somnifera* accumulate a range of steroidal lactones called withanolides (Frank et al. 1980; Glotter 1991). Most of the pharmacological activities of *W. somnifera* have been accredited to a few main withasteroids viz.withaferin A, withanolide A, etc. (Chakraborty et al. 1974; Chaurasiya et al. 2012). Metabolic origin of withanolides is from triterpenoid pathway, and both methyl-erythritol-4-phosphate and mevalonate pathways contribute to withanolide biosynthesis; however, the contribution of mevalonic acid (MVA) pathway is four times higher than the other one (Chaurasiya et al. 2012).

Hairy root cultures of *W. somnifera* have been established with an aim to study the accumulation of withanolides. It was reported earlier that withanolide content in hairy root culture was higher than root cultures established in vitro by hormone supplementation (Ray et al. 1996). The present work describes the effects of expressing a ß-cryptogein gene in hairy root cultures of *W. somnifera* established by genetic transformation with *Agrobacterium rhizogenes*. It was conceivable that the endogenously produced ß-cryptogein would be confronted by the hairy roots own signaling systems, which in turn might trigger hypersensitive response and could enhance secondary metabolite production. Enzyme assays and gene expression studies exhibited a metabolic shift from withasteroid production to phenylpropanoid accumulation in cryptogein-cotransformed hairy roots of *W. somnifera*.

Materials and methods

Plant material

Axenic cultures of *W. somnifera* available at the tissue culture collection at the Department of Botany of the University of Calcutta, Kolkata, India, were used in this study.

Agrobacterium rhizogenes-mediated transformation

Transformation method for obtaining hairy root cultures was described earlier in the literature (Majumdar et al. 2012; Ray et al. 1996). The excised leaves of *W. somnifera* were infected with *A. rhizogenes* strain LBA9402 with an empty vector pBIN19, and in another event, the excised leaves were infected with *A. rhizogenes* strain LBA9402-CRYPT containing pBIN19 vector harboring the synthetic β-cryptogein gene under the control of CaMV promoter. The synthetic gene encoding β-cryptogein was designed by O'Donohue et al. (1995) based on the previously determined amino acid sequence of β-cryptogein (Ricci et al. 1989). This synthetic β-cryptogein gene was cloned under the 35S CaMV promoter and a nos terminator in a pBIN19 vector (Tepfer et al. 1998). This pBIN19 vector harboring the cryptogein gene was transferred to *A. rhizogenes* strain and subsequently used for plant transformation purposes (Tepfer et al. 1998; Chaudhuri et al. 2009; Majumdar et al. 2012).

Hairy roots generated from the wound sites were cultured on Murashige and Skoog medium (MS) (Murashige and Skoog 1962) with 3 % (*w*/*v*) sucrose (SRL, India) and

solidified with 0.75 % (*w*/*v*) regular grade agar (Sigma, USA). The hairy root lines were subcultured in every 28-day period for maintenance. For our experiments, hairy roots were grown in 20-mL solid medium on 90-mm sterile Petri plates in the semidark condition. The cryptogein-cotransformed hairy roots containing both Ri-T DNA and cryptogein gene were selected on the basis of their tolerance to kanamycin (50 mg/L) supplemented in MS medium.

Growth of cotransformed and normal hairy root cultures

Growth studies were done with 15 root clones, 3 clones each from 5 randomly selected independent root lines in both LBA9402-transformed and LBA9402*crypt*-cotransformed root cultures. Root tips with laterals (approximately 100-mg fresh weight) of each root clone were cultured on solid MS medium (20 mL) in Petri dishes and grown in the dark. The roots were harvested, washed with deionized water, blotted dry and the fresh weight (FW) determined after 7, 14, 21, and 28 days. The roots were then oven-dried (55 °C) to obtain the dry weight (DW). Growth was measured as an index of dry weight (growth index, GI=harvested DW/inoculum DW).

DNA isolation and PCR analysis

Total genomic DNA extracted from the transformed roots according to the published methods (Dellaporta et al. 1983). The cryptogein-cotransformed hairy root genotypes were verified by polymerase chain reaction (PCR) according to a published report (Majumdar et al. 2012).

Withasteroid extraction and analysis

Extraction and analysis of withanolides in cultured hairy roots were done according to previously published methods (Glotter et al. 1973; Roja et al. 1991). Roots were oven-dried at 40 °C, crushed into fine powder, and extracted in methanol. The extracts were evaporated to dryness and dissolved in 1 mL of methanol (HPLC grade) and clarified through nylon filter (0.45 μ, Phenomenex, USA) for analysis of alkaloids by HPLC (Ray et al. 1996). HPLC analysis was performed on Waters HPLC system on a reverse phase Prodigy™ ODS2 C_{18} column with isocratic elution using methanol water (57:43), at a flow rate of 1 mL/min. Detection was done at 254 nm on Windows XP platform using integrated *BREEZE*™ software version 3.2 (Waters). The system was calibrated with standard samples of withaferin A (Sigma-Aldrich) and withanolide A (Chromadex). Both the withasteroids were detected in normal hairy roots and in cryptogein-cotransformed hairy roots. Withaferin A and withanolide A were identified and quantified on the basis of retention time, peak areas, and spiking with standards (Ray et al. 1996; Ray and Jha 1999).

Extraction of soluble and wall-bound phenolic acids

Soluble and cell wall-bound phenolic acids were extracted as described in the literature (Chakraborty et al. 2008). Fresh hairy root tissues (1 g each) were homogenized in 2 mL of 50 % methanol and then centrifuged at 10,000 rpm for 10 min. Supernatant was collected and used as a source of soluble phenolics. For cell wall-bound phenolics, centrifuged pellet was treated with 1.5 % (*w*/*v*) SDS and 5 mM sodium thiosulfate for 20 min at 26 °C and centrifuged at 10,000 rpm for 10 min. Supernatant was discarded, and pellet was again treated with 0.5 % SDS and 3 mM sodium thiosulfate for 2 h at 26 °C. This was centrifuged again, and collected pellet was extracted with boiling ethanol. Then, the pellet was washed for thrice with acetone and finally air-dried at room temperature. This dried cell wall material was then subjected to alkaline hydrolysis with 2 M NaOH for 24 h in dark for release of wall-bound phenolics (Parr et al. 1997). This extract was then acidified with 1 N HCl (pH 1–2) and extracted with equal volume of ethyl acetate. The ethyl acetate fraction was evaporated to dryness under reduced pressure and resuspended in 0.5 mL of aqueous MeOH (50 %, *v*/*v*) for HPLC analysis to detect all phenolic acids (both soluble and wall-bound) as described in the literature (Sircar et al. 2007). Accumulation of phenolic acids was calculated in terms of nanogram per gram of fresh weight of the sample.

Tissue localization of the fluorescence phenolic compounds

To localize the deposition of cell wall-bound phenolic compounds in hairy root tissues, fluorescence microscopy was carried out as essentially described in the literature (Parr et al. 1996). Epifluorescence micrographs of normal and cryptogein-cotransformed hairy roots were observed under Axioskop2 (Zeiss) fluorescence microscope operated by the ProgRes Capture ProV2.8.8 software. Filter set for Chromomycin A3 (CMA) was used for the excitation at 436 nm and emission at 470 nm. All images were recorded under identical conditions using Prog Res MF^{scan} camera (Zeiss).

HPLC analysis of phenolic acids

Phenolic acids were separated on an RP-Hydro Phenomenex™ (Torrance, USA) C-18 column (4 μm, 250 mm×4.6 mm) using a Waters HPLC system (Milford, USA) equipped with dual absorbance detector as described before (Sircar et al. 2007). An isocratic linear solvent system comprising 1 mM TFA in water (68 %) and methanol (32 %) with a flow rate of 1 mL/min for 30 min was used to separate the phenolic compounds. Chromatograms were analyzed on Windows XP platform with *BREEZE*™ software version 3.2 (Waters).

Protein extraction and determination of total protein concentration

Fresh biomass of normal and cryptogein-cotransformed hairy roots (1 g each) was harvested and grinded into fine powder using mortar and pestle in liquid nitrogen in presence of 20 % (*w*/*v*) Polyclar AT™ (Merck, Germany) to minimize the phenolic oxidation. For PAL and pyruvate kinase assays, proteins were extracted at 4 °C with 100 mM HEPES buffer, pH 8.0. For guaiacol peroxidase assay, proteins were extracted at 4 °C with 2 mL of 0.1 M potassium phosphate buffer (pH 7.0). Protein extracts were centrifuged at 16,000 rpm for 30 min at 4 °C. The supernatants were desalted and concentrated using Amicon™ filters. The clarified crude protein extracts were used for determination of total protein contents using standard method (Bradford 1976).

Assay of phenylalanine ammonia lyase

PAL (EC 4.3.1.24) activity was determined according to a previous report (Sircar and Mitra 2008). The reaction mixture consisted of 400 μL of 100 mM Tris-HCl (pH 8.6) buffer and 500 μL of 20 mM L-phenylalanine and crude protein extract (100 μg). Reaction mixture was incubated for 60 min at 37 °C and was stopped by adding 500 μL of a mixture of chilled glacial acetic acid and methanol (1:9, *v*/*v*). The mixture was then centrifuged at 10,000 rpm for 10 min, and the supernatant was taken for HPLC analysis. The control reaction mixture contained boiled enzyme extract. Reaction product (cinnamic acid) of PAL was determined by HPLC using Waters Symmetry™ (Milford, MA, USA) C-18 reverse phase column (3.5 μm, 75 mm×4.6 mm). An isocratic linear solvent system comprising 1 mM TFA in water (55 %) and methanol (45 %) with a flow rate of 1 mL/min for 10 min was used to elute the reaction product. Reaction product was monitored at 280 nm and quantified on the basis of peak areas and linear regression curve of *trans*-cinnamic acid standard.

Assay of guaiacol peroxidase

Peroxidase (EC 1.11.1.7) enzyme activities were determined by measuring the increase of absorbance at 436 nm for 5 min relative to guaiacol oxidation to tetraguaiacol ($\varepsilon = 25.5 \times 10^3$ L/mol/cm) as originally described in the literature (Chance and Maehly 1955). The assay (1 mL) contained 50 μg protein, 0.3 mM guaiacol, and 0.1 mM H_2O_2 in 0.1 M potassium phosphate buffer (pH 7.0).

Assay of pyruvate kinase

Pyruvate kinase (2.7.1.40) assay was performed for 15 min at 37 °C in a total volume of 1 mL containing 100 mM potassium phosphate buffer (pH 7.6), 17 mM phosphoenol pyruvate, 1.3 mM NADH, 100 mM $MgS0_4$, 44 mM ADP, lactic acid dehydrogenase, and enzyme extract (Andre et al. 2007).

Isolation of core cDNA fragment of *PAL* gene

Total RNA from the normal and cotransformed hairy roots of *W. somnifera* was isolated using RNeasy mini plant kit (Qiagen, USA). Oligo (dT)-primed reverse transcription was carried out at 42 °C with Revert Aid™ M-MuLV reverse transcriptase (Fermentas GmbH). Core cDNA fragment was amplified by PCR using *Taq*-DNA polymerase (Fermentas GmbH) and degenerate primers 1 and 2 (see Table 1) derived from conserved regions of plant PAL available in public database. The PCR cycle with degenerate primer was conducted at 94 °C for 3 min, followed by 10 cycles of touchdown PCR consisted in the following: denaturation, 94 °C for 45 s; annealing starting at 55 °C and ending 45 °C for 45 s and extension, 72 °C for 1 min; after the touchdown, 30 cycles of PCR were used with an annealing temperature of 51.3 °C and with final extension of 72 °C for 10 min. The RT-PCR products were separated by electrophoresis in a 1 % agarose gel. The bands were stained by ethidium bromide, extracted out from the gel, and purified by using QIAquick gel extraction kit (Qiagen). The purified DNA was cloned in pDrive vector using Qiagen PCR cloning kit (Qiagen) following manufacturer's instructions.

Table 1 Primer sequences used for cloning of WsPAL core cDNA (1, 2), PCR analysis of cryptogein (3, 4), RT-PCR analysis of PAL (5, 6), HMGR (7, 8), FPPS (9, 10), SGT (11, 12), and actin (13, 14)

Primer	Sequence
1	5′-CCNAARCARGAYVGNTAYGC-3′
2	5′-TCYTG RTTRTGYTGYTCNGC-3′
3	5′-CCATGTCGACCTACAAGGAAGAGCACTTGT-3′
4	5′-TCCGGTCGACATGGCTTGCACTGCTAC-3′
5	5′-GTGAATTGTGCGGCCATTTAG-3′
6	5′-TGGACTGCCCTGGAAATTG-3′
7	5′-ATCCTCCTCTACCTCTTCCCTC-3′
8	5′-CCCTATTGGCATCTCGCAAC-3′
9	5′-TCATTGCCTATCCATCGCC-3′
10	5′-TGCCAATCTTACCCAGCA-3′
11	5′-ACACATCCACTACAGCCTTC-3′
12	5′-TCAACCTCCACTGTTCCATC-3′
13	5′-TCCATAATGAAGTGTGATGT-3′
14	5′-GGACCTGACTCGTCATACTC-3′

The cloned product was sequenced at Eurofin Genomic India Pvt. Ltd (Bangalore, India).

Expression analysis of PAL, HMGR, FPPS, and SGT

Total RNA was isolated from 21-day culture of normal and cotransformed hairy roots of *W. somnifera* using RNeasy plant mini kit (Qiagen) by following manufacturer's instructions. Gene-specific primers for PAL, hydroxymethylglutaryl-CoA reductase (HMGR) (EC 1.1.1.34), farnesyl diphosphate synthase (FPPS) (EC 2.5.1.10), and sterol 3beta-glucosyltransferase (SGT) (EC 2.4.1.173) genes were designed using online software from MWG Operon (Germany). Gene-specific primers for PAL, HMGR, FPPS, and SGT are shown in Table 1. PCR reaction for PAL was performed under following conditions: 94 °C for 3 min followed by 25 cycles of amplification (94 °C for 45 s, 57 °C for 45 s, 72 °C for 1 min) and by final extension of 72 °C for 10 min. Similar program was used for amplification of HMGR, FPPS, SGT, and actin, except that the annealing temperatures were 54.4, 51.7, 52.3, and 54.5 °C, respectively. The housekeeping gene actin was used as control of RT-PCR. PCR-amplified products were separated on 1 % agarose gel stained with ethidium bromide and photographed by using MicroBis gel-imaging system (DNR Bio-Imaging System Ltd). ImageJ software, which analyzes the pixel intensity of PCR bands, was used to compare the BADH, PAL, and CHS expression levels relative to that of actin.

Statistical analysis

All experiments were performed in biological triplicate, with at least three technical replicates. 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 using IBM-SPSS 20.0 software.

Results

Establishment and growth of cotransformed and normal hairy root cultures

In this study, cryptogein gene was expressed under the control of 35S CaMV constitutive promoter which was placed in a binary vector within *A. rhizogenes* strain LBA 9402 (Majumdar et al. 2012). Several hairy root lines were established by transformation of *W. somnifera* with *A. rhizogenes* strain LBA9402 (normal hairy roots) and strain LBA9402 cryptogein (cryptogein-cotransformed hairy roots). The normal hairy root lines did not contain any cryptogein gene, and therefore used as controls in this study. Presence of β-cryptogein gene in cryptogein-cotransformed hairy roots was confirmed by PCR analysis. The PCR product was of the expected size and identical with that of corresponding positive control (plasmid DNA of LBA9402crypt) (Supplementary Fig. 1). Expression of cryptogein gene in cotransformed roots was confirmed by semiquantitative RT-PCR analysis using the same set of primers for detecting the presence of cryptogein gene. As expected, cDNA of normal hairy roots of *W. somnifera* did not show any amplification, whereas a band of around 320 bp in size was detected from the amplified cDNA of cotransformed roots (Fig. 1).

Both normal hairy roots and cryptogein-cotransformed roots showed a profusion of root hairs and a high degree of lateral branching with a doubling time of ca. 14 days (Fig. 2a, b). The root clones showed an exponential growth from day 7 to day 21 (Fig. 2c). Deceleration of growth was detected after 28 days of culture, and roots turned brown rapidly. No significant difference in growth index was observed between normal hairy root lines and cryptogein-cotransformed root lines after 7, 21, and 28 days of culture (Fig. 2c). Therefore, these 21-day old normal and cryptogein-cotransformed hairy roots were used for all subsequent biochemical and molecular analyses.

Comparison of withasteroid production between normal and cryptogein-cotransformed hairy roots

Quantitative analysis of time course study revealed maximum accumulation of withaferin A and withanolide A in 21-day-old normal hairy root culture which were 0.989 μg/mg of dry weight (DW) and 1.765 μg/mg DW, respectively. On the contrary, cryptogein-cotransformed hairy roots accumulated diminished amount of withaferin A and withanolide A with a

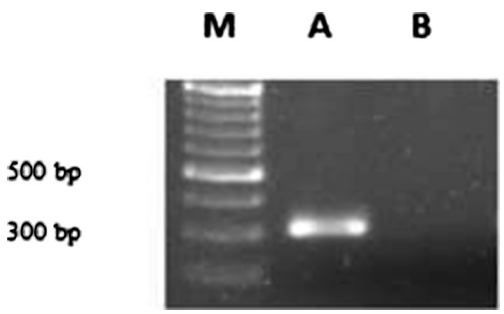


Fig. 1 Detection of cryptogein transcripts in cryptogein-cotransformed (*A*) and normal hairy roots (*B*) of *W. somnifera* by semiquantitative RT-PCR. Total RNA was reverse transcribed and used as the template for RT-PCR analysis. Molecular marker lane is also shown (*M*)

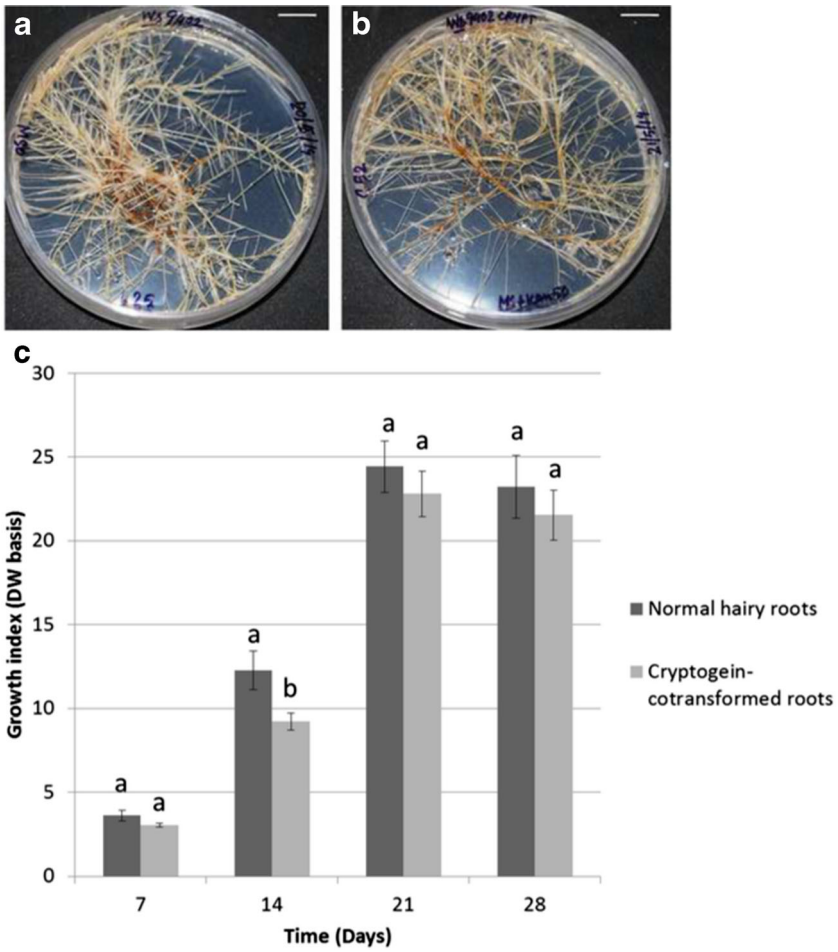


Fig. 2 **a** *Agrobacterium rhizogenes* strain LBA9402-transformed normal hairy root and **b** *Agrobacterium rhizogenes* strain LBA9402CRYPT-cotransformed hairy root cultures of *W. somnifera* after 21 days of incubation (*bar*=1.5 cm). **c** Growth index on the basis of DW in normal hairy roots and cryptogein-cotransformed hairy roots of *W. somnifera* during 4 weeks of growth

yield of 0.147 μg/mg DW and 0.085 μg/mg DW, respectively. (Fig. 3a, b). However, in nontransformed root cultures, withaferin A and withanolide A contents were 0.181 μg/mg DW and 0.479 μg/mg DW, respectively, after 21 days of culture. These observations indicated that cryptogein might have a masking effect on the withasteroid production in root cultures in vitro.

Comparison of soluble and cell wall-bound phenolic acid accumulation in normal and cryptogein-cotransformed hairy roots

It was observed that total phenolic content was nearly 2-fold higher in cryptogein-cotransformed hairy roots as compared to normal hairy roots (Fig. 4a). Subsequently, the contents of major wall-bound phenolic acids from these hairy roots were also examined. Out of the several phenolic acids detected in the wall-bound fraction, ferulic acid was found to the dominant metabolite in both normal and cryptogein-cotransformed hairy root lines in *W. somnifera*. The content of ferulic acid was found to be 2-fold higher in cryptogein-cotransformed hairy roots as compared to control (Fig. 4b).

Autofluorescence of accumulated wall-bound phenolic acids in normal hairy roots and cryptogein-cotransformed hairy roots

Longitudinal sections of hairy root explants were examined with fluorescence microscope and CMA fluorescence filter. Epifluorescence micrograph shows a large amount of phenolic depositions on the cell wall of cryptogein-cotransformed hairy roots. Cortical parenchymatous cells in longitudinal view show yellowish-green fluorescence signal in cryptogein-cotransformed hairy roots when examined under ×40 objective lens (Fig. 5a, b). Ferulic acid present in the lignified cortical cells of the hairy roots was shown to fluoresce yellowish-green when mounted in distilled water. The cortical cell walls of cryptogein-cotransformed hairy roots exhibit uniform pH-independent autofluorescence without any chemical treatment as illustrated in Fig. 5a, b.

Guaiacol peroxidase activity in normal and cryptogein-cotransformed hairy roots

Antioxidants play a key role to protect the plant from both biotic and abiotic stresses. Activities of antioxidant enzymes

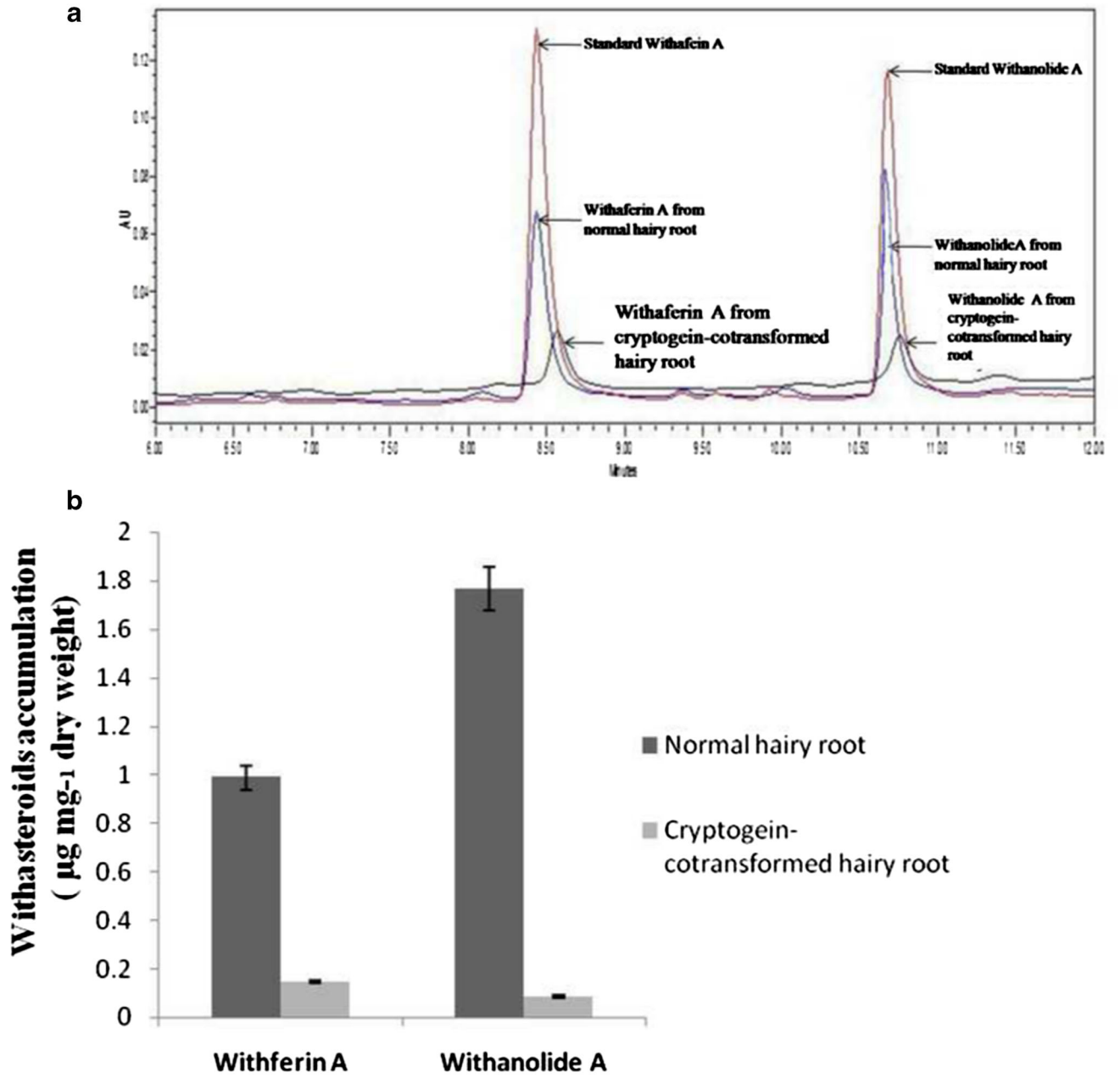
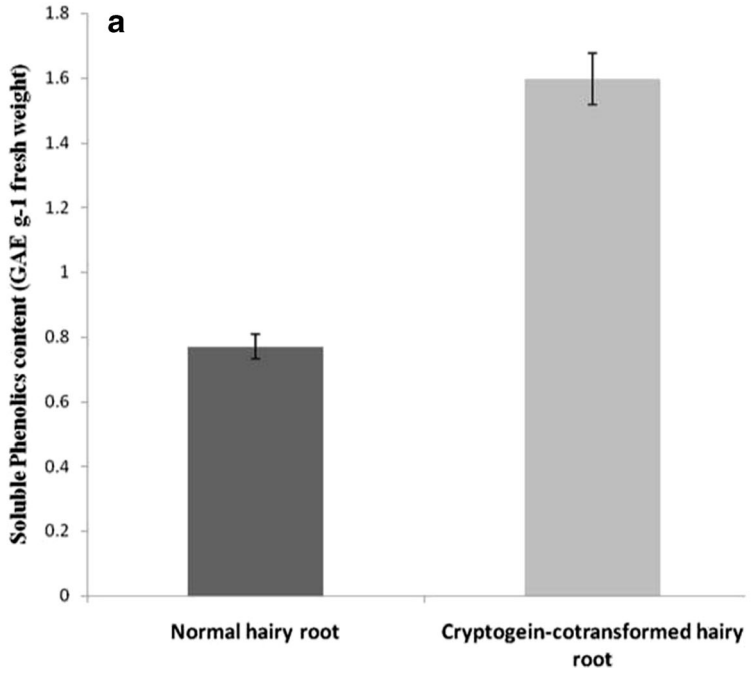
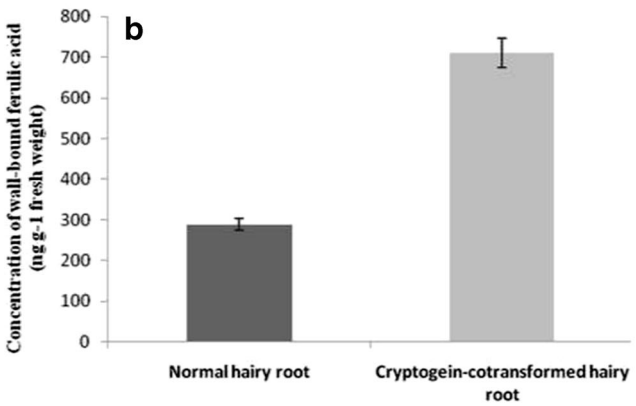


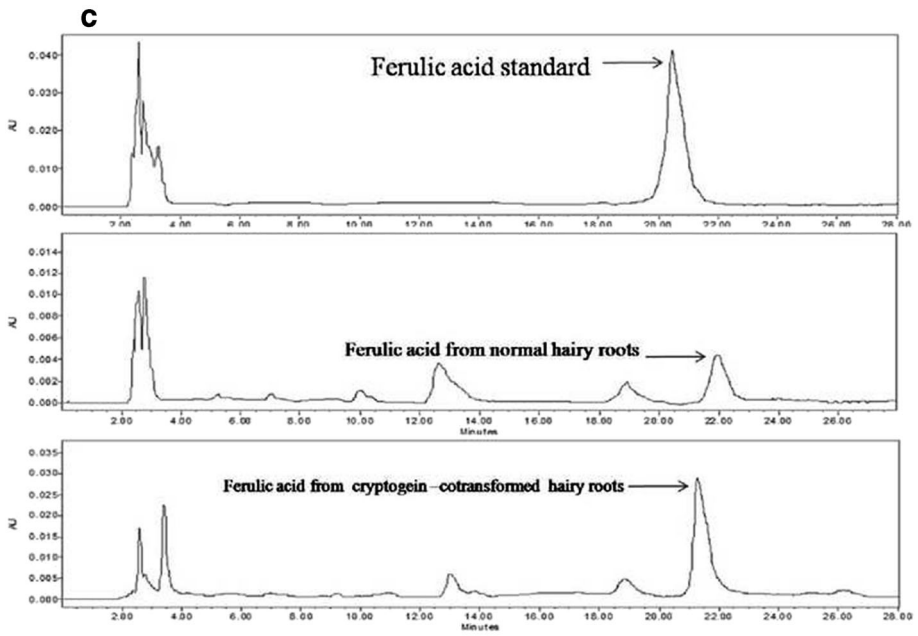
Fig. 3 Detection of withaferin A and withanolide A in the methanol extract of *W. somnifera* normal hairy roots and cryptogein-cotransformed hairy roots at 254 nm. Major peaks of withaferin A and withanolide A detected in 21-day-old hairy root cultures. **a** Stacking of HPLC chromatogram showing the major peaks of withaferin A and withanolide A in normal hairy roots and cryptogein-cotransformed hairy roots comparing with the standard samples. **b** Withasteroid accumulation in microgram (µg) per mg dry weight of hairy roots was measured. Determination and detection were done in triplicate. *Error bars* indicate ±standard deviation

correlate positively with the increasing contents of secondary metabolites (Blokhina et al. 2003). These metabolites, including several simple and complex phenolic compounds, act as major antioxidants in plants during stress (Bariuari et al. 2005). As cryptogein is an oomycetal pathogenic elicitor protein, it was anticipated that the expression of β-cryptogein protein in hairy roots could possibly exert biotic stress on hairy root tissues.

Thus, the activities of an antioxidative enzyme guaiacol peroxidase were monitored in both normal and cryptogein-cotransformed hairy root lines in *W. somnifera*. Conversion of guaiacol to tetraguaiacol was immediate after adding protein extracted from *W. somnifera* hairy roots in the reaction mixtures. Therefore, the crude protein extracts were diluted by 50 times in its respective buffer before measuring guaiacol peroxidase activities. Cryptogein-cotransformed root cultures


a
Soluble Phenolics content (GAE g-1 fresh weight)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Normal hairy root
Cryptogein-cotransformed hairy root


b
Concentration of wall-bound ferulic acid (ng g-1 fresh weight)
800
700
600
500
400
300
200
100
0
Normal hairy root
Cryptogein-cotransformed hairy root


c
Ferulic acid standard
Ferulic acid from normal hairy roots
Ferulic acid from cryptogein–cotransformed hairy roots
AU
Minutes

◀ **Fig. 4** Levels of total soluble phenolics and wall-bound phenolic accumulations in normal hairy roots and cryptogein-cotransformed hairy root tissue of *W. somnifera*. **a** Total soluble phenolic content was estimated spectrophotometrically according to the Folin-Ciocalteu method using gallic acid as a standard for calibration curve. Absorbance was measured at 765 nm. Total soluble phenolic content in hairy root tissue was expressed as mg gallic acid equivalents (GAE g^{-1} fresh weight). **b** Wall-bound ferulic acid (FA) accumulation is expressed in nanogram (ng) g^{-1} fresh weight hairy root tissue. For the analysis of phenolics, 21-day-old cultures were harvested. Determinations were in triplicate; detection of ferulic acid in the methanol extract of *W. somnifera* normal hairy roots and cryptogein-cotransformed hairy roots at 254 nm. Major peaks of ferulic acid detected in 21-day-old hairy root cultures. Overlay of HPLC chromatograms showing the major peak of ferulic acid in normal hairy roots and cryptogein-cotransformed hairy roots comparing with the standard sample. **c** Ferulic acid accumulation percentage nanogram (ng) g^{-1} weight of hairy roots was measured. Determination and detection were done in triplicate. *Error bars* indicate ±SD

showed nearly 2-fold higher guaiacol peroxidase activity than the control hairy root lines (Fig. 6a).

Pyruvate kinase activity in normal and cryptogein-cotransformed hairy roots

The fact that cryptogein-cotransformed hairy roots showed reduced withasteroid accumulation raised an important question about the levels of activities of pyruvate kinase in these hairy roots. Desalted and concentrated protein extracts from both normal and cryptogein-cotransformed hairy roots were used for spectrophotometric determination of pyruvate kinase activities. Around 3-fold reduced pyruvate kinase activity was noticed in cryptogein-cotransformed hairy root lines as compared to normal hairy roots (Fig. 6b). The carbon pool involved in isoprene biosynthesis mostly enters the biosynthetic pathway as pyruvate precursor. Thus, the role of pyruvate kinase activity in controlling the isoprenoid biosynthesis was evident.

PAL activities in normal and cryptogein-cotransformed hairy roots

An enhanced accumulation of soluble and wall-bound phenolics was observed in cryptogein-cotransformed hairy roots. Phenylpropanoids are derived after deamination of phenylalanine by the enzyme PAL. Previous studies had indicated PAL activity as the major control point for regulating the phenylpropanoid pathway (Bate et al. 1994). Therefore, it was found necessary to measure the activities of PAL. Hairy roots of 21 days old from both normal and cryptogein-cotransformed lines were harvested to determine the PAL activities (Fig. 7). It was found that the activity of PAL was nearly 3-fold higher in cryptogein-cotransformed hairy roots than normal roots (Fig. 7).

Gene expression analysis of *PAL* in normal and cryptogein-cotransformed roots using core cDNA fragment isolated and cloned from *W. somnifera* hairy roots

Core cDNA of *PAL* gene was isolated using degenerate primers from conserved regions of PAL amino acid sequences from other plants. After cloning and sequencing, the resulting amplified band of 481 bp was found to be a core cDNA

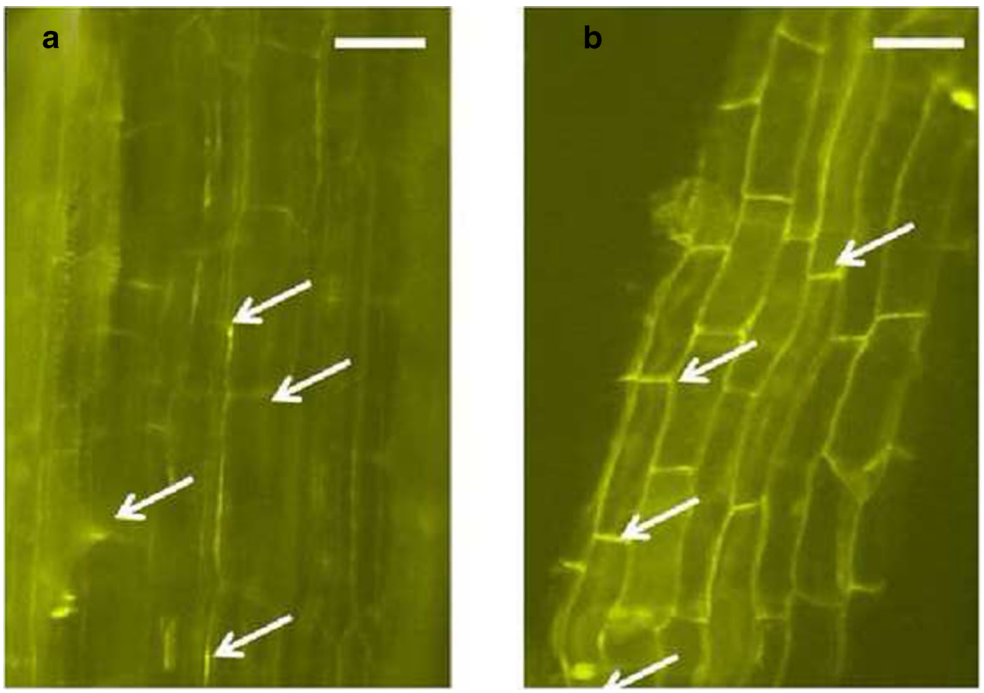


Fig. 5 Autofluorescence image of *W. somnifera* hairy roots. Fluorescence micrographs of the transverse longitudinal section of the normal hairy root (**a**) and cryptogein-cotransformed hairy root (**b**). Sections immersed for 2 min in a solution of 10 % KOH and washed with distilled water. *Arrow heads* indicate cortical cell wall junctions with phenolic depositions. In **b**, note the increase in fluorescence emitted from the rectangular cortical cells of cryptogein-cotransformed hairy root. Note the decreased intensity of the fluorescence emitted from normal hairy root (**a**). *Bars* are 160 μm

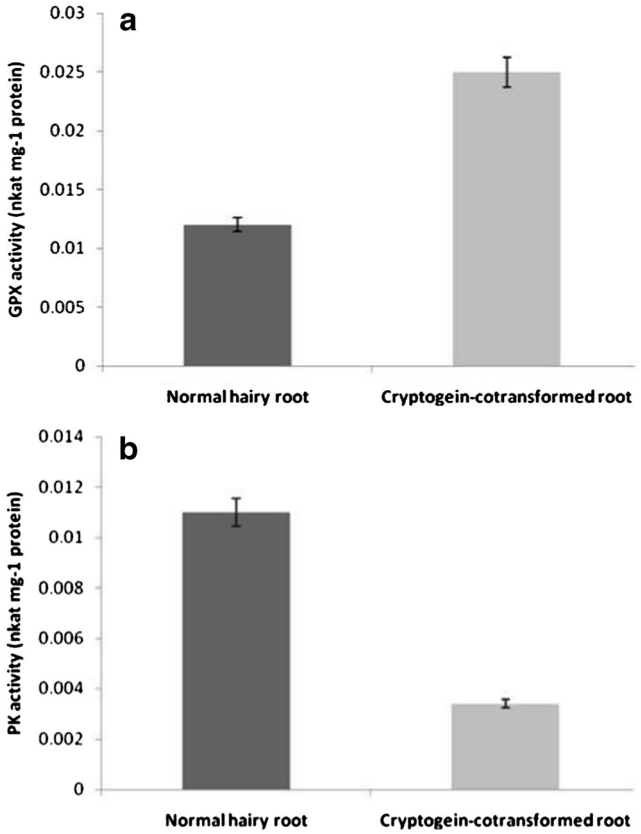


Fig. 6 Comparison of guaiacol peroxidase (GPX) and pyruvate kinase (PK) activities in normal and cotransformed hairy roots. **a** GPX activity was measured by monitoring the increased absorbance at 436 nm relative to oxidation of guaiacol to tetraguaiacol. **b** PK activity was determined by measuring the oxidation of NADH by enzyme at 340 nm. Normal and cryptogein-cotransformed hairy roots used for all assays were of same age. Enzyme activities are expressed in nanokat (nkat) mg^{-1} protein. Each value is the mean±SD from at least three independent experiments

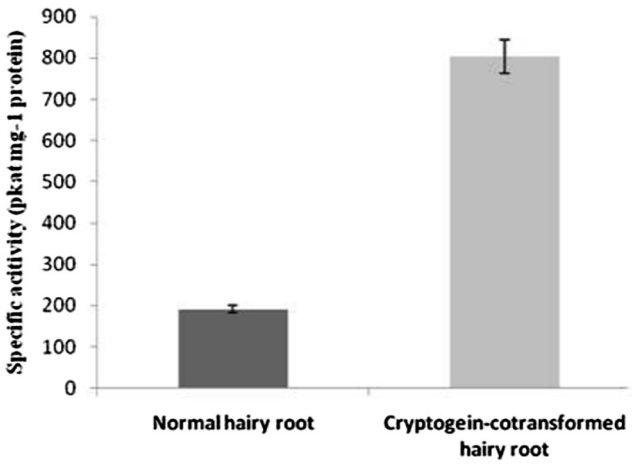


Fig. 7 PAL activities in normal and cryptogein-cotransformed hairy roots of *W. somnifera*. PAL activity was determined by measuring the accumulation of *trans*-cinnamic acid at 280 nm. Enzyme activity is expressed in picokat (pkat) mg^{-1} protein. Each value is the mean±SD from at least three independent extractions

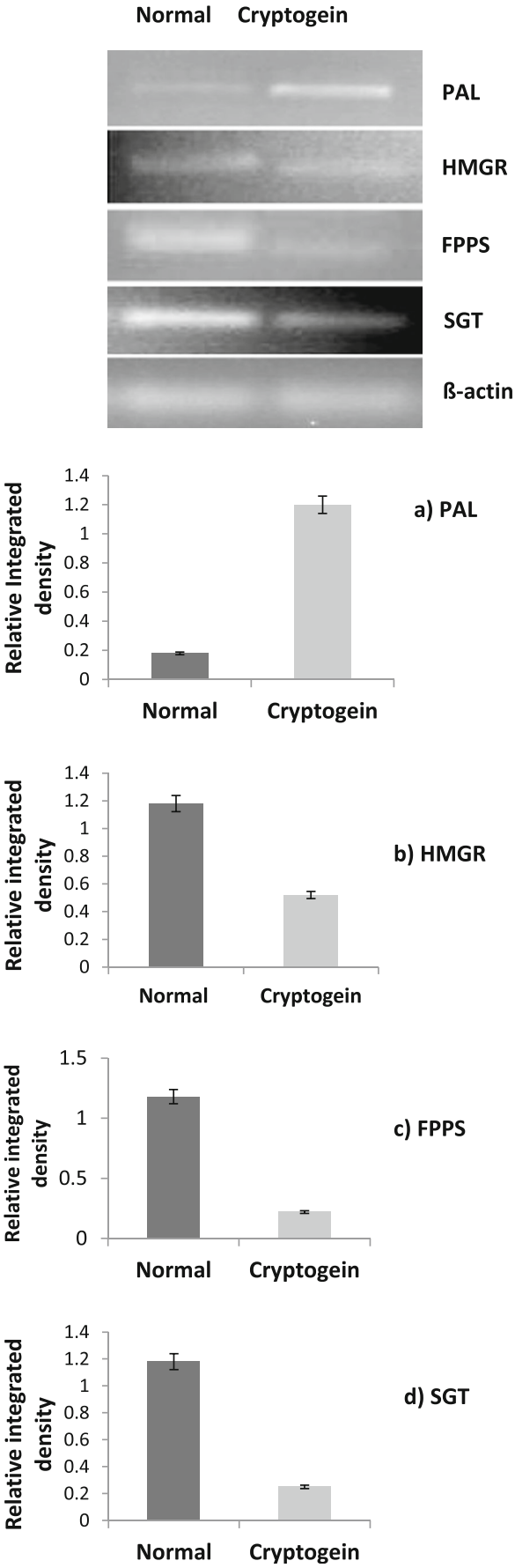
fragment of *PAL*, showing strong homology with other plant PAL available in the public database with amino acid sequence identity of 96 % (Supplementary Fig. 2). Therefore, the resulting sequence was further used for designing gene-specific primers to study the expression of *PAL* in *W. somnifera* hairy roots. Semiquantitative RT-PCR analysis for *PAL* transcript accumulation revealed that the *PAL* transcript accumulation was nearly 6-fold higher cryptogein-cotransformed hairy roots as compared to normal hairy roots (Fig. 8a).

Expression study of *HMGR*, *FPPS*, and *SGT* genes in normal and cryptogein-cotransformed hairy roots

Accumulation of *HMGR*, *FPPS*, and *SGT* transcripts in both cryptogein-cotransformed and normal hairy roots was studied using semiquantitative RT-PCR. Gene-specific primer pairs (Table 1) resulted in amplification of 287-, 324-, and 300-bp fragments of *HMGR*, *FPPS*, and *SGT* transcripts, respectively (Fig. 8b–d). The level of ß-actin served as control for equal RNA template amounts. As compared to normal hairy roots, the accumulation of *HMGR*, *FPPS*, and *SGT* transcripts in cryptogein-cotransformed roots was found to be lower by 2.3-fold, 4.6-fold, and 4-fold, respectively (Fig. 8b–d). Apparently, these low expression levels of *HMGR*, *FPPS*, and *SGT* genes in cryptogein-cotransformed hairy roots of *W. somnifera* supported the reason for such scanty accumulation of withasteroids in these roots as compared to normal hairy roots.

Discussion

Cryptogein-cotransformed hairy root cultures showed a clear suppression of withasteroid accumulation as compared to normal ones. This diminished accumulation of withasteroids which raised question about the possible consequence on other metabolite pathways in both types of hairy root cultures under study. It appears that expression of cryptogein gene in *W. somnifera* has channelized the major carbon pool of withanolide biosynthesis to unknown directions in cryptogein-cotransformed hairy root lines. To investigate for a possible shift metabolites in hairy roots of *W. somnifera* as a result of cryptogein expression, it was therefore necessary to check the total phenolic acid contents in both normal and cryptogein-cotransformed hairy roots. In order to progress further in this line of investigation, it appeared necessary to analyze the results obtained with tobacco cell lines challenged with cryptogein protein as elicitor. Through calcium-mediated process, cryptogein reshaped phenylpropanoid metabolism in tobacco cell lines which culminated in enhanced amount of 5-hydroxyferulate accumulation as compared to untreated ones



◀ **Fig. 8** Expression analysis of *PAL*, *HMGR*, *FPPS*, and *SGT* in normal and cotransformed hairy roots by semiquantitative reverse transcription (RT)-PCR. Total RNA (2 μg) was reversed transcribed and used as template for RT-PCR analysis. Both types of hairy roots used in this study were of same age group. *PAL*, *HMGR*, *FPPS*, and *SGT* transcript levels were normalized on the basis of the actin transcript levels (**a**–**d**)

(Amelot et al. 2011). These findings with tobacco cell lines encouraged us to examine the contents of soluble and cell wall-bound phenolics in both cryptogein-cotransformed and normal hairy root lines.

Cryptogein-cotransformed hairy roots accumulate considerably more amount of wall-bound phenolics (mainly ferulic acid) than the normal hairy roots. A similar trend in soluble phenolic accumulation was observed in cryptogein-cotransformed roots of *C. blumei* (Vuković et al. 2013). Upliftment of ferulic acid accumulation was also reported in tobacco cell lines challenged with cryptogein protein (Amelot et al. 2011). To accumulate such quantity of ferulic acid, it was suggested that a reverse monolignol pathway was operative in cryptogein-elicited tobacco (Amelot et al. 2011).

Autofluorescence study with longitudinal sections of root tissues also indicated the higher deposition of ferulic acid, mostly deposited in the cortical cell wall of cryptogein-cotransformed hairy roots as compared to normal hairy root tissues. Ferulic acid is one of the major hydroxycinnamates deposited in the cell walls. Ferulic acid appears to play a significant role in modifying the mechanical properties of cell

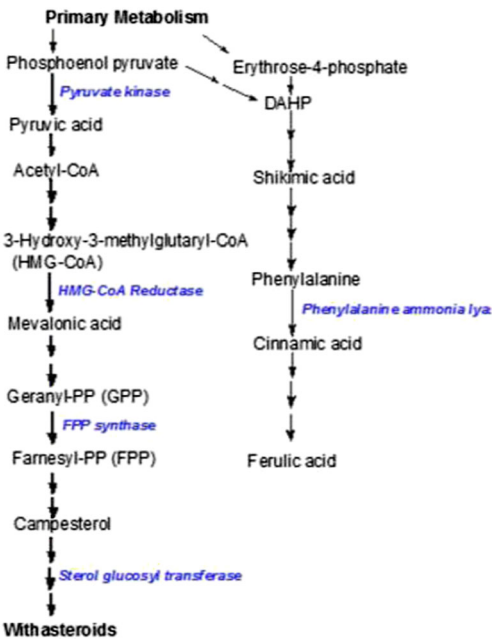


Fig. 9 Shifting of metabolite production from withasteroid biosynthesis (*bold arrows*) to phenylpropanoids (*light arrows*) in cryptogein-cotransformed hairy roots of *W. somnifera*. The targeted enzymes are shown in *italics*

walls by acting as a cross-link between polysaccharides and lignin (Parr et al. 1996).

Guaiacol peroxidase (GPX) mainly plays an important role in lignin biosynthesis and in biotic stress (Gill and Tuteja 2010). GPX reduces H_2O_2 by using aromatic electro donor such as guaiacol. Endogenous elicitor treatments were also shown to enhance GPX activities in different plants (Gill and Tuteja 2010). In our study, GPX activity was found to be much higher in crypt-cotransformed hairy roots as compared to normal hairy roots. This observation appeared consistent with the increased ferulic acid accumulation in cryptogein-cotransformed hairy roots. As ferulic acid originates via phenylpropanoid pathway, an enhancement of GPX activity, in turn, points toward the possible shift of metabolism from terpenoid biosynthesis in cryptogein-cotransformed roots leading to more phenolic accumulation. An analogous trend was recently observed with green hairy roots of *Daucus carota*, where suppressed accumulation of soluble phenolic metabolites correlates well with the reduction of guaiacol peroxidase activities, as compared to normal hairy root lines (Mukherjee et al. 2014).

The activity of PAL was nearly 3-fold higher in cryptogein-cotransformed hairy roots than normal roots (Fig. 7). Plants under stress condition rapidly induced PAL activity for accumulation of phenylpropanoids (Dixon and Paiva 1995). Thus, in cryptogein-cotransformed hairy roots, cryptogein itself acting as internal elicitor and uplifted the phenylpropanoid pathway as compared to normal hairy roots, where cryptogein was absent. Analysis for PAL transcript accumulation revealed that the PAL transcript accumulation was nearly 6-fold higher cryptogein-cotransformed hairy roots as compared to normal hairy roots (Fig. 8a). In an earlier study, upon expression of a heterologous protein of phenylpropanoid chain cleavage in tobacco, the steady-state mRNA levels for the phenylpropanoid pathway enzymes such as PAL were clearly increased, and such increment had a direct consequence of changes in phenylpropanoid metabolite levels (Mayer et al. 2001). In our study, as compared to normal hairy roots, the expressed β-cryptogein protein act as internal elicitor and uplift the accumulation of phenolic acids in cryptogein-cotransformed hairy roots. Thus, increased transcript accumulation of PAL in cryptogein-cotransformed hairy roots was correlated with enhancement of phenolic acid accumulation and PAL activity.

Plastidic isoprenoid biosynthesis initiates from pyruvate and glyceraldehyde-3-phosphate through an enzymatic reaction catalyzes by 1-deoxy-D-xylulose-5-phosphate synthase (DXS). Pyruvate kinase, the final enzyme of glycolysis, converts phosphoenol pyruvate (PEP) into pyruvate with the concomitant production of ATP, and thus occupies one of the most connected nodes in the plant metabolic network (Andre et al. 2007). Generally, the ratio of phosphoenol pyruvate to pyruvate is essential to maintain the flow of several biochemical pathways (Andre et al. 2007). Pyruvate is responsible for synthesis of acetyl-CoA, which in turn leads to the production of 24 methylene cholesterol via squalene pathway route, a precursor required for the synthesis of withasteroids in *W. somnifera* (Lockley et al. 1976). In cryptogein-cotransformed hairy roots, pyruvate kinase activity was reduced by 3-fold than normal hairy roots. This seems plausible as the metabolic response in cryptogein-cotransformed hairy roots turned to more phenylpropanoid accumulation.

HMGR, FPPS, and SGT are the main regulatory enzymes in MVA pathway. Previous study indicated that withanolide biosynthesis probably involves 24-methylene cholesterol as a metabolic intermediate (Glotter 1991). 24-Methylene cholesterol originated from a triterpenoid (C_{30} terpenoid) skeleton of isoprenoid pathway that operates via classical cytosolic mevalonate (MVA) pathway and/or plastidic 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (Chaurasiya et al. 2012). HMGR catalyzes irreversible conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) into MVA that constitutes the key regulatory step of isoprenogenesis leading to the synthesis of IPP and its isomer, DMAPP, the progenitors of homologous series of isoprenoids (Chappell 1995). Most of bioactive molecules from *Withania* are probably synthesized from 24-methylene cholesterol through mevalonate (MVA).

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG) into MVA and constituted the upstream pathway for withanolide biosynthesis in *W. somnifera*. HMGR gene was cloned earlier from the young leaves of *W. somnifera* (Akhtar et al. 2013). A study on HMGR transcript accumulation and withanolide contents was carried out in different chemotype of *W. somnifera*; the levels of HMGR transcript accumulation showed positive correlation with accumulation of withanolide in leaves (Akhtar et al. 2013). In our experiment, as compared to normal roots, cryptogein-cotransformed hairy roots showed low levels of HMGR expression which correlates well with the diminished amount of withasteroid accumulation. Similar outcome was also found with the expression levels of farnesyl pyrophosphate synthase (FPPS) gene.

Farnesyl diphosphate (FPP) is synthesized by catalytic action of the enzyme farnesyl diphosphate synthase (FPPS), which serves as a substrate for first committed reaction of several branched pathways (Chappell 1995). FPPS-catalyzed reaction occurs in two consecutive steps: condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) to form 10-C intermediate geranyl diphosphate (GPP) and condensation of GPP with another molecule of IPP which results into 15-C FPP (Gupta et al. 2011). Overexpression of ginseng FPPS in *Centella asiatica* hairy roots enhanced phytosterol and triterpene biosynthesis (Kim et al. 2010). FPPS gene was cloned earlier from young leaves

of *W. somnifera* (Gupta et al. 2011), and it was observed that the contents of major withanolide accumulation in different chemotype of *W. somnifera* were strongly correlated with the levels of transcript accumulation of FPPS. In our experiment too, reduced accumulation of FPPS correlates well with the low withanolide content in cryptogein-cotransformed hairy roots.

Most of the higher plant sterols possess b-OH group at C-3 (A-ring) and are transformed into their glycoconjugates by sterol glucosyltransferases (SGTs) (Sharma et al. 2007). In plants, sterols are biosynthesized by mevalonate (Dewick 2001) and nonmevalonate pathways (Rohmer 1999). They occur in highly diversified skeletal and structural forms that are finally glycosylated. Some of these (e.g., sitosterol, stigmasterol, brassinosteroids) are ubiquitous in plants while others (e.g., withanolides, limonoids) are highly restricted in occurrence. Glucosylation of sterol is catalyzed by sterol glucosyltransferase. Withasteroids are glucosylated sterols (Mishra et al. 2005). One member of 3β-hydroxy sterol glucosyltransferase (SGT) gene family was cloned recently from *W. somnifera* (Sharma et al. 2007). Differential expression of SGT gene was observed in different plant parts of *W. somnifera*. Elicitor-treated plants showed more SGT transcript accumulation than nonelicited plants (Sharma et al. 2007). Thus, it was anticipated that reduced amount of withasteroid accumulation in cryptogein-cotransformed hairy roots should possess low levels of SGT transcript accumulation. In our experiment, as compared to normal hairy roots, SGT transcript accumulation was 4-fold lower in cryptogein-cotransformed hairy roots.

Carbon flow shifting in secondary metabolism is very difficult, probably due to the complexity of the diverse pathways involved. Our approach was to mimic natural stimuli that cause a predictable change in metabolism, using a gene encoding a natural pathogen elicitor of a plant defense response. The gene was inserted into the host's genome, producing the elicitor protein endogenously. Most stimuli function at the cell's outer surface through signal transduction pathways. In our study, the signal was on the inside that perhaps leaked out and interacted with other cells via their surfaces. This is the first report of this approach, where an effect of the cryptogein gene has been assigned to a shift of carbon from one major pathway to another. This work may lead to new ways of understanding and manipulating secondary metabolism in plants and might be explored as a way to enhance the production of phenolic compounds.

Conclusion

In this study, expression of cryptogein in cotransformed hairy root lines of *W. somnifera* led to reduced accumulation of withasteroids (such as, withaferin A, withanolide A), as compared to normal hairy root lines. A correlation between suppressed expression of selected genes of withanolide biosynthetic pathway and diminished accumulation of withasteroids was evident. However, concomitant enhancement of PAL gene expression, enzyme activity, total soluble phenolics, and wall-bound phenolic acid accumulation was observed in cryptogein-cotransformed lines as compared to normal hairy root cultures. Thus, the potential of cryptogein expression in possible shifting of metabolite accumulation, from terpenoid biosynthesis toward the formation of phenylpropanoids, was indicated (Fig. 9) for the first time in *W. somnifera*-cotransformed hairy root lines.

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Conflict of interest The authors declare that they have no conflict of interest.

Author contribution AM, BS, and CM conceived and designed research. BS did all the experiments. BS and CM analyzed the data. SJ guided the genetic transformation experiments and took the overall responsibility. AM wrote the final manuscript. All authors read and approved the manuscript.

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