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ORIGINAL PAPER

Expression of *Withania somnifera* Steroidal Glucosyltransferase gene Enhances Withanolide Content in Hairy Roots

Vibha Pandey[1] · Rakesh Srivastava[1] · Nehal Akhtar[1] · Jyoti Mishra[1] · Pratibha Mishra[1] · Praveen C. Verma[1]

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Abstract In *Withania somnifera*, sterol molecules of immense medicinal value are diversified by means of glycosylation. Identifying sterol glycosyltransferases provides an imperative insight of diverse sterol modifications, thereby helping to comprehend the underlying plant mechanisms. In the present study, one of the *W. somnifera* sterol glycosyltransferase-4 (*Ws-Sgtl4*) gene was transformed into the *W. somnifera* leaf explant through *Agrobacterium rhizogene*. Transformed *W. Somnifera Ws-Sgtl4* leaf explants were subjected to hairy root induction and analyzed for biomass accumulation. The analysis of *Ws-Sgtl4* gene expression was performed at different time exposures with the application of salicylic acid and methyl jasmonate. The elicitation of *W. somnifera* hairy root expressing the *Ws-Sgtl4* gene was also evaluated for the enhancement if any, in the total withanolide yield as well as the withanolides-A contents. The results suggested that *Ws-Sgtl4* gene expression enhanced the production of total withanolide yield and withanolides-A in the hairy root culture of *W. somnifera* in the response to the elicitors.

Keywords Sterol glycosyltransferases · Withanolides · Stress · Gene expression · Hairy root culture

Vibha Pandey and Rakesh Srivastava contributed equally to this work.

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✉ Praveen C. Verma
praveencverma@yahoo.com

[1] Plant Molecular Biology and Genetic Engineering Division, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow 226001, Uttar Pradesh, India

Introduction

Withania somnifera (L.) Dunal is an important Ayurvedic and indigenous medicinal plant. In the interest of its wide-ranging therapeutic prospective, it has also been the issue of extensive modern scientific and clinical studies (Khedgikar et al. 2013). Withanolide biosynthesis terminates with biochemical conjugative reactions like glycosylation and acylation. Notably, more than 80 metabolites were obtained from metabolic profiling of *W. somnifera*, comprising alkaloids, withanolides, and several sitoindosides (Chatterjee et al. 2010). Among these metabolites, withanolides are a group of naturally occurring C28-steroidal lactone triterpenoids (Chaurasiya et al. 2012; Mirjalili et al. 2009). Glycosylation of withanolides is brought about by glycosyltransferases, which constitute a superfamily of enzymes that catalyses the conjugation of carbohydrate moieties to oligo/polysaccharides, proteins, lipids, terpenoids, flavanoids, alkaloids, and other small molecules (Bowles et al. 2006). Most of the higher plant sterols possess β-OH group at C-3 and are transformed into their glycosterols by sterol glucosyltransferases (SGTs) (Grunwald 1974; Madina et al. 2007a, b). Glycosylation of withanolides results in glycowithanolides with enhanced medicinal properties (Bhattacharya et al. 1997, 2001; Mote et al. 2010).

The regulation of gene expression is an exceedingly multilayered and regulated process being modulated by various factors such as light, elicitors, development, and many other environmental cues (Srivastava et al. 2014a, b). Additionally, this regulation of gene expression is also coupled with many cellular processes. Importantly, elicitors regulated gene expression induces many signaling and metabolic pathways through several intracellular signaling cascades (Srivastava et al. 2014b; Thakur and Sohal 2013). The elicitor treatment to plant cells or organs

is one of the most effective strategies for improving in vitro secondary metabolite production (Sivanandhan et al. 2013; Smetanska 2008). Intriguingly, secondary metabolites from root extract of *W. somnifera* have been used in various drug formulations (Khedgikar et al. 2013; Mohan et al. 2004; Mulabagal et al. 2009; Rai et al. 2003; Winters 2006). Roots are reported to possess a diverse number of glycosterols with much higher content than other tissues. Hairy roots can be a good alternative for the extraction of withanolides. Normally, wild grown plants of *W. somnifera* are the only source of withanolides in order to produce such an important medicinal compound. Genetic engineering techniques involving hairy root culture can be considered as a putative way for facilitating the production of withanolides with elevated volumes. The main objective of our study is to enhance the production of withanolides using hairy root culture by expressing *W. somnifera Ws-Sgtl4* (Chaturvedi et al. 2012; Tuli and Sangwan 2009). The expression profile of the *Ws-Sgtl4* gene in over expressed transgenic hairy root line and its physiological modulation by elicitation has been examined in comparison to the control hairy root line. In this study, the expression of *Ws-Sgtl4* leads to increase the production of total withanolide yield and withanolide-A contents in transformed hairy roots.

Material and Methods

Phylogenetic Analysis of Ws-SGTL4 Protein

The domain organization of representative *W. somnifera* sterol glycosyltransferases proteins was set up using conserved domain database (Marchler-Bauer et al. 2003). The phylogenetic relationship of Ws-SGTL4 proteins with various members of sterol glycosyltransferases of the dicot plants was established. To examine the phylogenetic relationship of Ws-SGTL4 proteins, an un-rooted tree was constructed from the alignments of the full-length protein sequences along with sterol glycosyltransferases proteins of dicots, monocots, tracheophyte, and bryophyta. The obtained sequence alignments were used as an input to construct a phylogenetic tree with the neighbor-joining algorithm within MEGA5.0 (Tamura et al. 2011).

Cloning of *W. Somnifera Ws-Sgtl4* Gene

The in vitro plants of *W. somnifera* were obtained from the germplasm that is maintained in experimental plot of CSIR-National Botanical Research Institute, Lucknow, India. Gene-specific primers for *Ws-Sgtl4* (EU342374), (Supplementary Table 1) were designed and used for PCR and RT-PCR analysis (Pandey et al. 2015). The RT-PCR products were electrophoresed on 1 % agarose gel. The resolved fragments were purified and cloned into pBluescript SK^+ vector following standard protocols (Sambrook et al. 1989). The expression cassette was constructed using CaMV35S with double enhancer promoter and *Ws-Sgtl4* gene in the destination vector pYL436 (GenBank accession numbers AY737283). *Agrobacterium rhizogenes* A4 (Courtesy Dr. David Tepfer, France) strain was transformed with expression cassette through electroporation (Singh et al. 2015; Verma et al. 2002, 2007). The wild and transformed strains of bacteria were grown for 48 h in dark at 25± 2 °C. The bacterial suspension cultures were raised by inoculating a single bacterial colony in 10-ml liquid YMB medium with spectinomycin (100 mg l^{-1}; for bacterial selection) for transforming *A. rhizogenes* strain and was grown for 12 h at 25±2 °C on a gyratory shaker at 220 rpm and used for infecting the explants (Hooykass et al. 1977).

Establishment and Maintenance of the Test Plant System

The shoots of *W. somnifera* were maintained in vitro through apical and axillary bud multiplication on MS medium (Murashige and Skoog 1962) supplemented with 2 mg l^{-1} BAP and 0.1 mg l^{-1} IAA and served as the explant source for all future transformation experiments. The cultures were maintained at 25±2 °C under controlled humidity and 16-/8-h light/dark period. Light of 60 µmol photon m^{-2} s^{-1} was supplied by fluorescent tube. Sub-culturing was generally done at 3-week intervals.

Hairy Root Induction and Maintenance of Transformed Hairy Roots

Leaves of in vitro grown shoots of *W. somnifera* were used as explant. Transformation of *Ws-Sgtl4* gene in the 3rd and 4th leaves from the tip of the shoots was performed with sterile needle dipped in the bacterial suspension of *A. rhizogenes* strain A4 using a protocol of Verma et al. (2007), with required modifications (Verma et al. 2007). Fully expanded leaves were infected individually with transformed (carrying *Ws-Sgtl4* gene) and wild type *A. rhizogenes* strain A4 in separate sets of 7–10 explants. All the infected explants were co-cultivated with bacteria on solid, hormone-free MS medium with 3 % sucrose, and 0.8 % agar and incubated at 25±2 °C, in dark. The leaves were pricked with a sterile needle without the bacterial suspension, which were treated as controls and were cultured under identical conditions. After co-cultivation period of 3 days, the infected as well as control explants were transferred to the selection media (MS medium supplemented with the 250 mg l^{-1} augmentin and 50 mg l^{-1} gentamycin). The cultures were examined up to 1 month for the emergence of hairy roots at the site of inoculation. The resultant hairy root

clones were excised individually from the explants upon attaining 1–1.5 cm length and were sub cultured on MS media of half strength as well as liquid B5 medium (Gamborg et al. 1968).

Detection of Transgenes in *Agrobacterium*-Induced Hairy Roots

Genomic DNAs were isolated from non-transformed and transformed hairy root culture using DNeasy Plant Mini Kit (Qiagen). Isolated DNA was used to detect presence of transgenes transferred through T-DNA into genomic DNA. Gene-specific primers were used in PCR analysis to screen the presence of *Ws-Sgtl4*, *Adgt* (amino adenosylglycosyltransferase), and *Rol C* genes (Supplementary Table 1) in different transgenic and non-transgenic hairy roots.

Growth Index Analysis

The growth index of hairy root clone was performed according to earlier published work (Verma et al. 2002). The growth performance of the best growing transgenic hairy roots of *W. somnifera* was determined (in triplicate) at 3rd, 4th, and 5th weeks interval of culture following the formula:

$$\textbf{Growth index}\ (\textbf{G.I.}) = (\textbf{Final fresh weight} - \textbf{Initial fresh weight}) / \textbf{Initial fresh weight}$$

The root tissues from three replicated flasks were harvested individually, washed thoroughly with distilled water to eliminate the traces of the medium, blotted on filter paper, and their fresh weights were determined. They were then air dried/ oven dried at 40 °C and kept in vacuum desiccators for uniform drying and dry weight determination.

Elicitor Treatment on Biomass and Withanolides Productivity in Hairy Root Culture

Methyl Jasmonate (MeJA) of 100 μM and salicylic acid (SA) of 1 mM (Sigma, St. Louis, USA) were used for elicitation studies. The flasks containing 50-ml half strength MS liquid culture medium with B5 vitamins were inoculated with 0.18 g fresh weight (FW) of hairy roots from 10 days old cultures. Elicitors were added to the hairy root culture after 21 days of inoculation. The same quantity of sterile distilled water was added in the culture and used as control. The flasks were then incubated at 25±2 °C on a continuous shaker (90 rpm) under a 16-/8-h photo period and harvested at 0, 3, 6, 9, 12, 24, and 48 h after elicitor treatment. At the termination of the experiment, the fresh weight was determined after the roots were washed in deionized sterile water to remove the traces of the medium and blotted dry between layers of absorbent paper in aseptic condition. Blotted dried hairy roots were subjected to comparative gene expression analysis. The dried root samples were ground to form a powder and subjected to secondary metabolite analysis through the extraction and quantitative estimation of the desired metabolites (Chaurasiya et al. 2008).

RNA Extraction, RT-PCR, and Real-Time PCR Analysis

Total RNA was isolated from the hairy roots, after DNase (Ambion) treatment, the integrity of RNA was tested by electrophoresis. Two microgram of total RNA was used for first-strand cDNA synthesis using the Superscript II RT kit (Invitrogen). Expression profiles of *Ws-Sgtl4* under normal and elicitor treatment were studied using real-time PCR. Real-time PCR was carried out by using ABI's 7500 Fast Real-time PCR machine. Gene-specific forward and reverse primers were designed by using ABI-Primer express v2.0 software (Table S1). The transcripts were normalized with regard to Ubiquitin (AJ309010) transcript that served as an endogenous control. At least two to three independent biological replicates and three technical replicates of each biological replicate were made for real-time PCR analysis. The relative expression levels were analyzed using ΔΔCt method, and the data were analyzed statistically.

Estimation of Total Withanolide Yield

In order to extract the total withanolide yield, the dried tissue (hairy roots 4.0 g) was finely powdered in liquid nitrogen and extracted overnight in 20 ml of methanol: water (25:75, *v/v*) at room temperature (25 °C) on a rocking platform and filtered. The filtrate was collected and the residue was extracted twice at 4 h intervals with the same volume of extractant. The filtrates were pooled and extracted with *n*-hexane (3×60 ml). The *n*-hexane fraction was discarded, and the methanol:water fraction was further extracted with chloroform (3×60 ml). The chloroform fractions were pooled and concentrated to a dry powder. The weight of this dried extract was recorded as crude extract weight. Dried crude extracts (10 mg) of control and elicitor-treated *Ws-Sgtl4* expressing hairy roots were dissolved in HPLC-grade methanol (1.0 ml), filtered through a Millipore (Bangalore, India) sample clarification kit (Millex GV; 13 mm, 0.22 μm), and subjected to HPLC analysis.

Extraction of Withanolide-A from Hairy Roots and Quantification Using HPLC Analysis

Quantification of withanolide-A by expression of *Ws-Sgtl4* in transgenic hairy roots as compared with control non-transformed hairy root clone was determined by HPLC. Extraction of withanolides from dried hairy roots (4 g) was performed following steps as described (Chaurasiya et al. 2008). Dried crude extract (10 mg) was dissolved in 2 ml of methanol and used for HPLC analysis after filtration through

0.22-μm nylon filter. The solution was further diluted to tenfolds and used for HPLC analysis. HPLC–PDA analysis was performed on LC-10 system (Shimadzu, Kyoto, Japan) using ($CHCl_3$ fraction) hairy root extract. The separation was carried out using waters reverse phase column (Nova-Pak, 4 μm, 3.9× 150 mm) and binary gradient elution at 27 °C. The gradient and composition of solvent system along with flow rate or run time for the quantitative estimation of withanolide-A were set according to established protocol (Chatterjee et al. 2010; Chaurasiya et al. 2008). The chromatograms were recorded at 227 nm after injection of 20 μl of extract. Standard of withanolide-A was obtained from Chromadex Inc. (Laguna Hills, CA, USA).

Results

Molecular Evolution Analysis of Ws-SGTL4

The present study was intended to comprehend the impact of sterol glycosyltransferases on the production of secondary metabolite under the influence of defense and stress signaling elicitor molecules. *Ws-Sgtl4* is not predominately expressed in root unlike *Ws-Sgtl1* gene (Chaturvedi et al. 2012; Tuli and Sangwan 2009). Notably, similar to Ws-SGTL1 proteins, Ws-SGTL4 also comprises the conserved domains of glycosyltransferases GT-B family (Fig. S1). Interestingly, phylogenetic analysis showed that Ws-SGTL4 protein was present in same clade with *Solanum lycopersicum* (XP_004237491), *Ricinus communis* (XP_002512608), and *Arabidopsis* sterol glucosyltransferase UGT80A2 (At3g07020; NP_850529), whereas Ws-SGTL1 and *Arabidopsis* UGT80B1 (AT1G43620; NP_001077674) were present in different clade (Fig. S1). The protein similarity between *Arabidopsis* UGT80A2 and Ws-SGTL4 protein is 74 %, whereas similarity between Ws-SGTL4 and Ws-SGTL1 is 59 % (Fig. S2). Thus, Ws-SGTL4 has a glycosyltransferases domain, no expression in root, slight difference properties at the sequence level, and different evolutionary prospect from Ws-SGTL1, which marks Ws-SGTL4 as a potential candidate to study the role of sterol glycosyltransferases on secondary metabolite production in root.

Development of Transgenic *W. Somnifera* Hairy Roots Expressing *Ws-Sgtl4*

The transgenic *W. somnifera*, expressing *Ws-Sgtl4* cDNA under the control of CaMV 35S promoter, was developed through *A. rhizogenes*-induced hairy roots. *W. somnifera* leaf explants were used for hairy root induction. The optimum time for the maximum emergence of hairy roots from the leaf explants was between 3rd and 4th week. *A. rhizogene* infected hairy roots were subjected to selection media, and individual root clones were obtained. On the basis of growth and profuse branching on semi solid medium, one non-transgenic wild type hairy root clone (A4nt) and five different transgenic hairy root clones (*Ws-Sgtl4*), namely T1 to T5, were selected for further growth index analysis.

Transgenic Analysis *Ws-Sgtl4* in Hairy Roots

To confirm the integration of T-DNA from the *A. rhizogene* into the hairy root, isolated genomic DNA from transformed hairy roots was subjected to PCR analysis using primers specific to the *Adgt*, *Rol* C and *Ws-Sgtl4* genes. PCR amplification confirms the presence of 750, 930, and 2096-bp gene fragment in

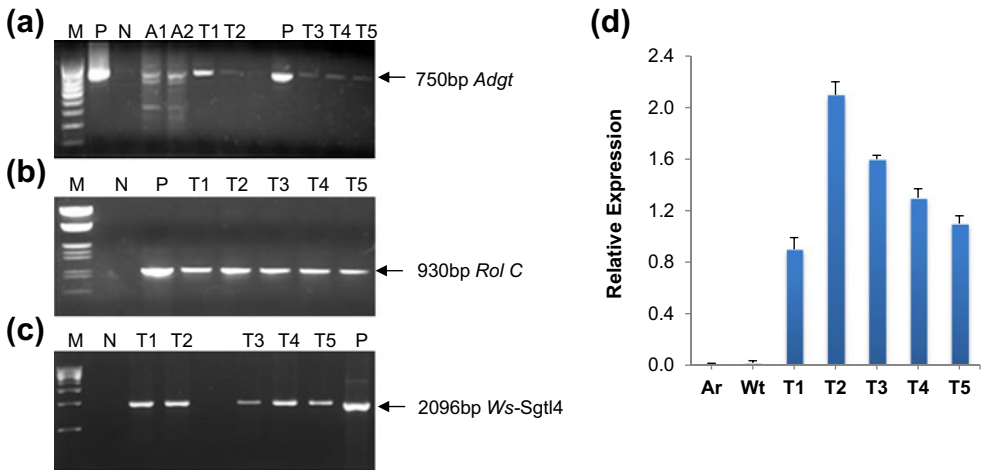

Fig. 1 Transgenic analysis *Ws-Sgtl4* in the hairy root of *W. somnifera*. **a**–**c** PCR analysis of *Adgt*, *Rol C*, and *Ws-Sgtl4* genes from transformed hairy *W. somnifera* roots; *M* Marker, *N* negative sample, *P* positive control, T1–T5: randomly selected transgenic hairy root lines expressing *Ws-Sgtl4*, A1-A2 transformed clones of *A. rhizogenes* showing 750 bp band of *Adgt* gene after amplification. **d** Expression analysis of *Ws- Sgtl4* gene from control adventitious root (Ar), wild type hairy root (Wt), and *Ws-Sgtl4* transformed hairy roots (T1-T5) clone through real-time PCR. The values above the bars indicate the standard error from the means of two independent experiments and statistically significant differences ($P<0.05$)

the case of *Adgt*, *Rol C*, and *Ws-Sgtl4* genes, respectively, in transformed hairy roots of *W. somnifera* in comparison to the absence of *Adgt* and *Ws-Sgtl4* genes in non-transformed control roots (Fig.1a–c). The expression profile of *Ws-Sgtl4 gene* in transgenic hairy root lines as compared to non-transgenic hairy roots was obtained through quantitative real-time PCR. The gene expression of *Ws-Sgtl4* was significantly high in transgenic hairy roots culture as compared to relatively no expression in the wild type control and adventitious roots clone culture (Fig. 1d).

Growth Index and Biomass Analysis of Hairy Root Clones Expressing *Ws-Sgtl4*

The comparative growth index was determined in different transgenic hairy root clones during the next set of experiments. All the selected hairy roots showed the typical phenotypes of hairy roots, such as rapid growth and plagiotropism. The non-transformed hairy root clone (A4nt) and five selected transformed hairy root clones (T1-T5) were further transferred to liquid medium to study their growth characteristic during different growth phases, i.e., 3rd, 4rth, and 5th weeks (Fig. 2a–c). Though the morphological appearance of these clones was quite similar initially on the semi-solid medium, but they behaved differently in liquid medium. A gradual increase in fresh and dry weight up to 4th week of culture could be noted with all the hairy root clones, amongst which clones (T1) continued to grow even further up to 5th week of culture while the rest four (A4nt, T2, T4, and T5) exhibited a decline in their growth after the 4th week of culture and clone T3 was at a saturation level after the 4th week of culture. However, the fresh weight remained same (Fig. 2a–b). Clone number T2 could be distinguished from the rest four by increased lateral branching and healthy appearance and attained a maximum 29.64 GI at 4th week followed by that of the T4 and T3 over the same period of time (Fig. 2c). The non-transformed roots also grew healthy and attained a maximum of 22.6-fold increase in biomass on the 4th week of culture (Fig. 2c).

Influences of *Ws-Sgtl4* Expression on Secondary Metabolite Production at Different Growth Phases

The five selected hairy root clones and non-transformed control roots were analysed to estimate total withanolide yield, which was determined in the form of crude extracts produced during three different growth phases, i.e. 3rd, 4rth, and 5th weeks. In the selected hairy root lines (T1–T5), the synthesis of total withanolide in the form of crude extract was found to be correlated to growth. The maximum yield of the total withanolides coincided with the exponential growth phase. The optimal level of total withanolide content of the clones T2 and T3 was recorded on the 4th week of culture, which was higher than the maximum of non-transformed roots noted on its 5th week of culture. Two of the selected hairy root lines, T2 and T3 exhibited distinct superiority over other lines both in terms of growth index and crude metabolite (total withanolides) productivity, were subjected to more detailed examinations of elicitation and production profiles along with the gene expression analysis.

Effect of the Elicitor Treatment on *Ws-Sgtl4* Gene Expression in Transgenic Hairy Roots

The defense and stress molecules SA and MeJA are known to modulate many physiological events in plants including

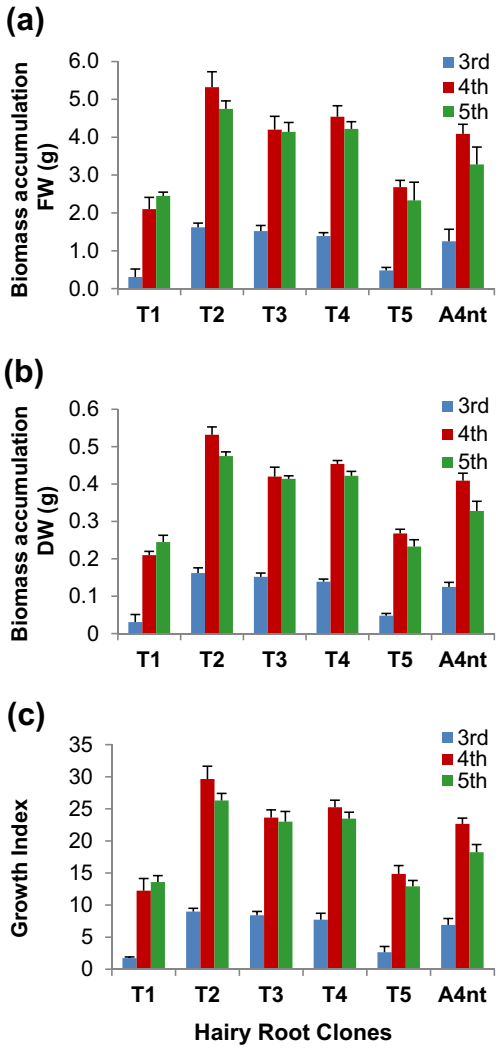

Fig. 2 Growth kinetics and biomass accumulation of different clones of hairy root cultures of *W. somnifera*. Comparative analysis of the biomass yields growth index (GI) production potential of five independent hairy root clones (T1–T5) as compared to the non-transformed (A4nt) in vitro grown control roots during three different growth (3rd, 4th, and 5th weeks) phases. **a** Biomass accumulation fresh weight (FW), **b** biomass accumulation dry weight (DW), **c** growth index. Values are the mean of triplicate results and error bars show standard deviations statistically significant differences ($P<0.05$)

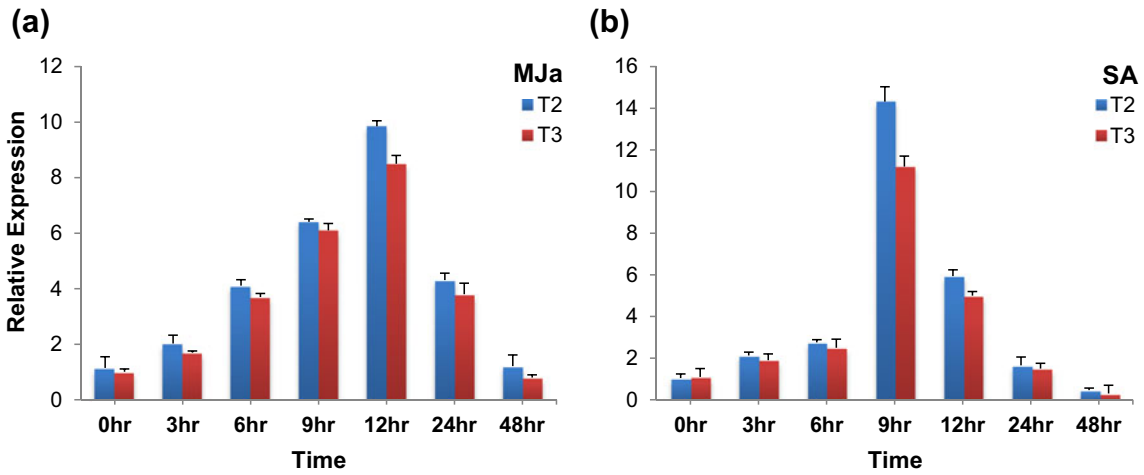

Fig. 3 Expression analysis of *Ws-Sgtl4* gene in hairy root clones T2 and T3 of *W. somnifera*. **a** Effect of MeJA and **b** effect of SA on *Ws-Sgtl4* gene expression at different intervals of time. The values above the bars indicate the standard error from the means of two independent experiments

defense responses by affecting the expression of several genes and are ultimately responsible for the biosynthesis of secondary metabolites (Kim et al. 2011; Srivastava et al. 2014b; Sun et al. 2011). The native *Ws-Sgtl4* gene is not expressed in roots of *W. somnifera*, which was confirmed by the real-time PCR analysis of field grown root (Tuli and Sangwan 2009). The effect of SA and MeJA on *Ws-Sgtl4* gene expression was analyzed at 0, 3, 6, 9, 12, 24, and 48 h after 4 weeks of culture, in two selected hairy root clones T2 and T3 of *W. somnifera*. After the application of MeJA, the transcript level of *Ws-Sgtl4* in clones T2 and T3 increased gradually and reached maximum up to 9.9- and 8.5-folds at 12 h (Fig. 3a), while in response to SA, transcript level of *Ws-Sgtl4* started increasing up to 14.3- and 11.2-folds at 9 h and then started declining, respectively (Fig. 3b). These results suggested that expression of *Ws-Sgtl4* in hairy roots increases with the application of the elicitors MeJA and SA.

Effects of Elicitor Treatment and Expressing *Ws-Sgtl4* on Withanolides Productivity in Hairy Root Culture

The effects of elicitors on the total withanolide production potentials at the optimum culture period of 4th week on the selected T2 and T3 hairy root clones of *W. somnifera* were investigated at 0, 3, 6, 9, 12, 24, and 48 h. Treatment of MeJA affects the production of total root withanolide accumulation increased after 3 h, reached to maximum after 12 h, and declined thereafter. In case of SA treatment, total root withanolide accumulation augmented after 3 h and reached a maximum at 9 h and then reduced subsequently. Production of withanolide-A after MeJA and SA treatment was also analyzed in T2 and T3 hairy root clones of *W. somnifera* at 0, 3, 6, 9, 12, 24, and 48 h. Results showed that the production of withanolide-A increases up to approximately 23- and 20-folds with the treatment of MeJA, whereas in the case of SA,

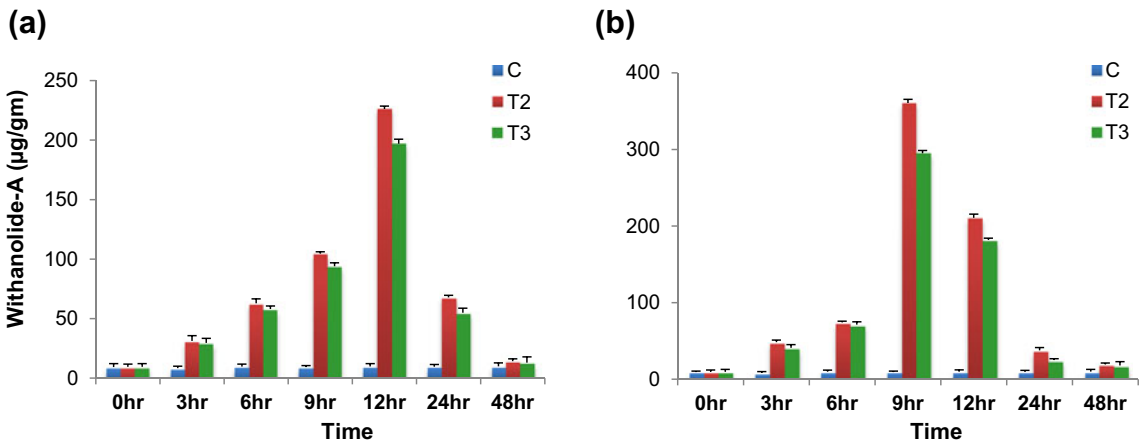

Fig. 4 Effects of withanolides-A production in T2 and T3 hairy root clones expressing *Ws-Sgtl4* gene. **a** Effect of MeJA and **b** effect of SA on withanolides-A production at different intervals of time in transgenic hairy roots as compared with the control (**C**) non-transgenic hairy root. The values above the bars indicate the standard error from the means of two independent experiments

withanolide-A increases up to about 39- and 32-folds in T2 and T3 hairy root clones as compared with the control non transgenic hairy root (Fig. 4). These results indicated that the effect of *Ws-Sgtl4* and the application of defense molecules MeJA and SA in hairy roots increase with production of total withanolide yield and withanolide-A.

Discussion

In the bio-production of several secondary metabolites and therapeutic molecules, transgenic hairy roots have already shown significant potential (Singh et al. 2014, 2015; Verma et al. 2002; Zhou et al. 2011). Hairy root culture of *W. somnifera* has already been reported to enhanced production of withanolides (Bandyopadhyay et al. 2007; Mirjalili et al. 2009; Murthy et al. 2008) and different factors like hormones, carbohydrates as well as elicitation of MeJA and SA also enhance withanolides production in *W. somnifera* (Doma et al. 2012; Sivanandhan et al. 2012a, b, 2013). Withanolide-A production in *A. rhizogene*-infected *W. somnifera* was found elevated with the elicitor treatment as hairy root cultures are well-known to upregulate the levels of secondary metabolites in several plant species like *Picrorhiza kurroa* (Verma et al. 2002), *Catharanthus roseus* (Vazquez-Flota et al. 2009), *Panax ginseng* (Kim et al. 2009; Zhou et al. 2007), *Pueraria candollei* (Udomsuk et al. 2011), *Glycyrrhiza glabra* (Shabani et al. 2009), and *Salvia sclarea* (Kuzma et al. 2009).

The various elicitors, such as SA and MeJA, have been shown to affect the metabolic flux in diverse metabolism pathways, including the biosynthetic pathways of secondary metabolites and production of ATP. The regulation of biosynthetic activity pathway by the elicitors is governed by an enhancement in biosynthesis-related enzyme activities and upregulation of the gene expression encoding these enzymes, which ultimately yields to the enhanced production of secondary metabolite and plant natural products (Ruiz-May et al. 2011; Suzuki et al. 2005). The fold increase in metabolite flow in the 'activated' pathway, in the form of 'metabolic flux', is estimated quantitatively for understanding the regulatory mechanisms of elicitors for secondary metabolite pathway. The activation and inactivation of the conversion process are expressed, as an increase and decrease of flux values. The SA and MeJA are reported to modulate many physiological events in higher plants, including defense responses by affecting the transcription of several genes and are ultimately responsible for the biosynthesis of secondary metabolites and adaptation of plants to biotic stress (Kim et al. 2009; Martin et al. 2002; Rivas-San Vicente and Plasencia 2011; Srivastava et al. 2014b; Sun et al. 2011). In addition, Mizukami et al. (1993) reported that JA and MeJA have been involved in a signal transduction pathway that induces particular enzymes to catalyze biochemical reactions to form defense compounds of low molecular weight in plants, such as polyphenols, alkaloids, quinines, terpenoids, and polypeptides (Mizukami et al. 1993).

Many studies suggest that introduction of transgenes can interact with homologous genes effect the expression of both genes or inactivation of transgenes (Hart et al. 1992; Napoli et al. 1990; Smith et al. 1990). Notably, this homology-mediated effect or inactivation can be transcriptional or post-transcriptional level (Matzke et al. 2002; Meyer and Saedler 1996). Nevertheless, the expression of *Ws-Sgtl4* was enhanced under the influence of (MeJA and SA) elicitation and similarly, withanolide production also found to be enhanced during these elicitor treatments. This higher expression of *Ws-Sgtl4* may lead to change the cellular and physiological metabolisms and affects the production of withanolides in hairy roots. In the case of another medicinal plant *P. ginseng*, Kim et al. (2009) reported that MeJA elicitation induces the glucosyltransferase gene expression of EST IDs PG07020C06, PG07025D04, and PG07029G02 in hairy roots (Kim et al. 2009). The expression of an important gene of sterol pathway, squalene synthase gene has also been reported to accumulate several phytosterols in adventitious transgenic roots of *Bupleurum falcatum* L., where the expression of squalene synthase as well as quantity of phytosterols were found to be further upregulated after MeJA elicitation (Kim et al. 2011). Altogether, one interesting strategy to boost biosynthesis of plant natural bioactive molecules is the expression of biosynthetic gene and use of external compounds, which are able to mimic in vitro effect of physical stresses and force plant cells to synthesize secondary metabolites. As evident from the results no *Ws-Sgtl4* expression was in normal wild type roots, therefore, hairy roots expressing sterol glycosyltransferases serve as a good system to enhance the secondary metabolite production.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no competing interests.

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