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ARTICLE *in* PLANTA · JUNE 2014

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ORIGINAL ARTICLE

*WsSGTL*1 gene from *Withania somnifera*, modulates glycosylation profile, antioxidant system and confers biotic and salt stress tolerance in transgenic tobacco

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Received: 9 October 2013 / Accepted: 7 February 2014

Abstract Glycosylation of sterols, catalysed by sterol glycosyltransferases (SGTs), improves the sterol solubility, chemical stability and compartmentalization, and helps plants to adapt to environmental changes. The SGTs in medicinal plants are of particular interest for their role in the biosynthesis of pharmacologically active substances. *WsSGTL*1, a SGT isolated from *Withania somnifera*, was expressed and functionally characterized in transgenic tobacco plants. Transgenic *WsSGTL*1-*Nt* lines showed an adaptive mechanism through demonstrating late germination, stunted growth, yellowish-green leaves and enhanced antioxidant system. The reduced chlorophyll content and chlorophyll fluorescence with decreased photosynthetic parameters were observed in *WsSGTL*1-*Nt* plants. These changes could be due to the enhanced glycosylation by *WsSGTL*1, as no modulation in chlorophyll biogenesis-related genes was observed in transgenic lines as compared to wildtype (WT) plants. Enhanced accumulation of main sterols like, campesterol, stigmasterol and sitosterol in glycosylated form was observed in *WsSGTL*1-*Nt* plants. Apart from these, other secondary metabolites related to plant's antioxidant system along with activities of antioxidant enzymes (SOD, CAT; two to fourfold) were enhanced in *WsSGTL*1-*Nt* as compared to WT. *WsSGTL*1-*Nt* plants showed significant resistance towards *Spodoptera litura* (biotic stress) with up to 27 % reduced larval weight as well as salt stress (abiotic stress) with improved survival capacity of leaf discs. The present study demonstrates that higher glycosylation of sterols and enhanced antioxidant system caused by expression of *WsSGTL*1 gene confers specific functions in plants to adapt under different environmental challenges.

Keywords Adaptation · Antioxidant · Glycosylation · Environmental stress · Secondary metabolites

Abbreviations

NTPH	*Nicotiana tabacum* cv. Petit Havana
UGT	Uridine diphosphate glycosyltransferase
*WsSGTL*1	Sterol glucosyltransferase gene (Clone1) of *Withania somnifera*
*WsSGTL*1-*Nt*	*WsSGTL*1 expressing transgenic plants of *Nicotiana tabacum*
WT	Wild type

Introduction

Withania somnifera (Family Solanaceae), also known as winter cherry, Indian ginseng and 'Ashwagandha', is widely used in traditional medicine systems of Ayurveda and Unani. All parts of this plant like root, stem, leaves, fruits and seeds possess diverse pharmacological properties, therefore, are used in medicinal formulations since ancient Ayurvedic era. Therapeutic aspects of *W. somnifera* include antioxidant, anti-stress, anti-tumour, hepatoprotective, immunomodulatory, anticonvulsant, antiproliferative, cardioprotective, radio-sensitizing, hypoglycaemic, diuretic, hypocholesterolemic, thyroid stimulant, adaptogenic and antiosteoporotic properties (Mishra et al. 2000;

Electronic supplementary material The online version of this article (doi:10.1007/s00425-014-2046-x) contains supplementary material, which is available to authorized users.

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Published online: 08 March 2014

Kulkarni and Dhir 2008; Sharma et al. 2011; Ahlawat et al. 2012; Alam et al. 2012; Uddin et al. 2012; Ihsan-ul-Haq et al. 2013; Khedgikar et al. 2013). Metabolic profiling of roots and leaves of *W. somnifera* identified more than 80 compounds from *W. somnifera* including alkaloids and steroids like withanone, withanosides, withaferins, withanolides, sterol glycosides, acyl steryl glucosides, etc. (Chatterjee et al. 2010; Chen et al. 2011; Sharma et al. 2011; Chaturvedi et al. 2012).

Withanolides (C-28 steroidal lactones) are the most important pharmaceuticals obtained from *Withania* spp. These are synthesized from isoprenoids (triterpenoid pathway intermediates) via isoprenogenesis using isopentenyl diphosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP; Gupta et al. 2013a, b). In plants, isoprenoid synthesis is governed either by cytoplasmic mevalonate (MVA) pathway or by non-mevalonate/DOXP/MEP pathway in plastids, resulting in the biosynthesis of 24-methylene cholesterol (C-30 terpenoid) (Chaturvedi et al. 2011; Chaurasiya et al. 2012). Various biochemical reactions including oxidation/reduction or transfer of different moieties on 24-methylene cholesterol lead to withanolide synthesis in a tissue-specific manner. Enzymes which catalyse specific withanolide biosynthesis belong to cytochrome P450s (CYP450s), glycosyltransferases (GTs) and methyltransferases (MTs) gene families. Conversion of 24-methylene cholesterol to withanolide A, withanolide D, withaferin A and withanone is catalysed by CYP450s through oxidation/hydroxylation. Biosynthesis of glycosylated forms of these steroidal lactones, withanosides and sitoindosides requires enzymatic action of GTs (Gupta et al. 2013b).

Glycosylation is the most important mechanism transferring sugar moieties from sugar donors, catalysed by ubiquitous GTs, in all living organisms. Among 94 classes of reported GTs (http://www.cazy.org/), GT1, the largest family in plants, is also called uridine diphosphate glycosyltransferases (UGTs) as members of this family use activated UDP-sugar donor. Family 1 GTs contain the Plant Secondary Product Glycosyltransferase (PSPG) motif and a conserved UGT-defining motif near C-terminal end and glycosylate secondary metabolites of plants. UDP-glucose, UDP-xylose, UDP-arabinose, UDP-galactose, UDP-rhamnose and UDP-glucuronic acid are the major identified activated sugar donors for this family. Lipophilic acceptor molecules are glycosylated at –OH, –COOH, $–NH_2$, –SH, C–C groups, etc. through single or multiple glycosylation (Chaturvedi et al. 2011; Kim et al. 2013; Veljanovski and Constabel 2013).

Sterol glycosylation carried out by sterol UDP-glycosyltransferases (SGTs) confers important biological functions and regulates bioactivity of molecules by serving cellular homeostasis, lipid metabolism, enhanced water solubility, molecule stability and modified properties of cell membrane to enable transport system access, stress tolerance and host defence against pathogens and developmental events by altering its movement within the cell (Jones and Vogt 2001; Caputi et al. 2012; Shimamura 2012). Till date, hundreds of UDP-glycosyltransferase genes have been cloned and characterized from bacteria, fungus, model and nonmodel crops, ornamental and medicinal plants (Caputi et al. 2012).

Medicinal importance of *Withania* (Kulkarni and Dhir 2008; Sharma et al. 2011; Ahlawat et al. 2012; Uddin et al. 2012) led to the initiation of studies related to metabolic profiling of this important plant (Madina et al. 2007a, b; Chatterjee et al. 2010; Chen et al. 2011; Alam et al. 2012; Chaurasiya et al. 2012; Bhatia et al. 2013; Ihsan-ul-Haq et al. 2013), genetic transformation (Pandey et al. 2010a; Mishra et al. 2013b), generation of ESTs and various other studies (Sharma et al. 2007; Chaturvedi et al. 2012; Akhtar et al. 2013; Gupta et al. 2011, 2012, 2013a, b; Mishra et al. 2013a; Razdan et al. 2013). Steroidal lactones, i.e. withanolides and their glycosides, i.e. withanosides are the major sources of medicinal properties of *W. somnifera*. These withanosides are the results of enzymatic glycosylation of withanolides. Few members of sterol glycosyltransferases (*WsSGT*s) have been identified and characterized from *W. somnifera* (Sharma et al. 2007; Chaturvedi et al. 2012; Mishra et al. 2013a). Enzymatic activities of WsSGTs towards various substrates have also been analysed (Madina et al. 2007a, b). Differential expression of these *WsSGT*s in different parts of the plant as well as to different stress conditions indicated their significance in plant growth and stress response (Sharma et al. 2007; Chaturvedi et al. 2012; Mishra et al. 2013a).

The present study examines functional role of one member of *SGT* gene family of *W. somnifera*, i.e., *WsSGTL*1 gene (DQ356887.1) through the development of transgenic lines of *Nicotiana tabacum* cv. Petit Havana (tobacco), a heterologous system, under the control of constitutive promoter CaMV 35S. Difference in transgenics *N. tabacum* expressing *WsSGTL*1 (*WsSGTL*1-*Nt*) and nontransgenics (WT) was analysed for germination and growth pattern of the seedlings, photosynthetic parameters along with difference in phytochemical properties and antioxidant system. In addition, responses of *WsSGTL*1-*Nt* to biotic (*Spodoptera litura*) and abiotic (salt stress, NaCl) stress have also been studied. Results suggested that *WsSGTL*1 confers an adaptive behaviour in transgenic plants under normal condition along with tolerance towards *S. litura* and salt stress through modulation of phytochemical profile.

Materials and methods

*WsSGTL*1 gene cloning and construct preparation

The plants of *Withania somnifera* were obtained from the germplasm being maintained at CSIR-National Botanical

Table 1 List of primers used in PCR and qRT PCR analysis

L1ORF1 F	GTCACCATGGAAATGGACAGTAATGGGCATAATG
L1ORF1 R	CCATCGATGTCGACAGCTTAAGACCCACAA GGCAGG
NtUBI F	TCCAGGACAAGGAGGGTAT
NtUBI R	GAGACCTCAGTAGACAAAGC
RTL1 F	GCCGAGTGCCCTCATGATT
RTL1 R	GTTGGACACCCAGCACGTAGTC
NPT II F	CTGAATGAACTGCAGGACGAGG
NPT II R	GCCAACGCTATGTCCTGATAGC
RBCS F	AGTCAGCTGCCTCATTCCCTGTTTC
RBCS R	TGGGTATGCCTTCTTCGCCTCTC
CAB F	CTGAGCAAACTCCATCATACCTC
CAB R	CGAGGATGCTTTGGGCATGGAC
PSBO F	TCACAGTCAAGGCAGAGAGTGTG
PSBO R	GCTCTCAAATACTCCAATGACCTC
PSBP1 F	TCTAGCTCAGCAGCTAGAACAAC
PSBP1 R	CGTAGTCAGTGATGGATTTCTTATC
PSBP2 F	CTGGTCAGGTTCTTAGATTTG
PSBP2 R	ACCATCTCTTGTCACCAGCTTG

Research Institute, Lucknow, India. Hot phenol method (Pawlowski et al. 1994) was used to extract total RNA from young leaves of greenhouse grown mature plants. First-strand cDNA was synthesized using Superscript II RNase H reverse transcriptase (Thermo Fisher Scientific, Vilnius, Lithuania), according to the manufacturer's instructions. The full-length open reading frame of the *WsSGTL*1 (DQ356887.1) cDNA was isolated using oligonucleotide primers L1ORF F and L1ORF R (Table 1) having recognition sites for NcoI and SalI restriction endonucleases (Sharma et al. 2007). The reaction conditions for PCR started with initial 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 60 °C for 30 s, 72 °C for 2 min and the final 5 min extension at 72 °C. Plant expression construct was prepared through cloning PCR product (*WsSGTL*1 cDNA) under transcriptional control of cauliflower mosaic virus 35S double enhancer promoter in pBI101 (Clontech, Mountain View, CA, USA) replacing β-glucuronidase gene.

Plant transformation and selection of transgenic lines

Binary vectors harbouring *WsSGTL*1 gene under the control of CaMV 35S double enhancer promoter were transferred by electroporation into LBA4404 strain of *Agrobacterium tumefaciens*. Plants of *Nicotiana tabacum* cv. Petit Havana (tobacco) were grown at 25 °C and 16-h-light/8-h-dark periods in glasshouse. Leaf disc cocultivation method (Horsch et al. 1985) was used to transform *N. tabacum* for generating transgenic plants, which were selected on medium containing 200 mg l^{-1} kanamycin. Antibiotic-resistant transgenic plants were shifted to glass house. In these transgenic plants, self-fertilization was carried out by bagging and the seeds were harvested; sterilized and grown on half strength MS media (Murashige and Skoog 1962) supplemented with kanamycin. *WsSGTL*1 transgenic and WT *N. tabacum* plants were grown at 26 °C on a 16-h-light/8-h-dark cycle. Several homozygous transgenic lines (T3 generation) constitutively expressing *WsSGTL*1 gene were obtained and selected through PCR and RT-PCR. Further experiments were performed from the plants of T3 generation.

Gene expression analysis

Young leaves of 1-month-old *N. tabacum* were used to extract total RNA by using Spectrum Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO, US) which was subsequently treated with RNase-free DNase (Thermo Fisher Scientific, Vilnius, Lithuania). RNA was subjected to reverse transcription to generate first-strand cDNA using oligo dT primers (Thermo Fisher Scientific). To equalize cDNA, ubiquitin gene primers of tobacco (Table 1) were used as an internal control.

Relative expression of *WsSGTL*1 transcript was analysed in transgenic and WT *N. tabacum* plants using real-time PCR. Real-time PCR was performed in 25 µl for set of selected genes using Power SYBR Green PCR Master Mix (ABI, Foster City, CA, USA). The reactions were performed using the cycle conditions of, initial 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and the final 5 min extension at 72 °C. After obtaining ct value for each reaction, the fold change was calculated by using Delta-Delta ct method (Livak and Schmittgen 2001; Dubey et al. 2010). Three independent experiments were conducted using three biological replications.

To investigate the expression pattern, mRNA of *WsSGTL*1-*Nt* and WT were subjected to semi-quantitative RT-PCR analysis using gene-specific primers (Table 1) for some selected nuclear-encoded chlorophyll-related genes, CAB (AY219853), RBCS (AY220079), PSBO (AY220076), PSBP1 (X64347) and PSBP2 (X64348). RT-PCR analysis was carried out using PCR Master-mix (Thermo Fisher Scientific). The selected oligonucleotide primers used in the study are listed in Table 1. PCR was carried out using the following cycle conditions: an initial denaturation at 94 °C for 2 min, 30 cycles at 94 °C for 15 s, 59 °C for 20 s, and 72 °C for 45 s, followed by a final 5-min extension at 72 °C. Three independent experiments using three biological replications were performed.

Phenotypic analysis

Seeds of *WsSGTL*1-*Nt* lines (T3 generation) and WT were collected. Seed weight (per 100 seeds) of

*WsSGTL*1-*Nt* and WT were compared as the first step towards phenotypic observations. Sterilized seeds were germinated on half strength MS media at 26 °C on a 16-h-light/8-h-dark cycle. Seedlings of all *WsSGTL*1-*Nt* lines and WT were analysed on different parameters like percentage of germination, root length, seedling weight and biomass.

Chlorophyll estimation

Young (3rd–4th) leaves (100 mg) of 1- and 3-month-old *WsSGTL*1-*Nt* and WT plants were crushed in 5 ml (80 %, v/v) chilled acetone (Arnon 1949). Homogenized tissues were centrifuged at 7,800*g* for 10 min. Supernatant was collected and absorbance was recorded at 663, 645, 510 and 480 nm. Total chlorophyll content along with its components, i.e. chlorophyll-A and chlorophyll-B, was calculated in mg g^{-1} fresh wt using formula of Machlachalan and Zalik (1963). Carotenoid content was calculated in mg g^{-1} fresh weight using formula given by Duxbury and Yentsch (1956). Three independent experiments using three biological replications have been performed.

Physiological measurements

Chlorophyll fluorescence of 20-day-old seedlings of *WsSGTL*1-*Nt* and WT was measured by using Imaging PAM (Pulse Amplitude Modulator) Chlorophyll Fluorometer (Walz, Effeltrich, Germany). Maximum photochemical efficiency (F_v/F_m) of photosystem II (PSII) for various chlorophyll parameters in seedlings of *WsSGTL*1-*Nt* and WT was calculated according to Maxwell and Johnson (2000); where, F_0 is minimum fluorescence, F_m is maximum fluorescence, F_v is variable fluorescence and equal to $F_m - F_0$ (Mishra et al. 2013a).

Fully expanded (young) 3rd–4th leaves from the tip of 4-month-old *WsSGTL*1-*Nt* and WT mature plants were used for leaf gas exchange net photosynthetic rate (A_P, μmol m^{-2} s^{-1}), respiration rate (A_R, μmol m^{-2} s^{-1}) and stomatal conductance (g_s, mmol m^{-2} s^{-1}), at PPFD (600 μmol m^{-2} s^{-1}) using a combined gas exchange fluorescence system (GFS-3000; Walz; Pandey et al. 2010b). Saturating flash of 10,000 mol m^{-2} s^{-1} for 800 ms was given at every 3–4 min for the determination of gas exchange parameters. During gas exchange measurements, the flow rate of air through the cuvette was maintained such that conditions in the cuvette approximated ambient CO_2 (which ranged between 375 and 385 μmol mol^{-1}). Three independent experiments using three biological replications have been performed.

Extraction of secondary plant metabolites

As WsSGTL1 is known for glycosylation of secondary metabolites (Sharma et al. 2007), glycosylation pattern of various secondary metabolites was analysed through HPLC in *WsSGTL*1-*Nt* and WT plants. Seeds of *WsSGTL*1-*Nt* and WT plants were used to analyse glycosylation pattern of sterols whereas young leaves of 3-month-old plants for analysis of modulation of flavonoids and alkaloids. Plant materials (~100 mg) were homogenized in 1 ml of acetone (80 %, v/v) for seeds (Bush and Grunwald 1972) and 1 ml of methanol (80 %, v/v) for leaves (Misra et al. 2010b) followed by incubation at 40 °C for 2 h. Glycosylated and nonglycosylated sterols were analysed quantitatively from acid-hydrolysed or non-hydrolysed extracts of *WsSGTL*1-*Nt* and WT plants, respectively. For acid hydrolysis seeds were homogenized in 1 ml of acetone containing 1 % HCl and incubated at 40 °C for 2 h. Acid hydrolysis of extract was required to obtained free sterols by breaking of bonds between sterols and sugar moiety. Homogenous extract obtained was filtered through 0.2-μM filter (Millipore, India). Acetone/methanol was evaporated and dried extract was dissolved (1 mg ml^{-1}) in 100 % methanol before HPLC analysis. Free sterols were quantified before and after hydrolysis, and the difference between hydrolysed and un-hydrolysed sterols would be due to the glycosylation action of WsSGTL1.

HPLC analysis

Qualitative and quantitative analysis of sterols was performed by HPLC–PDA with a Shimadzu (Japan) LC-10 system comprising an LC-10AT dual pump system, an SPD-M20A PDA detector and rheodyne injection valve furnished with a 20-μl sample loop. Compounds were separated on a Merck Purospher star® RP-C18 column (250 × 4.6 mm, 5 μm pore size) protected by guard column containing the same packing (Misra et al. 2010a, b; Pandey et al. 2012) with a flow rate of 2 ml min^{-1} and injecting 20 μl of extract. Sterols were eluted by isocratic solution of acetonitrile and water (95:5, v/v). Quantification of sterols was performed at 202 nm at 34 °C, 60 min retention time with 2 ml min^{-1} flow rate delivery of mobile phase by injecting 20 μl of extract. Sterols were quantified by calculating the area of individual peak and compared with peak from standards (Sigma-Aldrich). To analyse glycosylation of sterols, three main sterols, sitosterol, stigmasterol and campesterol (Liu et al. 2007) were analysed due to established activity of WsSGTL1 for these sterols (Sharma et al. 2007). As no activity of WsSGTL1 was observed with cholesterol by Sharma et al. (2007),

quantification of cholesterol was not considered in current study. Flavonoids and phenolics quantification was performed by using mobile phase of gradient prepared from 1 % (v/v) acetic acid in HPLC-grade water (component A) and acetonitrile (component B). The gradient was from 20 to 35 % of component B in 0–14 min then from 35 to 50 % of component B in 14–40 min. Quantification was carried out at 254 nm at room temperature and 42 min retention time with flow rate of 0.6 ml min^{-1}.

Parameters like specificity, linearity, peak purity, precision and accuracy, limits of quantification and detection and robustness were followed to quantify the three sterols and eight phenolic compounds by HPLC. Linear calibration plots for all metabolites were obtained over the concentrations of range 0.1–50 μg ml^{-1}. The correlation coefficient ranged from 0.96 to 0.99. The precision of the proposed method was investigated with respect to repeatability and intermediate precision. In order to measure repeatability, ten consecutive injections were made with the same standard solution. A high repeatability in the retention time was obtained with RSD values lower than 2 %, both for single standard and mixture of compounds, even at high concentration. The accuracy was determined by recovery of known amounts of standards added in the plant matrix prior to extraction and sample preparation. The recovery in case of sterols was ranged from 97.97 to 98.32 % while for phenolics observed recovery was 85.66–100 %. Sensitivity was expressed in the terms of LOD and LOQ. Values of LOD for sterols ranged from 1.01 to 1.16 μg ml^{-1} while for phenolics ranged from 1.19 to 1.48 μg ml^{-1} and values of LOQ for sterols ranged from 2.30 to 3.05 μg ml^{-1} and in case of phenolics ranged from 1.46 to 2.67 μg ml^{-1}, which suggested full agreement for the quantification of each compounds investigated.

Data were integrated by Shimadzu class VP series software and results were obtained by comparison with standards (Niranjan et al. 2009). The results were the mean values from three independent experiments with three biological replicates.

Antioxidant enzyme activities

Young leaves of 2-month-old *WsSGTL*1-*Nt* and WT plants were used for enzymatic assay. Leaves (~500 mg) were homogenized in 1.5 ml of cold 100 mM phosphate buffer (pH 7.8) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 % (w/v) polyvinyl-polypyrrolidone (PVP) and 0.5 % (v/v) Triton X-100; in a pre-chilled mortar and pestle. The homogenate was filtered and centrifuged at 4 °C, 17,600*g* for 30 min. The supernatant was recentrifuged at 4 °C, 17,600*g* for 30 min before determination of antioxidant enzyme activities. Bradford (1976) method was used to determine protein concentration in enzyme extracts using bovine serum albumin (BSA) as a standard. Antioxidant enzyme activities were determined by the methods specific to each enzyme with some modifications as described below. All enzyme assays were performed in three independent experiments using three biological replicates at 25 °C.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by monitoring the inhibition of phytochemical reduction of nitro blue tetrazolium (NBT) according to the protocol (Beyer and Fridovich 1987). One unit of SOD enzyme activity is defined as the amount of an enzyme required to inhibit NBT reduction by 50 % at 560 nm. Guaiacol peroxidase (GPX, EC 1.11.1.7) activity was determined by measuring the change in absorption at 470 nm due to guaiacol oxidation, following Pütter's (1974) protocol. GPX activity was assayed for 5 min in a reaction mixture of 50 mM potassium phosphate buffer (pH 7.0), 20.1 mM guaiacol, 12.3 mM H_2O_2 and suitable amount of enzyme extract. For measuring catalase (CAT, EC 1.11.1.6) activity, the reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0), 2 mM H_2O_2 and 1 mM EDTA. Oxidation of H_2O_2 was measured at 240 nm following protocol of Aebi (1974).

Insect toxicity bioassay

The genetically homozygous larvae of *S. litura* were maintained in the laboratory on semisynthetic diet at 26 ± 2 °C and 70 % relative humidity on a 14-h-light/10-h-dark cycle. To examine anti-feeding behaviour of *WsSGTL*1-*Nt* and WT plants, young leaves were picked from the 3rd and 4th nodes of 2-month-old plants. These leaves (3–4) were washed thoroughly with distilled water, blotted dry, placed in a plastic beaker and fed to first instar larvae (ten in numbers) of *S. litura* for 7 day. Observations for percentage mortality and survival rate were made on 3, 5 and 7 days after start of the experiment as per Misra et al. (2010b) and Pandey et al. (2012). After completion of experiment, comparative larval weight was recorded. Each treatment was repeated for three times.

Treatment with salt stresses

Young leaves from 2nd and 3rd nodes were excised from 1-month-old *WsSGTL*1-*Nt* and WT plants to analyse comparative response during the leaf discs salt assay. These leaves were transferred into salt solution of 200 mM. The treatment was carried out at 25 °C for 6 days. In order to investigate the effect of salinity stress, survival of leaves and chlorophyll content of NaCl treated leaves were analysed. All the data were recorded from 0 to 6 day of NaCl treatment. The experiment was repeated three times.

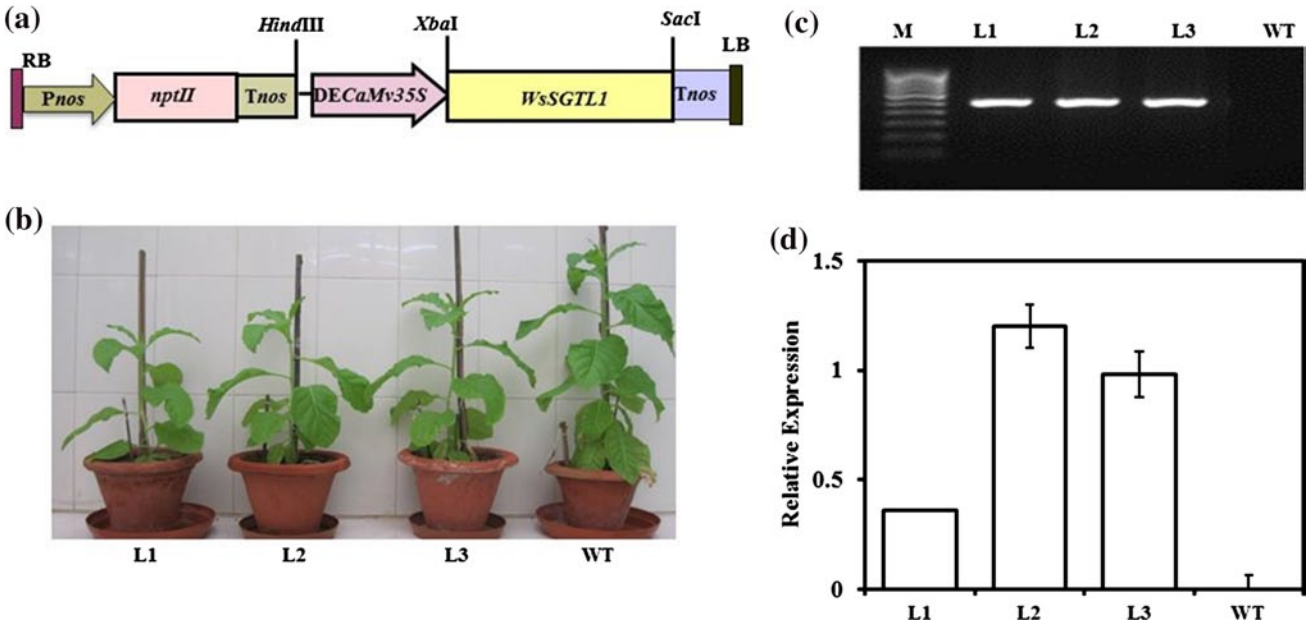

Fig. 1 Transformation of *WsSGTL*1 gene in *Nicotiana tabacum*. **a** T-DNA of pBI101 having *WsSGTL*1 (2.1 kb) gene under the control of double enhancer CaMv 35S promoter. **b** Stunted phenotype of selected *WsSGTL*1-*Nt* (L1, L2, L3) transgenic lines along with nontransgenic *N. tabacum* (*WT*). **c** Genomic-DNA PCR confirmed T-DNA insertion in *WsSGTL*1-*Nt*. **d** *WsSGTL*1 gene expression was confirmed through real-time PCR

Statistical analysis

Each experiment was carried out under a completely randomized design with three independent experiments using three biological replications. Morphological changes were recorded by visual observations. The values of data are mean ± standard deviation of three replicates. The data were analysed by one-way analysis of variance (ANOVA) to confirm the variability and validity of results. Duncan's multiple range test (DMRT) was performed to determine significant difference between treatments (Gomez and Gomez 1984). Comparisons with P values ≤ 0.05 were considered significantly different.

Results

Phenotypic analysis of transgenic *N. tabacum* plants expressing *WsSGTL*1

Transgenic lines of *N. tabacum*, expressing *WsSGTL*1 under the control of CaMV 35S double enhancer promoter (Fig. 1a), were selected on kanamycin containing media. The homozygous overexpressing lines (T3 plants) were confirmed through genomic PCR and qRT-PCR analysis (Fig. 1c, d).

Phenotypically transgenic plants (*WsSGTL*1-*Nt*) showed relatively stunted growth and yellowish-green leaves as compared to green leaves of WT plants (Fig. 1b). However, leaf orientation, leaf size, flower colour, seed setting, number of seeds, etc. were similar for both *WsSGTL*1-*Nt* transgenic and WT plants.

Physiological difference in *WsSGTL*1-*Nt* and WT plants

Stunted growth and slight yellowing in the middle and lower leaves of *WsSGTL*1-*Nt* plants suggested a need to examine the difference in *WsSGTL*1-*Nt* and WT plants on the basis of physiological parameters like photosynthesis, respiration and fluorescence. Chlorophyll fluorescence imaging in 20-day-old seedlings showed reduced fluorescence as well as absorbance in *WsSGTL*1-*Nt* as compared to WT (Fig. 2a). Significantly decreased physiological parameters were observed in *WsSGTL*1-*Nt* lines with up to twofold lower photosynthetic rate, twofold lower stomatal conductance, 6.4-fold lower respiration rate and up to 1.4-fold lower photochemical efficiency (F_v/F_m) as compared to WT plants (Fig. 2c and Fig. S1a). Chlorophyll content on the basis of total chlorophyll, chlorophyll-A, chlorophyll-B and carotenoid was up to 1.5-fold lower in leaves of 1-month-old *WsSGTL*1-*Nt* plants, while up to 1.4-fold lower at the age of 3 months (Fig. S1b) as compared to WT (Fig. 2b) plants.

Expression analysis suggested that genes related to chlorophyll biogenesis were not affected by expression of *WsSGTL*1 gene. This suggested that decreased photosynthetic

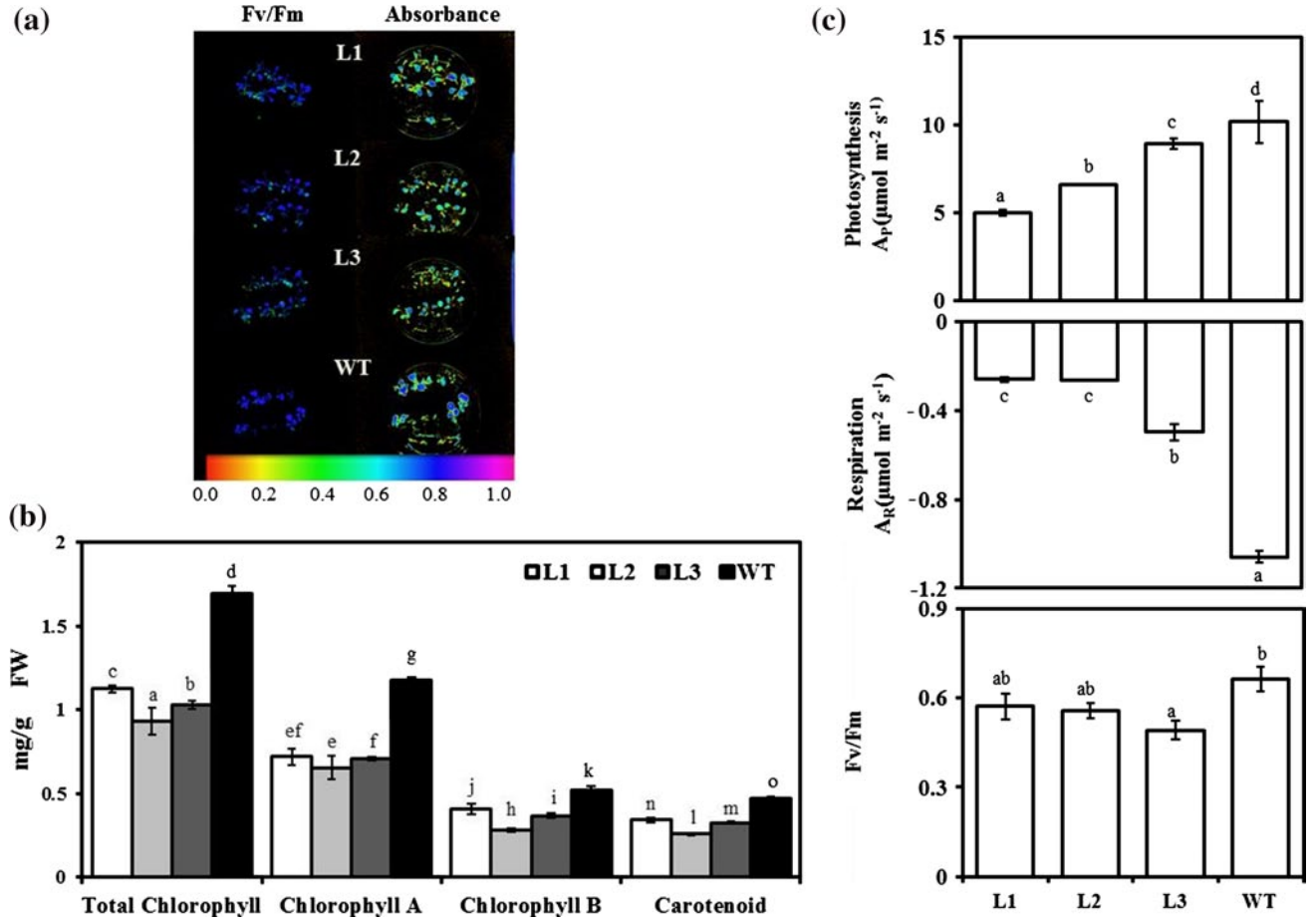

Fig. 2 Physiological measurements of *WsSGTL*1-*Nt* and WT plants. **a** Chlorophyll fluorescence imaging of 15-day-old seedlings. **b** Chlorophyll quantification from leaves of 1-month-old *WsSGTL*1-*Nt* (L1, L2, L3) and WT plants. **c** 4-month-old mature plants were used to determine photosynthesis (A_P) and respiration rate (A_R) along with chlorophyll fluorescence (F_v/F_m). Data are expressed as mean ± SD of three independent experiments. *Different letters* indicate significantly different values in a particular tissue (DMRT, $P \leq 0.05$) and ANOVA significant at $P \leq 0.01$

rate was not associated with modulated expression of chlorophyll-related genes. These results confirmed that expression of *WsSGTL*1 indirectly modulates the growth pattern of transgenics as well as their physiological properties by influencing adaptive machinery of plants similar to reduced growth and photosynthetic rate of plants of severe climatic conditions.

Germination and growth pattern of *WsSGTL*1-*Nt* and WT plants

Growth and morphology of seedlings of *WsSGTL*1-*Nt* and WT were compared on the basis of comparative seed weight, percentage germination, cotyledon emergence, fresh weight and dry weight. No significant difference was observed in the average seed weight of *WsSGTL*1-*Nt* and WT (Fig. S3a) suggesting that *WsSGTL*1 expression did not affect the yield of plants. Percentage germination of *WsSGTL*1-*Nt* lines was significantly lower (1.4-fold) on 3rd day of seed inoculation as compared to WT (Fig. 3b). After 3rd day, no significant difference was observed in germination between *WsSGTL*1-*Nt* lines and WT. There was no significant difference in cotyledon emergence between *WsSGTL*1-*Nt* lines and WT (Fig. S3b). Seedlings (15-day old) of *WsSGTL*1-*Nt* showed comparatively smaller roots (1.4-fold), lesser fresh weight (1.2-fold) and biomass (1.4-fold) as compared to seedlings of WT (Fig. 3a, c). Adaptive strategy for abiotic stress tolerance in the form of reduced plant growth has been suggested by Leyva-González et al. (2012) and Han et al. (2012).

WsSGTL1-mediated modulated glycosylation

WsSGTL1 is known to glycosylate variety of sterols having 3β-OH group (Madina et al. 2007a; Sharma et al. 2007). Involvement of *WsSGTL*1 in response to salt, heat and cold

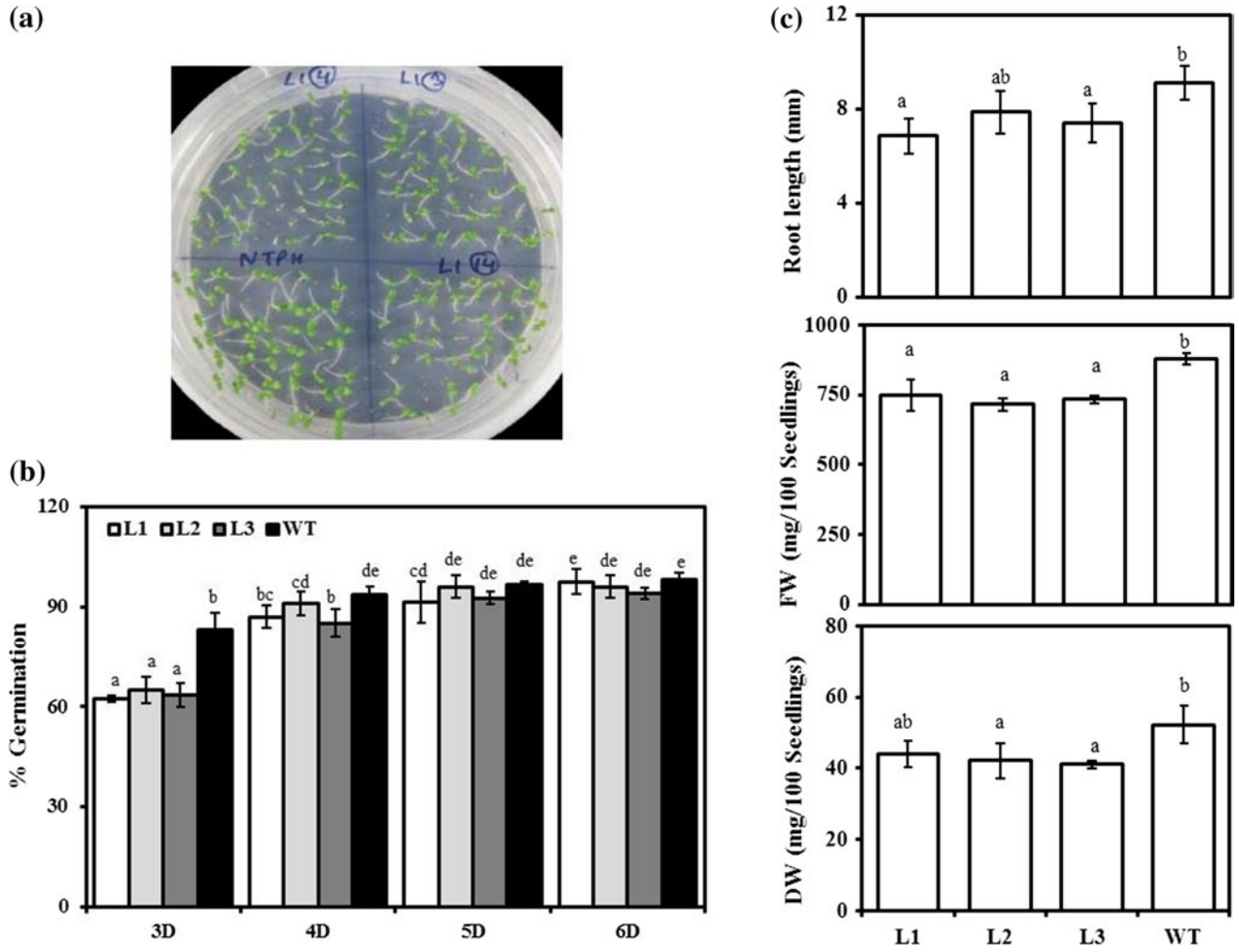

Fig. 3 Germination and growth pattern in *WsSGTL1-Nt* and WT seedlings. **a** Germinated seeds after 10 days. **b** Percentage germination from 3rd (radicle emergence) day to 6th day. **c** Average root length, fresh weight and dry weight of 20-day-old seedlings. Data are expressed as mean ± SD of three independent experiments. *Different letters* indicate significantly different values in a particular tissue (DMRT, $P \leq 0.05$) and ANOVA significant at $P \leq 0.01$

stress has already been elucidated through heterologous expression in *Arabidopsis thaliana* (Mishra et al. 2013a).

Role of WsSGTL1 in glycosylation of sterols of heterologous system was determined by analysis of sterols in free and glycosylated form. Glycosylated sterols were quantified as free sterols after acid hydrolysis. Campesterol, stigmasterol and sitosterol (having 3β-OH group) are the major sterols found in plant system (Liu et al. 2007), targeted by WsSGTL1 (Sharma et al. 2007). Earlier studies suggested decrease in content of glycosides of these sterols during seed germination due to their conversion into free sterols (Bush and Grunwald 1972). Also, among other sterols, glyco-sitosterol was found to be more abundant in seeds of *N. tabacum* (Bush and Grunwald 1972). Hence, seed can be considered as a store house of glycosides of these sterols and the difference in glycosylation pattern by WsSGTL1 can easily be quantified in seeds. This was confirmed by quantitative estimation of three major plant sterols: sitosterol, stigmasterol and campesterol in seed extract through HPLC analysis (Fig. S4). To quantitate modulation in glycosylation profile, amount of sterols was measured in hydrolysed and non-hydrolysed extracts. Differences in two values of sterols were considered as glycosylated sterols. Glycosylated amount of campesterol was 164–207 μg g^{-1} DW, stigmasterol was 67–119 μg g^{-1} DW and sitosterol was 13.14–21.6 mg g^{-1} DW in *WsSGTL1-Nt* while in WT glycosylated amount of campesterol, stigmasterol and sitosterol were relatively lower, i.e. 46.7, 3.9 μg g^{-1} DW and 11 mg g^{-1} DW, respectively (Fig. 4).

Effect of *WsSGTL1* on flavonoids and phenolic accumulation

N. tabacum is a rich source of various secondary metabolites like flavonoids and phenolics. Through in vitro

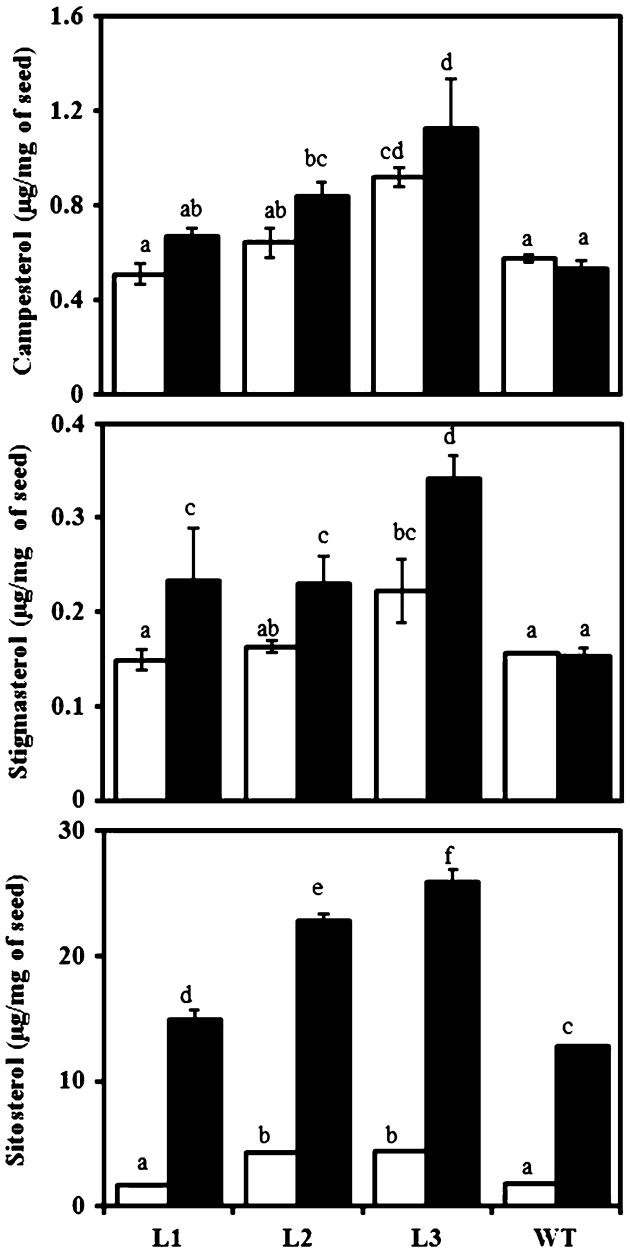

Fig. 4 Quantitative estimation of glycosylation of sterols due to enzymatic activity of WsSGTL1. Free sterols were measured by breaking bond between sterols and sugar moiety after acid hydrolysis of extract and compared with free sterols before hydrolysis (*white bar* represents sterols before hydrolysis and *black bar* represents sterols after hydrolysis). Difference between free sterols before and after hydrolysis resulted in glycosylated amount. This amount calculated in *WsSGTL1-Nt* lines for campesterol, stigmasterol and sitosterol was 164–207 µg g^{-1} DW, 67–119 µg g^{-1} DW and 13.14–21.6 mg g^{-1} DW, respectively; while in WT were relatively lower, i.e. 46.7, 3.9 µg g^{-1} DW and 11 mg g^{-1} DW, respectively. Data are expressed as mean ± SD of three independent experiments. *Different letters* indicate significantly different values in a particular tissue (DMRT, $P \leq 0.05$) and ANOVA significant at $P \leq 0.01$

studies, many metabolites have been reported as substrates for WsSGTL1, (Sharma et al. 2007). Besides sterols, these substrates may include flavonoids, phenolics, etc. To study the modulation due to expression of *WsSGTL*1 in quantity of these metabolites, quantitative estimation of phenolics and flavonoids was performed using HPLC.

HPLC analysis of methanolic leaf extract of *WsSGTL*1-*Nt* and WT provided the quantification of these metabolites (Fig. S5). Quantification of flavonoids revealed that, amount of quercetin and its glucosylated form rutin in *WsSGTL*1-*Nt* plants was up to fivefold and eightfold higher, respectively, as compared to WT (Fig. 5); whereas kempherol was up to sixfold lower in *WsSGTL*1-*Nt* as compared to WT plants (Fig. S6). These results suggested that *WsSGTL*1 expression shifted flavonol biosynthesis pathway towards increased production of glycosylated products, quercetin and rutin.

Flavonoids like kempherol, quercetin, rutin, etc. also contribute to the antioxidant system, which suggested that the antioxidant system may also be affected by *WsSGTL*1. This was confirmed by quantification of antioxidants from both *WsSGTL*1-*Nt* and WT through HPLC. Two major antioxidant polyphenols, chlorogenic acid (CGA; glycosides of caffeic acid, CA) and protocatechuic acid (PA) were significantly accumulated up to 25-fold and twofold, respectively, in *WsSGTL*1-*Nt* plants as compared to WT (Fig. 5). Quantification of gallic acid showed no significant difference in *WsSGTL*1-*Nt* and WT. Comparatively lower amount of caffeic acid (up to fourfold) and ferulic acid (up to fivefold) might be associated with higher amount of chlorogenic acid in *WsSGTL*1-*Nt* plants (Fig. 5 and Fig. S6). These results suggested that CGA and PA amongst all other antioxidant phenolics were present in remarkably enhanced quantities in *WsSGTL*1-*Nt* plants. Studies of Mucciarell et al. (2000), Ozyigit et al. (2007) and Abdelwahd et al. (2008) reported reduction in regeneration, differentiation and proliferation of plant tissues resulting in reduced growth and development due to accumulation of phenolics. This indicates that an enhanced quantity of these phenolics might be responsible for reduced growth pattern, i.e. stunted growth.

Effect of *WsSGTL*1 on antioxidant enzyme

Modulation in expression of *WsSGTL*1 gene under different environmental challenges suggested its functional importance during stress conditions (Sharma et al. 2007; Chaturvedi et al. 2012; Mishra et al. 2013a) by affecting antioxidant system. Antioxidant system comprises not only antioxidant metabolites but also antioxidant enzymes. Therefore, leaves of *WsSGTL*1-*Nt* and WT were subjected

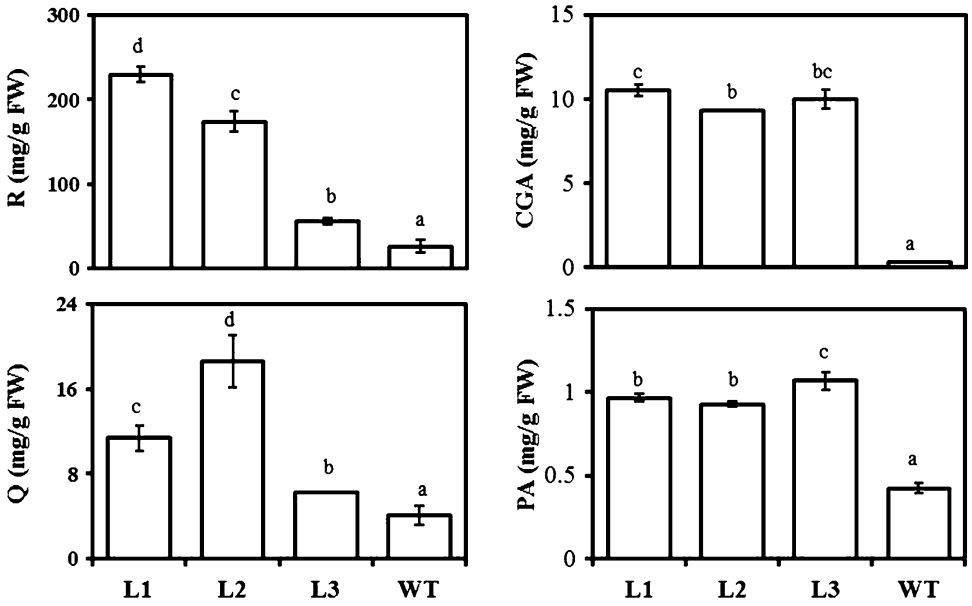

Fig. 5 Expression of *WsSGTL*1 modulates accumulation of other flavonoids and phenolics in leaves of *WsSGTL*1-*Nt* as compared to WT (*R* rutin; *Q* quercetin; *CGA* chlorogenic acid; *PA* protocatechuic acid). Data are expressed as mean ± SD of three independent experiments. *Different letters* indicate significantly different values in a particular tissue (DMRT, $P \leq 0.05$) and ANOVA significant at $P \leq 0.01$

to analyse activity of antioxidant enzymes (SOD, CAT). SOD being first line of defence (Alscher et al. 2002) is an important antioxidant enzyme, which showed three- to fourfold higher activity in *WsSGTL*1-*Nt* as compared to WT (Fig. 6a). CAT also had two- to fourfold higher activity in *WsSGTL*1-*Nt* plants as compared to WT (Fig. 6b).

Increased rutin accumulation leads to insect resistance in plants

Certain secondary metabolites of plants, including flavonoids (Misra et al. 2010b; Pandey et al. 2012), have been reported to confer insect resistance in plants. Experiment conducted with the leaves of *WsSGTL*1-*Nt* and WT plants suggested insect-resistant quality of *WsSGTL*1-*Nt* from insect bioassay using *S. litura*. This insect-resistant quality of *WsSGTL*1-*Nt* plants (Fig. 7 a, upper panel) may be due to enhanced production (two- to eightfold) of rutin (Misra et al. 2010b) as compared to WT (Fig. 5). Mortality rate of *S. litura* was not affected by *WsSGTL*1 expression, but 23–27 % weight loss (Fig. 7b) was observed with surviving *S. litura* larvae fed on *WsSGTL*1-*Nt* leaves (Fig. 7a, lower panel).

Salt stress tolerance

To analyse the response of *WsSGTL*1 under salt stress, leaves of *WsSGTL*1-*Nt* and WT were subjected to 200 mM of NaCl solution. Leaf disc assay was performed to analyse comparative effect of NaCl on leaves of *WsSGTL*1-*Nt* and WT. Higher chlorosis was observed in the leaves of WT from 3rd day, and reached maximum (complete decay) up to 6th day. Chlorosis was significantly lower in

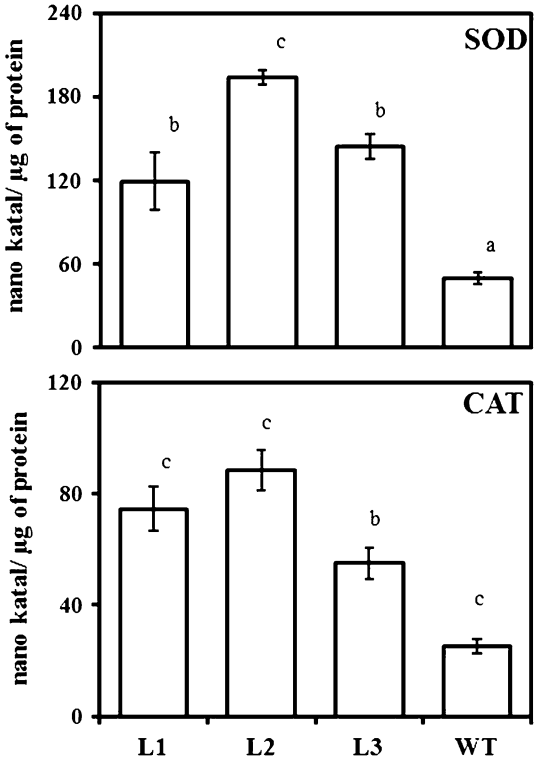

Fig. 6 Enhanced enzymatic activity of superoxide dismutase (SOD) and catalase (CAT) in *WsSGTL*1-*Nt* plants show modulated stress-tolerant capacity of transgenics as compared to WT. Data are expressed as mean ± SD of three independent experiments. *Different letters* indicate significantly different values in a particular tissue (DMRT, $P \leq 0.05$) and ANOVA significant at $P \leq 0.01$

*WsSGTL*1-*Nt* leaves as compared to WT (Fig. 8a). Chlorosis occurred as a result of salinity, which ultimately affected chlorophyll content. Survival of leaves was measured by

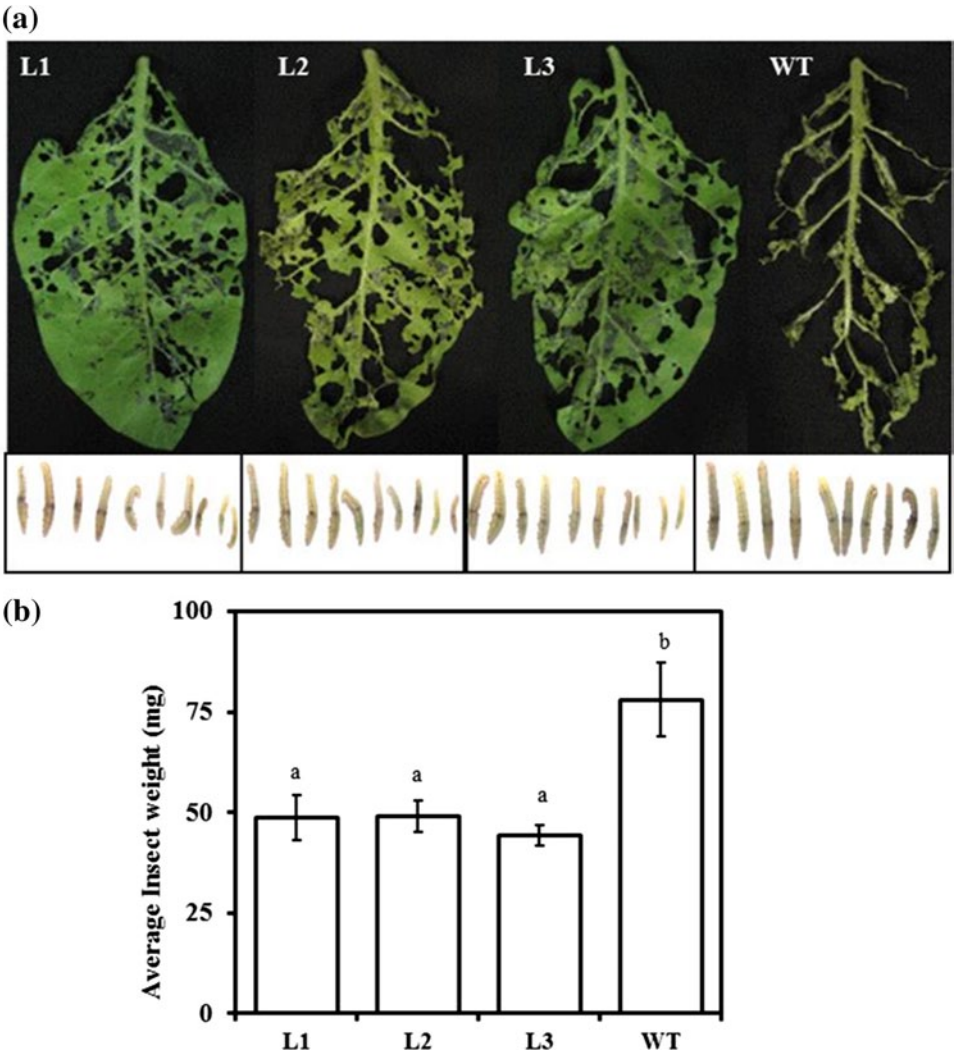

Fig. 7 Insect resistance due to expression of *WsSGTL*1. **a** Detached leaves (3–4) of 2-month-old *WsSGTL*1-*Nt* and WT plants were used for insect bioassay. Two-day-old *S. litura* larvae (ten in number) were released on detached leaves and analysed up to 7th day (*upper panel*). Reductions in growth of surviving insects were observed in different lines of *WsSGTL*1-*Nt* and WT of *S. litura* (*lower panel*). **b** Average larval weight (10 larvae) was observed after completion of experiment (after 7 days) with reduction in weight of surviving larvae fed on *WsSGTL*1-*Nt* leaves as compared to larvae fed on WT leaves. Data are expressed as mean ± SD of three independent experiments. *Different letters* indicate significantly different values in a particular tissue (DMRT, $P \leq 0.05$) and ANOVA significant at $P \leq 0.01$

quantifying total chlorophyll, chlorophyll-A, chlorophyll-B and carotenoid (Fig. 8b; Fig. S7). Estimation of chlorophyll also suggested that decay of *WsSGTL*1-*Nt* leaves was faster as compared to WT, while chlorophyll contents were lesser in *WsSGTL*1-*Nt* plants, under normal conditions.

Discussion

Plants have developed numerous adaptive mechanisms to tolerate various biotic and abiotic stresses. These mechanisms include expression of various genes in the face of stress condition, enhanced production of antioxidant metabolites (phenols, flavonoids, etc.) and enhanced activity of antioxidant enzymes (Siddiqui 2013). During stress conditions, enhanced expression of *WsSGTL*1 is reported to provide tolerance towards different kind of environmental stresses (Sharma et al. 2007; Chaturvedi et al. 2012; Mishra et al. 2013a). In this study, we functionally characterized *WsSGTL*1 by its expression in transgenic tobacco, and analysed various parameters including responses of transgenic lines towards biotic and abiotic stresses. We observed reduced height of mature plants and reduced root length, fresh weight and dry weight of seedling stage of *WsSGTL*1-*Nt* lines, which suggested that overall growth of the plant was affected. Retarded (stunted) growth of *WsSGTL*1-*Nt* plants as well as of the transgenic seedlings (as compared to WT) suggested that expression of *WsSGTL*1 in heterologous system can be credited for providing a kind of adaptation capability against stresses. Similar to our observations, various studies correlated reduced growth with enhanced survival capability of transgenic plants towards different types of environmental stresses after homologous or heterologous expression of various genes (Han et al. 2012; Leyva-González et al. 2012). It was suggested that the stunted growth is one of the kind of adaptations towards stresses. Reduced growth might be due to decreased photosynthetic rate of the transgenic lines as compared to WT plants. *WsSGTL*1-*Nt* plants possess yellowish-green leaves signifying lesser chlorophyll content along with reduction

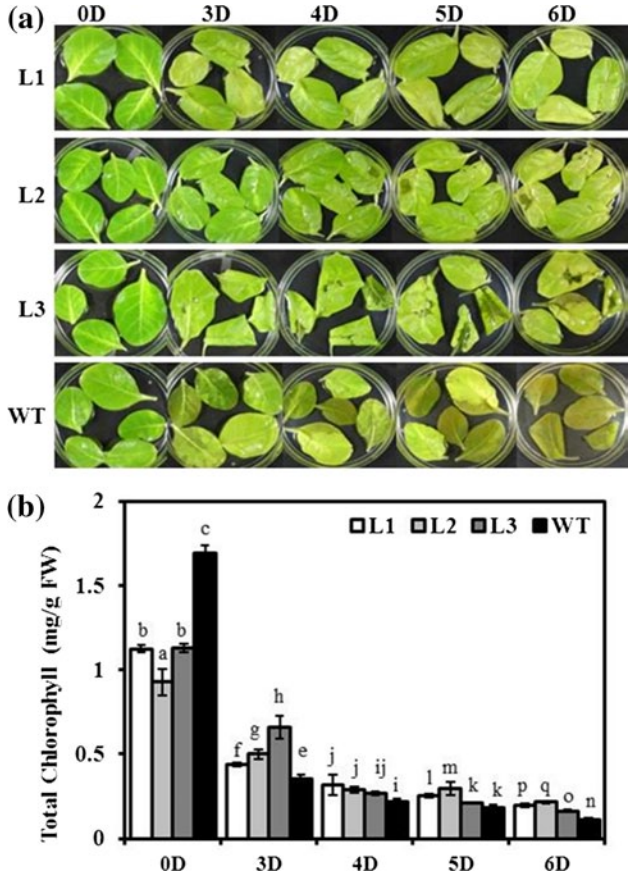

Fig. 8 *WsSGTL*1-*Nt* and WT plants were analysed for salinity tolerance via leaf disc assay. **a** Fully expanded leaves of 1-month-old plants were used to float on 6 ml of 200 mM NaCl solution (for salt stress) for 6 days. The effects of these treatments on leaf discs were assessed with phenotypic changes (chlorosis) from 0 day to 6 days. **b** These experimental leaves were also used to analyse change in total chlorophyll content (quantification of individual chlorophyll content in the leaf-disc assay was also performed; Fig. S7). Data are expressed as mean ± SD of three independent experiments. *Different letters* indicate significantly different values in a particular tissue (DMRT, $P \leq 0.05$) and ANOVA significant at $P \leq 0.01$

in their chlorophyll fluorescence and photosynthetic rate. This reduction in chlorophyll fluorescence and photosynthesis is not due to reduction in chlorophyll biogenesis since there was no significant reduction in expression of genes related to chlorophyll biogenesis; instead, the photosynthetic rate was reduced due to increased quantity of secondary metabolites (Ibrahim and Jaafar 2012). Photosynthetic rate increased with increasing level of irradiance from 225 to 900 μmol m^{-2} s^{-1}, while plant secondary metabolites were increased with decreasing level of irradiance from 900 to 225 μmol m^{-2} s^{-1} (Ibrahim and Jaafar 2012). Increase in several plant secondary metabolites in association with reduction in some other metabolites might cause reduction in availability of metabolic precursors for chlorophyll. Also, reduced photosynthesis is a kind of plant behaviour during stress condition to combat the stresses (Alves et al. 2002).

Reduced growth, physiological difference and modulation in metabolic profiles of transgenic plants might have none or weak correlation with the expression level of *WsSGTL*1. There are several reports from the area of metabolic engineering which analysed none or weak correlation between expression and phenotype (Misra et al. 2010b; Dubouzet et al. 2013; Pandey et al. 2013; Qiu et al. 2013). This suggested that though expression of *WsSGTL*1 might have enhanced the polypeptide synthesis, but due to limitation of substrates, the metabolite levels might not be different in different transgenic lines. While the expression of *WsSGTL*1 modulated the phenotype of transgenic lines significantly as compared to WT, it may not be necessarily parallel to expression of *WsSGTL*1. Therefore, similar pattern of stunted growth (Figs. 1, 3) has been observed in transgenic lines having significantly different transgene expression as compared to WT.

Enhanced glycosylation of secondary metabolites has been reported in this study through HPLC analysis. Sterols are precursors of various secondary metabolites including steroidal hormone and are also a component of cell membrane affecting its permeability and fluidity. Glycosterols, also known as saponins, are responsible for structural and functional diversity and act as antioxidants during growth and development by scavenging active oxygen species (Sayama et al. 2012). Earlier studies suggest that leaves of *N. tabacum* accumulate sterol glycosides in very low amount (Grunwald 1970, 1978) as compared to seed (Bush and Grunwald 1972). It has also been suggested that sterol glycosides present in seeds of *N. tabacum* are converted into free sterols during seed germination (Bush and Grunwald 1972). Based on these reports, only the seed extracts (not leaf extracts) were used to quantify glycosylated amount of sterols in the present study. Influence of *WsSGTL*1 on antioxidant system was analysed on the basis of enhanced glycosterols and flavonoids. Phenolic content and antioxidant enzymes were considered as the major contributors to the antioxidant properties of plants (Maizura et al. 2011). Considering this, glycosylation by WsSGTL1 of various secondary metabolites belonging to 3β-OH group, including sterols, flavonoids and phenolics, was analysed in this study through HPLC analysis, which revealed up to 2.3-fold more glycosylation of sterols and up to eightfold enhanced flavonoid contents in *WsSGTL*1-*Nt*.

Enzymatic (SOD, CAT and GPX) and non-enzymatic (flavonoids, phenolics) components of antioxidant system were quantified to confirm their increased production in *WsSGTL*1-*Nt*. Phenolics, (glycoside of caffeic acid, i.e. CGA and PA) which serve as antioxidants, increased 2- to 25-fold in the transgenic plants. The activities of antioxidant enzymes (SOD, CAT) were also enhanced

up to fourfold in *WsSGTL*1-*Nt*. The enhanced amount of these phenolic compounds may be one of the reasons for retarded growth of the transgenics in the present study. Antioxidant enzyme activity and phenolic contents were considered as the major contributors to the antioxidant properties of plants (Maizura et al. 2011). Study of Mucciarell et al. (2000) reported reduction in regeneration and proliferation of leaf tissue resulting in reduced growth due to accumulation of benzoic acid derivative (PA). It is well known in literature that the presence of phenolic compounds hinders growth and development and also differentiation in tissue culture (Ozyigit et al. 2007; Abdelwahd et al. 2008). Đinh et al. (2013) also reported accumulation of several phenolic compounds like chlorogenic acid and rutin in *N. tabacum* to minimize destructive effects caused by UVB radiation.

Such enhanced antioxidant system ought to provide adaptation and tolerance towards various kinds of biotic and abiotic stresses. *S. litura* larvae were allowed to feed on leaves of *WsSGTL*1-*Nt* and WT. A total of 23–27 % of weight loss in larvae of *S. litura* was observed under the influence of *WsSGTL*1 gene. Studies revealed that the insect resistance property in the form of larval growth and larval mortality resulted from enriched amount of phenylpropanoids, quercetin, chlorogenic acid (CGA) and rutin (Leiss et al. 2009; Misra et al. 2010b; Jadhav et al. 2012), which are environmentally friendly insecticides used for insect resistance in breeding programs (Misra et al. 2010b). However, involvement of altered levels of sterols in leaf tissues of transgenic lines causing insect tolerance cannot be ruled out and needs to be investigated in future. In the present study, correlation of retarded growth of larvae and altered sterol profile in leaf tissues was not analysed due to relatively lower quantity of glycosylated sterols in leaves of *N. tabacum* as reported by Grunwald (1970, 1978).

Mishra et al. (2013a) reported recently that expression of *WsSGTL*1 in heterologous system of *Arabidopsis thaliana* confers tolerance towards salt stress due to enhanced antioxidant system. The present study also analyses the differential content in active antioxidant system of *WsSGTL*1-*Nt* plants which helps to overcome negative effects of salt stress. As a result of tolerance behaviour, leaves of *WsSGTL*1-*Nt* plants survived more than a week in 200 mM NaCl. Besides other antioxidants, SOD leads to enzymatic antioxidant system being the first enzyme of the pathway (Alscher et al. 2002), along with CAT is supposed to play a major role to nullify salt stress with up to fourfold activity in *WsSGTL*1-*Nt* plants. The metabolic position of SOD makes it an important component in the direction of tolerant behaviour of mangroves like halophytic plants, which retain high level of SOD enzymatic activity (Margaret et al. 2010).

Acknowledgments The Director, Council of Scientific and Industrial Research, National Botanical Research Institute, is gratefully acknowledged by authors for the provided best facilities and moral support. PM is thankful to the Department of Biotechnology (Project No. GAP 231225), Govt. of India, for providing financial support to carry out the research work and CSIR-NMITLI project for identification of *WsSGTL*1 gene. VP is thankful to CSIR for the award of Senior Research Fellowship.

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