CHAPTER 3

OVEREXPRESSION OF WsSGTL1 GENE IN W. SOMNIFERA
AND CHARACTERIZATION OF TRANSGENIC LINES
3.1 INTRODUCTION

*W. somnifera* is especially attractive for studying the enzymes involved in steroidal transformations like glycosylation because it is a rich source of a variety of pharmacologically important withanolides and their derivatives such as withanosides, sitoindosides, withanomides etc. (Chatterjee et al. 2010; Chaturvedi et al. 2012; Chen et al. 2011; Jayaprakasam et al. 2003; Sharma et al. 2011). Withanolides are a group of naturally occurring steroids based on ergostane nucleus and characterized by a lactone-containing side chain (Abouzid et al. 2010). Involvement of steroid nucleus, side chain and additional ring formation are known for their structural diversity. The withanosides (saponins) are mainly comprised of withanolides with one or more glucose units attached to C-3 or C-27 positions (Bhattacharya et al. 2006; Matsuda et al. 2001). Withanolide biogenesis and accumulation is limited to specific genera of Solanaceae family, among them *Withania* shows maximum production of withanolide in more than 200 diversified forms, with or without functional groups (Chaurasiya et al. 2012; Chen et al. 2011; Misra et al. 2006). Glycosylation of sterols and their derivatives involves a glycosidic bond formation between sugar residue and a 3β-hydroxy group of sterols (Christie 2012; Shimamura 2012). Glycosylation reaction involves the transfer of sugar moieties to a wide range of acceptor molecules, mainly plant secondary metabolites. The reaction is catalyzed by glycosyltransferases (GTs) (EC 2.4.x.y) grouped in family 1 out of total the 94 families (http://www.cazy.org/GlycosylTransferases.html).

Pharmacological activities of withanolides, withaferin A and withanolide D (Khedgikar et al. 2013), get altered with respect to enhanced activity or specificity to specific disease after glycosylation, leading to synthesis of withanosides and sitoindoside (Bhattacharya et al. 1997, 2002; Bhattacharya et al. 2001; Jayaprakasam et al. 2003). This attracted the identification and characterization of members of GT family
in *W. somnifera* along with their functional importance. Family members of glycosyltransferases and their subfamily members have been highlighted in several studies for their involvement in response to several environmental stresses, development of vaccines, pigments, therapeutically important molecules etc. (Chang et al. 2011; Grille et al. 2010; Song et al. 2006). In this context, four members of sterol glycosyltransferases (*SGTs*) have been identified from *W. somnifera* (Chaturvedi et al. 2012; Sharma et al. 2007). *WsSGTL1*, a member of *SGT* of *W. somnifera* was cloned and characterized in *E. coli* by Sharma et al. (2007). Involvement, as well as importance of these genes, has been illustrated *in vitro* through enhanced expression during different stress conditions and during different elicitor treatments (Chaturvedi et al. 2012; Madina et al. 2007a, b; Sharma et al. 2007).

Previously, *WsSGTL1* was expressed in *E. coli* and enzyme activity of the partially purified recombinant enzyme was compared with the activity of *AtSGT* (Sharma et al. 2007). Various substrates including 5-α-cholestan-3β-ol, cholesterol, solasodine, brassicasterol, ergosterol, β-sitosterol, stigmasterol, pregnenolone, 3-β-hydroxy-16, 17-α-epoxypregnenolone, transandosterone, deacetyl 16-DPA and dehydroepiandrosterone were used to analyse the activity of the enzymes. Relative activities of enzymes were analysed, assuming 100% activity of *WsSGTL1* with dehydroepiandrosterone. *WsSGTL1* did not show any activity with cholesterol and 5-α-cholestan-3β-ol. Kinetic studies revealed that both the enzymes showed activity with sterols having a 3β-OH group and better reactivity of *WsSGTL1* against sterol without side chain (Sharma et al. 2007). Expression pattern of key biosynthetic *WsSGTL1* gene suggested that it is differentially expressed in response to different stresses and elicitors treatment exhibiting physiological role in stresses (Chaturvedi et al. 2012; Madina et al. 2007a, b; Sharma et al. 2007). Heterologous expression of *WsSGTL1* gene in model
plants *Arabidopsis* and tobacco demonstrated its role in glycosylation of sterols (Mishra et al. 2013; Pandey et al. 2014). Glycosylation not only stabilizes the products but also alters their physiological activities and governs intracellular distribution (Ullmann et al. 1993). Glycosylation increases the water solubility of lipophilic membrane sterols and, therefore, can lead to a change in cellular mobility, fluidity, permeability, hydration and phase behavior (Schuler et al. 1991; Webb et al. 1995). Glycosylation of secondary metabolites is considered as a promising option for improving their bioavailability and pharmacokinetics (Gachon et al. 2005; Ikeda et al. 2003).

Keeping in view the necessity to understand more about the steroidal pathway of *W. somnifera*, this present work was undertaken for functional characterization of the gene(s) related to the steroidal pathway. Hence, an important gene, sterol glycosyltransferase gene of *W. somnifera* (*WsSGTL1*) was targeted, as it catalyzes the glycosylation of phytosterols and related compounds (Madina et al. 2007). For functional analysis of *WsSGTL1* gene, transgenics of *W. somnifera* have been raised by overexpression of *WsSGTL1* via *Agrobacterium tumefaciens* mediated transformation. *A. tumefaciens* is the most reliable way to assess gene function by generating gain-of-function or loss-of-function mutants (Curtis et al. 2003). Developed overexpressed transgenic lines were analysed for the presence and expression of *WsSGTL1* gene. Also, study of current chapter is based on the analysis of transgenics in comparison to WT plants. Phenotypic differences, HPLC of withanolides (withaferin A, withanolide A, Withanone), withanoside V and three main sterols (sitosterol, stigmasterol, campesterol; to quantify glycosylated sterols) were analysed that reveals the enhanced growth and glycosylation of a range of secondary metabolites by *WsSGTL1*. In addition, a thorough analysis of the effect of abiotic stress (cold) and biotic stress (*Spodoptera litura*) was investigated on transgenic lines. *Spodoptera litura* was chosen for biotic stress because
it is an important polyphagous insect (Hadapad et al. 2001; Kandagal et al. 2012). Also, it is active throughout the year and fed a total of 112 cultivated crop plants in tropical and temperate zones (Sharma et al. 2005) and 40 species of wild plants (Kumar et al. 1993; Paulraj 2001), which makes it a model of a serious polyphagous pest (Sadek 2003). WsSGTL1 overexpressing lines of *Nicotiana tabacum* also showed significant resistance towards *S. litura* with up to 27% reduced larval weight (Pandey et al. 2014).

### 3.2 REVIEW OF LITERATURE

In the global market, *Withania* is well established as a positive health promoter due to its medicinal properties and its wide application as a therapeutic agent, which in turn, has attracted the interest of various phytochemists. With the result, the chemistry of *Withania* has been extensively studied, leading to the isolation and characterization of several groups of chemical constituents, which are of great biological and pharmacological interests. The biologically active chemical constituents are alkaloids, steroidal compounds, including ergostane type steroidal lactones (withaferin A, withanolides A-Y, withanone etc). Other constituents include saponins containing an additional acyl group (sitoindosides VII and VIII), withanolides with a glucose at carbon 27 (sitoindosides IX and X) and withanolide glycosides called withanosides I to VII (Ali 1997; Baraiya 2005; Gupta 2007; Matsuda 2001; Mishra et al. 2000; Misra et al. 2005). More than 200 major and minor primary and secondary metabolites have been identified from leaves, roots and fruits of *W. somnifera* and illustrated by Jayaprakasam et al. (2003), Chaurasiya et al. (2008), Misra et al. (2008), Chatterjee et al. (2010), Bharti et al. (2011), Sidhu et al. (2011), Bolleddula et al. (2012), Jain et al. (2012), Vanden et al. (2012), Bhatia et al. (2013) and Dhar et al. (2013). At present, more than 12 alkaloids, 40 withanolides, and several sitoindosides have been isolated and reported...
from *Withania* species (Mirjalili et al. 2009). Besides these, the plant carries a number of other secondary metabolites, including flavanol glycosides, sterols, phenolics, chemical constituents like withanol, acylsteryl glucosides, starch, reducing sugar, hantreacotane, ducitol, a variety of amino acids including aspartic acid, proline, tyrosine, alanine, glycine, glutamic acid, cystine, tryptophan, and high amount of iron (Gupta and Rana 2007).

Biosynthesis of secondary metabolite is a multistage and multilevel dynamic process prone to a range of intrinsic (developmental/physiological) and extrinsic (environmental) signals (Dhar et al. 2013). Metabolic modulation in plant species increases the fitness, sustainability and defence mechanism under different biotic and abiotic stresses. These activities are controlled by strict regulation of metabolic channels of biosynthetic, storage and transporter genes (Eldin et al. 2012; Yang et al. 2012). Withanolide biosynthesis involves a series of desaturation, hydroxylation, epoxidations, cyclization, chain elongation, and glycosylation steps. Identification and overexpression of key genes of withanolide biosynthesis pathway would be instrumental in understanding their synthesis and would also provide wealth information at the molecular level for metabolic engineering in this medicinal plant (Singh et al. 2015). Recently there has been a surge for the study of cloning and characterization of genes involved in withanolide biosynthetic pathway (Singh et al. 2015a). 3-Hydroxy 3-methylglutaryl CoA Reductase gene of *W.somnifera* (*Ws*HMGR) encodes a polypeptide of 575 amino acids which is differentially expressed in different tissues and chemotypes, this gene is significantly elevated when exposed to salicylic acid (SA), methyl jasmonate (MeJA), and mechanical injury (Akhtar et al. 2013). Farnesyl diphosphate synthase gene (FPPS) encodes a polypeptide of 343 amino acids, its amino
acid sequence homology and phylogenetic analysis suggest that $Ws_{FPPS}$ has close similarity to its counterparts from tomato ($Sl_{FPPS}$) and capsicum ($Caa_{FPPS}$) and its expression is significantly elevated in response to SA, MeJA and mechanical injury (Gupta et al. 2011). Squalene synthase ($SQS$) encoding a polypeptide of 411 amino acids is expressed in all tissues including roots, stem and leaves with the highest level of expression in leaves (Bhat et al. 2012). Silencing of $SQS$ causes reduced phytosterols, withanolides and biotic stress tolerance (Singh et al. 2015b). Squalene epoxidase ($SE$) encoding a protein of 531 amino acids with the highest transcript levels in leaves, as compared to stalk and root tissues also involved in various biotic and abiotic plant stresses (Razdan et al. 2013). Mutants of $SE$ gene have shown developmental abnormalities (Laranjeira et al. 2015). 1-deoxy-D-xylulose-5-phosphate synthase ($DXS$) and 1-deoxy-D-xylulose-5-phosphate reductase ($DXR$) encodes polypeptides of 717 and 475 amino acids residues, respectively. Its expression analysis suggested that $Ws_{DXS}$ and $Ws_{DXR}$ are differentially expressed with response to SA, MeJA, as well as mechanical injury in different tissues and chemotypes of Withania (Gupta et al. 2013). Cytochrome P450 reductase ($Cyt\_P450$) is the full-length paralogs of $Ws_{CPR1}$ and $Ws_{CPR2}$ encoding 685 and 713 amino acid residues, respectively. Phylogenetic analysis demonstrated that grouping of dual CPRs was in accordance with class I and class II of eudicotyledon CPRs (Rana et al. 2013). Glycosyltransferase gene of $W.\_somnifera$ ($UGT73A16$) showed 85-92 % homology with UGTs from other plants. HPLC analysis and hypsochromic shift indicated that UGT73A16 transfers a glucose molecule to several different flavonoids. Based on kinetic parameters, UGT73A16 shows more catalytic efficiency towards naringenin (Singh et al. 2013a). A member of 3β-hydroxysterol glucosyltransferase gene of $W.\_somnifera$ ($Ws_{SGTL1}$) amino acid sequence deduced from the 2103 bp open reading frame (ORF) showed homology (67-
45%) to the reported plant SGTs. The presence of two putative transmembrane domains suggested the association of SGTL1 with membrane and its relative expression is higher in roots and mature leaves (Sharma et al. 2007). Members of oxidosqualene cyclase (OSC) super-family: ß-amyrin synthase (OSC/BS), lupeol synthase (OSC/LS), and cycloartenol synthase (OSC/CS) were found to be spatially regulated at a transcriptional level. Empirical evidence suggested that repression of competitive branch OSCs like WsOSC/BS and WsOSC/LS possibly leads to the diversion of the substrate pool towards WsOSC/CS for increased withanolide production (Dhar et al. 2014; Singh et al. 2015a).

Table 3.1: Withanolides biosynthetic genes isolated from W.somnifera (Singh et al. 2015)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Gene size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-hydroxy-3-methylglutaryl coenzyme A reductase</td>
<td>HQ293119.1</td>
<td>2021</td>
<td>Akhtar et al. (2013)</td>
</tr>
<tr>
<td>Farnesyl pyrophosphate synthase</td>
<td>HM855234.1</td>
<td>1253</td>
<td>Gupta et al. (2011)</td>
</tr>
<tr>
<td>Squalene synthase</td>
<td>GU181386.1</td>
<td>1560</td>
<td>Gupta et al. (2012)</td>
</tr>
<tr>
<td>Squalene epoxidase</td>
<td>GU574803.1</td>
<td>1829</td>
<td>Bhat et al. (2012)</td>
</tr>
<tr>
<td>1-deoxy-D-reductase xylulose-5-phosphate reductase</td>
<td>JQ710679.1</td>
<td>1653</td>
<td>Gupta, et al. (2013)</td>
</tr>
<tr>
<td>1-deoxy-D-xylulose-</td>
<td>JQ710678.1</td>
<td>4162</td>
<td>Gupta et al. (2013)</td>
</tr>
</tbody>
</table>
3.3 MATERIALS AND METHODS

3.3.1 Plant material and culture conditions

The seeds of *Withania somnifera* (NMITLI-101) used in the present study were collected from the germplasm being maintained at CSIR-National Botanical Research Institute, Lucknow, India. The seeds were washed initially with 5% Teepol solution, 3-4 thorough washings with RO water and kept overnight in 0.1N HCl. Next day seeds were disinfected with 70% alcohol for 1 min and 0.1% (w/v) HgCl$_2$ for 15 min followed by 4-5 rinsed by sterile distilled water. Seeds were inoculated after a fine incision on the thicker seed coat region delicately under a microscope with the help of a blade protecting the embryo for germination on half-strength Murashige and Skoog’s (1962, MS) medium. Cultures were kept initially in the dark for 50-60 h and after germination, shifted to 16/8 h light/dark period at 25 ± 2 ºC. Segments of cotyledonary leaves were used as explants for transformation. For cold stress, the plants were shifted from 25 ºC.
in green house to illuminated growth chambers at 4 ºC for 24 hrs followed by recovery in glass house.

**3.3.2 Gene cloning and construct preparation**

Total RNA was extracted from the leaves of *W. somnifera*, using the Spectrum Plant Total RNA kit (SIGMA) and the first strand cDNA was synthesized using the Revert AID First Strand cDNA synthesis kit (Fermentas), according to the manufacturer’s instructions. The full-length open reading frame of the *WsSGTL1* (DQ356887.1) cDNA was isolated using oligonucleotide primers (Table 3.2). The PCR parameters were: initial denaturation at 94 ºC for 3 min, followed by 34 cycles of 94 ºC for 1 min annealing at 59 ºC (2 min), extension at 72 ºC (2 min) and final extension at 72 ºC for 5 min. Plant expression construct was prepared by ligating PCR product (*WsSGTL1* cDNA) into binary vector pBI121 at *XbaI* and *SacI* sites. The resultant pBI121 harboring *WsSGTL1* gene (2.1 kb) in sense orientation was mobilized into LBA4404 strain of *Agrobacterium tumefaciens* by electroporation (Figure 3.1). *Agrobacterium* cells harboring pBI121 *WsSGTL1* were selected on LB medium containing 50 mg/l kanamycin and stored as glycerol stocks at −80 ºC.

![Fig 3.1](image.png) Vector pBI121 used for transformation (pBI121:WsSGTL1). Promoter DECaMV35S (600 bp), WsSGTL1 (2.1 Kb) in sense orientation
3.3.3 *A. tumefaciens*-mediated transformation, selection and regeneration of transformed plants

The excised cotyledonary leaf from in vitro grown seedlings was transformed with LBA4404 strain harboring the binary vector pBI121-\textit{WsSGTLI} gene. The explants were infected with resuspended \textit{A.tumefaciens} cultures (OD$_{600}$ = 0.4) for 20 min under continuous shaking at room temperature and sonicated in a Branson sonifier (Branson-Ultrasonic Cleaner, USA, Model No. 3210EMTH) with three pulses of 4 seconds duration at the maximum output power (14 W). Thereafter transferred onto co-cultivation medium as MS medium having 1.0 mg/l benzyl amino purine (BAP) + 0.1 mg/l indole acetic acid (IAA) for 3 days at 25±2 °C. After transformation the explants were rinsed 3-4 times with sterilized distilled water supplemented with 250 mg/l cefotaxim to eradicate the bacterial suspension, blotted and cultured in shoot inducing MS medium supplemented with 1.0 mg/l BAP + 0.1 mg/l IAA + 0.25 mg/l gibberellic acid (GA$_3$). Selection of transformants was done on 50 mg/l kanamycin. The explants were subcultured at 15 d intervals. Cultures were incubated at 25 ± 2 °C under 3 klux light through fluorescent tubes for 16-h light: 8-h dark photo-cycle at a light intensity of 50-60 µmol m$^{-2}$ s$^{-1}$. All the phytohormones and antibiotics used for the preparation of media were from M/s Sigma Aldrich, USA.

Regenerated shoots that survived on kanamycin selection were rooted on MS medium containing indolebutyric acid (IBA, 1 mg/l) + 50 mg/l kanamycin. The plantlets with well developed roots were transferred to earthen pots for hardening and hardened plants were grown in the glass house.
3.3.4 Molecular confirmation of transgenics

PCR confirmation was done in the 9 putative transgenic (T₀) plants for the presence of \textit{NPTII} gene. Seeds obtained from 5 lines, were collected and T1 progeny were raised in pots in glass house. The plants at 3-4 leaf stage were used for molecular analysis. Genomic DNA was extracted from the leaves of five independent lines of T₁ generation and WT plants using DNeasy Plant Minikit, Qiagen. The PCR was conducted using \textit{NPTII} gene primers (Table 3.2) generating a fragment of about 790 bp. The reaction was performed as follows: 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec and 72 °C for 5 min as final extension step. The amplified products were analysed by 1% agarose gel electrophoresis.

3.3.5 RNA isolation, RT-PCR and relative expression

RNA was isolated 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) and subjected to reverse transcription to generate first-strand cDNA using oligo dT primers (Thermo Fisher Scientific).

To analyze the expression pattern of T₁ transgenics and WT, were subjected to semi quantitative RT-PCR and real time PCR, the primers are listed in Table 3.2. Semiquantitative PCR analysis was carried out using PCR Master-mix (Thermo Fisher Scientific) using the following cycle conditions: 94 °C for 2 min, 26 cycles at 94 °C for 30 s, 60 °C for 30 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. Real-time PCR was performed in 20 μl for set of selected genes using Power SYBR Green PCR Master Mix (ABI, USA). After obtaining ct value for each reaction, the
fold change was calculated by using Delta-Delta ct method. Three independent experiments were conducted using three biological replications.

3.3.6 Southern blot analysis
Genomic DNA from three highly overexpressing WsSGTL1 lines (L1, L3 and L6) and WT were isolated by C-TAB method (Doyle and Doyle, 1987). The genomic DNA (20 µg) was digested with EcoRI restriction enzyme and resolved on 0.8% agarose gel. After electrophoresis, DNA was transferred to Hybond-N+ membranes (GE Healthcare) using standard protocol (Sambrook et al. 2001). A radiaolabelled probe was prepared using 550 bp fragment of NPTII gene (primer given in Table 3.2) and overnight hybridization was performed (Sambrook et al. 2001). After hybridization, autoradiography was done and analyzed on Molecular Imager FX (Bio-Rad).

3.3.7 Phenotypic analysis
T1 transgenics from three independent transgenic lines as L1, L2, L3 and WT plants of W. somnifera, grown in a green house were collected for phenotypic study. Leaf area was determined immediately after harvest and plotted on graph paper. Different parameters like seed weight (per 100 seeds), height of the plant, no. of nodes, intermodal length, and fruit weight (per 10 fruits) were taken into account. Specific leaf area was calculated as area of fresh leaf divided by its oven dry weight. Leaves, stem and roots were dried at 60 °C until a constant weight was obtained (4 d) and final dry weight was determined.

3.3.8 Extraction and HPLC analysis of secondary plant metabolites
3.3.8.1 Withanolides
The leaves and roots of T1 transgenic lines and WT W. somnifera were crushed and dried into liquid nitrogen. The sample was extracted overnight in 10 ml of methanol water (25:75 v/v) at room temperature on orbital shaker and filtered. The filtrate was
collected and the residue was extracted twice at 4 h intervals with the same amount of extracts. The filtrates were pooled and extracted with n-hexane (3 X 30 ml). The n-hexane fraction was discarded and methanol-water fraction was further extracted with chloroform (3 X 30 ml). The chloroform fractions were pooled and was concentrated up to dryness (39 °C). The residue was dissolved into HPLC grade methanol (2 ml) and filtered through 0.45 µM nylon filter (Millipore). The solution was further diluted to 10 folds and injected into HPLC.

Separation for qualitative and quantitative analysis of the withanolides were performed by HPLC-PDA with a Shimadzu (Japan) LC-10A system comprising an LC-10AT dual-pump system, a SPD-10A PDA detector (operated at 227 nm), and Rheodyne injection valve with 20-µL sample loop. Compounds were separated on a (Merck) RP-C18 column (4.6 mm × 250 mm, 5-µm pore size) protected by a guard column containing the same packing. The mobile phase prepared from 0.1% (v/v) acetic acid in HPLC-grade water (component A) and 0.1% (v/v) acetic acid in HPLC-grade methanol (component B) in gradient mode. Before use, the components were filtered through 0.45-µm nylon filters and de-aerated in an ultrasonic bath. The gradient was from 40-60% B in 0-30 min, hold up for 2 min, then from 60% to 75% B in 32 min to 45 min, 75% to 95% B in 45 min to 54 min and 100% in 54 min to 55 min. The flow rate was 0.6 ml min\(^{-1}\). Data were integrated by Shimadzu class VP series software and results were obtained by comparison with the standards. Results are mean values from three replicate analyses of the same sample. All samples and solutions were filtered through 0.45-µm nylon filters before analysis by HPLC. Standard compounds, viz., withaferin A, withanolide A, withanone and withanoside V (Sigma) were accurately weighed (10 mg separately) and dissolved in 10 ml methanol to prepare stock solution.
of 1 mg/ml. These stock solutions were subsequently diluted to prepare solutions with concentrations in the range of 0.5 mg/ml to 50 mg/ml. These working standard solutions were used for quantification of samples.

3.3.8.2 Sterols

Dried leaf samples were homogenized in 1 ml of methanol (80% v/v) followed by incubation at 40 °C for 2 h. Glycosylated and nonglycosylated sterols were analysed quantitatively from acid-hydrolysed or non-hydrolysed extracts of WsSGTL1 and WT plants, respectively. For acid hydrolysis, leaf samples were homogenized in 1 ml of ethyl acetate in 1% HCl and incubated at 90 °C for 2 h. Acid hydrolysis of extract was required to obtain free sterols by breaking of bonds between sterols and sugar moiety. Homogenous extracts obtained were dissolved in high-performance liquid chromatography (HPLC)-grade methanol and drained through 0.2-µm filter (Millipore, India) before conducting HPLC analysis. Free sterols were quantified before and after hydrolysis, and the difference between hydrolysed and nonhydrolysed sterols would be due to the glycosylation action of WsSGTL1 (Pandey et al. 2014).

For the estimation of sterols the above HPLC system was used. Sterols (β-sitosterol, stigmasterol and campesterol) were separated by isocratic solution of acetonitrile and water (95:5, v/v) at flow rate of 2 ml/min at 34 °C for 40 min run time. The scanning of sterols was performed at 202 nm. Standard compounds, viz., β-sitosterol, stigmasterol and campesterol (Sigma) were accurately weighed (10 mg separately) and dissolved in 10 ml methanol to prepare stock solution of 1 mg/ml. These stock solutions were subsequently diluted to prepare solutions with concentrations in the range of 0.5–50 mg/ml. These working standard solutions were used for quantification in samples.
Parameters like specificity, linearity, peak purity, precision and accuracy, limits of quantification and detection and robustness were followed to quantify all compounds by HPLC (Pandey et al. 2014).

All data were integrated by Shimadzu class VP series software and results were obtained by comparison with standards. The results were the mean values from three independent experiments with three biological replicates.

3.3.9 Physiological studies

For each experiment, T<sub>1</sub> generation of three independent transgenic lines and WT, five or six plants per line were collected from green house. Leaf gas exchange was measured with Li-Cor 6400 gas exchange portable photosynthesis system (Li-Cor, Lincoln, Nebraska). The CO<sub>2</sub> levels inside the leaf cuvette was maintained at 400 ppm, photosynthetic photon flux density (PPFD) was 400 μmol m<sup>−2</sup> s<sup>−1</sup>, leaf temperature was 25 °C and leaf-air vapor pressure deficit was <3.0 kPa. Leaves were then exposed gradually to increasing PPFD to 400 μmol photons m<sup>−2</sup> s<sup>−1</sup> and measurements of steady-state A, gs, E, internal CO<sub>2</sub> concentration (C<sub>i</sub>), and quantum yield of PSII in the light (□) were made. Intrinsic water use efficiency (WUE) was calculated as the A/E ratio.

Fluorescence was measured with An Imaging-PAM, M-Series Chlorophyll Fluorometer (Walz, Effeltrich, Germany) used to study the chlorophyll fluorescence parameters of Fv/Fm during the control, cold stress and recovery stage of the plant. Before the measurement of maximum photochemical efficiency of photosystem II (PSII) the leaves were dark adapted at vapour pressure deficit (VPD) ranging between 0.5 and 1 kPa, leaf temperature of 25 °C, and CO<sub>2</sub> concentration of 400 μmol mol<sup>−1</sup>, and Fv/Fm measurements were made using a light pulse of 2000 μmol photons m<sup>−2</sup> s<sup>−1</sup>.
Light response curves for fluorescence (PSII) and P-700 (PSI) were measured with DUAL-PAM-100 (Walz) as described by Klughammer and Schreiber (2008). Maximal fluorescence and maximal P700 changes were obtained from dark adapted leaves (as described above) and then leaves were exposed to high light (i.e. 1200 to 1500 µmol photons m\(^{-2}\) s\(^{-1}\)) for 30 min to obtain a steady state before commencing measurements of several fluorescence parameters every 5 min at each PPFD (ranging from 11 to 2000 µmol photons m\(^{-2}\) s\(^{-1}\)). The quantum yield of PSI Y(I) is defined by the proportion of overall P700 that is reduced in a given state and not limited by the acceptor side. It is calculated from the complementary PSI quantum yields of non-photochemical energy dissipation due to donor-side limitation and acceptor-side limitation Y(ND) and Y(NA) (Schreiber and Klughammer 2008). The quantum yields of PSII, non photochemical quenching due to regulated energy dissipation, and non-photochemical quenching due to non regulated energy dissipation Y(II), Y(NPQ), and Y(NO) were calculated from the measurement of chlorophyll fluorescence, as described by Kramer et al. (2004). The electron transport rates ETRI and ETRII was calculated as ETR (I or II) = Y (I or II) × PPFD × 0.5 × abs, where Y is the apparent quantum yield, 0.5 is the proportion of absorbed light reaching PSI or PSII, and absI is absorbed irradiance, taken as 0.84 of incident irradiance. NPQ was calculated as (Fm – Fm’)/Fm’. Chlorophyll and carotenoid contents were measured by isolating chlorophyll, carotenoids from leaf discs and calculated according to Wellburn (1994).

Anthocyanin was extracted with 1% acidified methanol. Absorbance of the supernatant was measured at 530 and 650 nm and corrected values were calculated as, AA = A\(_{530}\) - (0.288 × A\(_{650}\)), where AA is corrected anthocyanin absorbance. Total anthocyanin content was then calculated using this corrected absorbance and a molar
absorbance coefficient for anthocyanin at 530 nm of 30000 L mol$^{-1}$ cm$^{-1}$ (Murray and Hackett 1991).

Control and cold treated leaf samples (200 mg) were incubated in 20 ml distilled water, vacuum infiltrated (3 times for 3 min each at 25 psi), and shaken well for 1 h at 250 rpm. Electrical conductivity was measured for each sample with a conductivity meter before and after autoclaving (121 °C for 20 min). Initial leakage was expressed as percent of the final conductivity and the percent leakage for each treatment temperature was converted to percent injury.

3.3.10 Estimation of stomatal density
Stomata were observed from leaf epidermis under a light microscope (Leica DM2500). Stomata were counted in an area of 1 mm$^2$ in three different regions from three independent leaves of the same position from three independent plants.

3.3.11 Insect bioassay
The larvae of *Spodoptera litura* were maintained in the laboratory on castor leaves at 26 ± 2 °C and 80% relative humidity and bioassays were also performed in similar conditions. For bioassay detached leaf of 2-months-old transgenic and WT *W.somnifera* plants were used. Leaves were washed in distilled water; air dried and placed in bioassay vials. The neonate larvae of *S.litura* were separately released on leaf (10 larvae/leaf) and observations were recorded for feeding and mortality of larvae. Three replicates were maintained for bioassay with each insect.

3.3.12 Data collection and statistical analysis
Morphological changes were recorded by visual observations. Each experiment was repeated three times using three biological replicates. The values of data are mean ± standard deviation of three replicates. All statistical analyses were performed by using ANOVA-INDOSTAT software.
Table 3.2 List of primers used in this study.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence of Primers</th>
<th>Forward/Reverse</th>
<th>Use and specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>WsSGTL1F</td>
<td>GCTCTAGAATGGACAGTAATGGGCATAATGGCA</td>
<td>Forward</td>
<td>For cloning of WsSGTL1 gene</td>
</tr>
<tr>
<td>WsSGTL1R</td>
<td>ATAGCGAGGCTCTAAGAACCACAGGC</td>
<td>Reverse</td>
<td>For cloning of WsSGTL1 gene</td>
</tr>
<tr>
<td>NPTII F1</td>
<td>ATGATTGAACAAGATGGATTGCACGC</td>
<td>Forward</td>
<td>For confirmation of overexpressed WsSGTL1 transgenic W.somnifera</td>
</tr>
<tr>
<td>NPTII R1</td>
<td>TCAGAAGAACACGTCAAGAACGC</td>
<td>Reverse</td>
<td>For confirmation of overexpressed WsSGTL1 transgenic W.somnifera</td>
</tr>
<tr>
<td>WsSGTL1F1</td>
<td>GACAGGACCATGTATGGTGTATTC</td>
<td>Forward</td>
<td>Gene specific primer for semiquantitative PCR analysis</td>
</tr>
<tr>
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<td>GCTCGAGGGTGGAACACACTAGAA</td>
<td>Reverse</td>
<td>Gene specific primer for semiquantitative PCR analysis</td>
</tr>
<tr>
<td>Actin F</td>
<td>GTATTGTGTTGGACTCTGGTGATGTTG</td>
<td>Forward</td>
<td>Housekeeping gene of Nicotiana for semiquantitative PCR analysis</td>
</tr>
<tr>
<td>Actin R</td>
<td>GATGGATCCCTCAATCCAGACACTGTA</td>
<td>Reverse</td>
<td>Housekeeping gene of Nicotiana for semiquantitative PCR analysis</td>
</tr>
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</table>
### 3.4 RESULTS

#### 3.4.1 Analysis of WsSGTL1 gene in overexpression lines of *W. somnifera*

To analyse the function of *WsSGTL1* gene of *W. somnifera*, transgenic lines of *W. somnifera* overexpressing *WsSGTL1* gene was generated. Several plants were regenerated from the kanamycin resistant cotyledonary leaf explant. Nine T₀ putative transformants were confirmed with PCR and showed amplification of the neomycinphosphotransferase (*NPTII*) gene (Fig. 3.2). All the nine T₀ transgenics grew well but among them 2 were nonfertile. Therefore, seeds were collected from all the seven T₀ lines and grown on kanamycin medium and T₁ progeny were raised, out of this five transgenic lines of T₁ generation were raised as shown in Fig.3.3 (A-G). All the transgenic plants appeared larger and bigger in phenotype as compared to WT control.


T<sub>1</sub> homozygous overexpression lines of WsSGTL1 <i>W.somnifera</i> plants were identified through PCR analysis using their genomic DNA analysis. <i>NPTII</i> gene was amplified in transgenic lines but not in the non-transformed WT plants (Fig. 3.4A). To assess the expression of the WsSGTL1 gene in transgenic <i>W.somnifera</i> plants, 7-weeks-old five healthy looking T<sub>1</sub> transgenic lines were subjected to RT-PCR (Fig. 3.4B). The actin gene which gave a band of 622 bp was used as internal control. A significantly higher levels of WsSGTL1 (2.8 to 29.6 fold) over non transformed WT seedlings was obtained through quantitative real time PCR. Real time PCR showed that 29.6 fold expression was obtained in one of the overexpressive WsSGTL1 line (Fig. 3.4C). Southern blot analysis confirmed the insertion of single copy of <i>NPTII</i> gene in L1 and L3 lines, while two copies were inserted in L6 line and no <i>NPTII</i> gene in WT (Fig. 3.4D). Therefore, on the basis of realtime PCR and southern blot analysis, three lines of overexpressive WsSGTL1 transcripts (L1, L3 & L6) were selected for further studies.

**Figure 3.2** PCR analysis for the detection of <i>NPTII</i> gene in <i>T<sub>0</sub></i> transformants. M 100-bp ladder, L1-L9 putative transformants, +C positive control, WT Wild type.
Figure 3.3 *Agrobacterium tumefaciens*- mediated transformation of *W. somnifera*. (A) Cotyledonary leaf explants. (B-D) Shoot induction on selection media. (E) Plantlet formation. (F) Putative transgenic plant under in vitro condition. (G) Putative transgenic plant in pot.

3.4.2 Effect of overexpression of *WsSGTL1* gene on plant growth and development

Transgenic *W. somnifera* plants overexpressing *WsSGTL1* grew vigorously than WT (Fig. 3.5 A, B, C) plants. The transgenic lines were taller and stronger than WT with higher number of nodes (Fig. 3.6 A, B, C). The total leaf area and biomass was observed to be four times higher than WT plants (Fig. 3.6 D, E). This was supported by the absence of any significant change in the specific leaf area (Fig. 2F). Significant increase
in growth rate and biomass production was evident mainly due to increase in leaf and stem dry weights (Fig. 3.6 E, G). In transgenic plants, shoot and root biomass increased 215.4 mg to 288.8 mg and 135.8 mg to 227.8 mg, respectively, as compared to WT, where shoot biomass was 161.4 mg and root biomass was 105.4 mg (Fig.3.6 H,I). The transgenics showed comparatively low seed yield without any significant difference in fruit weight and seed weight (Fig. 3.6 J, K, L).

![Figure 3.4. WsSGTL1 gene expression analysis in T1 transgenics of W.somnifera. (A) PCR analysis for the detection of NPTII gene (L to R); WT-wild type, C-nontransgenic control, L1 to L7 transgenics, M-100 bp ladder. (B) RT-PCR analysis for the detection of WsSGTL1 expression; WT, L1, L3, L4, L6 & L7-transgenics. (C) Relative expression of WsSGTL1 gene by real time PCR. (D) Southern blot analysis showing the stable integration of NPTII gene in T1 progeny of transgenic W.somnifera overexpressing WsSGTL1 gene; Lane 1-WT, Lane 2-L1, Lane 3-L3 and Lane 4-L6.](image)
Figure 3.5 Morphological characterization of T₁ transgenics of *W. somnifera*. A 8-weeks-old seedlings growing in the pot. B 4-months-old plants in the pot. C Morphological difference in the leaf size (L to R) of the transgenic lines and WT.

3.4.3 Overexpression of *WsSGTL1* reduces withanolides content and directs the withanoside V accumulation

To investigate the *WsSGTL1* overexpression effect on withanolides, major withanolides were analysed by HPLC using leaf and root tissues of transgenics and WT plants. It was observed that there was a drastic reduction of withanolides accumulation in transgenic lines than WT plants as evident from Fig. 3.7. Among the individual withanolides, withaferin A, the major withanolide present in leaves exhibited the decline of approximately 4.7 fold followed by withanolide A (approximately 3 fold) and
withanone (approximately 7 fold, Fig. 3.7 A-C). Further, to validate the results obtained in overexpressed lines, transgenic lines and WT leaves were subjected to analyse glycowithanolide i.e. withanoside V content. It was observed that transgenics exhibited drastically higher amount of withanoside V (approximately 10 fold, Fig. 3.7 D).

**Figure 3.6** Growth analysis of WT and transgenic plants. Plants were harvested after 14-weeks of growth. Results are mean ± SE of five different plants. Asterisks indicate that mean values are significantly different between WT and transgenic plants (*, P < 0.05; **, P < 0.01; ***, P <0.001).

Demonstrating the effectiveness of overexpression of WsSGTL1 gene, root sample were also analysed for withanolide content. Considerable decrease in withanolide A (approximately 7.3 fold), withanone (approximately 10 fold) and withaferin A content (approximately 6 fold, Fig. 3.7E-G) was observed. For further confirmation of results, accumulation of withanoside V was investigated which was observed higher
(approximately 10 fold, Fig. 3.7 H). The reduction of withanolides and increase in withanoside V in root samples of transgenic lines was similar to leaf samples of transgenic lines, demonstrating the effectiveness of WsSGTL1 gene involved in biosynthesis of withanolide thereby regulating glycosylation of withanolides.
Figure 3.7 Quantitative estimation of withanolides in WT and transgenic plants (L1, L2 and L3) illustrating enhanced glycosylation of withanolides A-D From samples of leaf extract. E-H From samples of root extract. I-L HPLC chromatogram Results are mean ± SE of three independent experiments. Asterisks indicate that mean values are significantly different between WT and transgenic plants (*, P < 0.05; **, P < 0.01; ***, P <0.001)

3.4.4 Synthesis of glycosylated sterols in transgenic plants

To determine the effect of WsSGTLI gene on sterol biosynthesis, the overexpressed WsSGTLI lines were examined by HPLC analysis for few sterols individually.
Quantification of sterols from HPLC analysis indicated a significant increase in the level of glycosylated sterols. Among individual sterols, increase in glycosylated campesterol was approximately 5 fold (Fig. 3.8A), glycosylated β-sitosterol was approximately 10 fold (Fig. 3.8B) and glycosylated stigmasterol was approximately 13.5 fold (Fig. 3.8C) was obtained in transgenic lines as compared to WT plants.

**Figure 3.8** 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 (*black bar* represents sterols before hydrolysis and *grey bar* represents sterols after hydrolysis) Difference between free sterols before and after hydrolysis resulted in glycosylated amount. Data are expressed as mean ± SE of three independent experiments. Asterisks indicate that mean values are significantly different between WT and transgenic plants (*, P < 0.05; **, P < 0.01; ***, P <0.001).
3.4.5 Physiological activity of transgenic plants under cold stress

3.4.5.1 Chlorophyll fluorescence image analysis: Chlorophyll fluorescence imaging for maximum photochemical quantum yield (Fv/Fm) was shown in Fig. 3.9. Under control conditions, WT and transgenic lines showed Fv/Fm near to 0.8 which is the maximum for healthy green plants. However, under cold treatment, WT plants showed reduction in Fv/Fm by 40%, whereas transgenic lines showed 10% to 20% reduction. Fig. 3.9E showed minimum heterogeneity due to maximum leaf area damaged, while 3.9F-H showed high heterogeneity because of uneven damaged reaction centers. Some areas showed damages while others were as good as untreated plants. After 10 days of recovery, L2 line showed maximum recovery in Fv/Fm followed by L1, L3 and WT plants (Fig. 3.9 I-L).

Figure 3.9 Chlorophyll Fluorescence Imaging for maximum photochemical quantum yield (Fv/Fm) for WT and WsSGTL1 transgenic lines (L1, L2 and L3) of W.somnifera. A-D Before cold stress. E-H After 1h of cold treatment (0 °C). I-L After recovery of 10

54
days. The false color code depicted on the right side of the images ranges from 0.000 (black) to 1.000 (pink).

3.4.5.2 Photosynthesis rate (A), transpiration rate (E) and stomatal conductance (gs):
Photosynthesis rate (A) showed significant reduction during cold stress condition (Fig. 3.10A). WT plants reduced A by 81%, while in transgenic lines (L1, L2, L3) it was reduced by 43%, 39% and 78%, respectively. Only L2 showed 100% recovery after 10 days of cold stress. Minimum recovery was recorded in WT plants. Transpiration rate (E) and stomatal conductance (gs) were also significantly reduced under cold stress but recovered after 10 days. L3 line showed maximum E and gs under control as well as recovery conditions. L2 showed significant reduction in E but not in gs under cold stress conditions (Fig. 3.10 B, C). However E and gs decreased in L1, L3 and WT plants under cold stress. E and gs fully recovered in all the lines under recovery condition except WT. Intrinsic water use efficiency (WUE) increased in all the lines except WT during cold stress and did not come back under recovery condition (Fig. 3.10 D).

Figure 3.10 Physiological parameters measured in WT and transgenic lines (L1, L2 and L3) of W.somnifera. A Photosynthesis rate. B Stomatal conductance. C Transpiration. D
Intrinsic water use efficiency was monitored in plants before cold stress, immediate after 1 hour of cold treatment (0 °C) and on 10th day of recovery. Data represent the means ±SD of five separate measurements. Asterisks indicate that mean values are significantly different between WT and transgenic plants (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

3.4.5.3 Analysis of chlorophyll, carotenoid, anthocyanin, water potential, electrolyte leakage and stomatal density: Since A depends on chlorophyll content of the leaf, therefore we have also estimated the chlorophyll content and observed significant reduction in chlorophyll and carotenoid contents after cold stress treatment. Chlorophyll content was decreased under cold stress and recovered after 10 days. Hundred percent recovery of chlorophyll could be observed in L1 under recovery condition. L3 line did not show significant reduction under cold stress but it was reduced under recovery condition. Under recovery condition, chlorophyll content recovered, whereas, carotenoids did not increase in any line (Fig. 3.11 A, B). Anthocyanin content increased by 2, 4, 7, 3 folds in WT, L1, L2, L3 lines, respectively, under cold condition and did not decrease again under recovery condition (Fig. 3.11 C). Water potential (WP) was increased by 2.2 and 1.7 folds in L2 and L3 lines, respectively, under cold stress, however in WT and L1 line it did not increase much. Under recovery condition, WP reached at their control level in all the four lines (Fig. 3.11 D). Membrane damages were studied as electrolyte leakage and expressed in relation to control. Maximum electrolyte leakage was observed in WT plants under cold stress condition (Fig. 3.12 A), while minimum increase was in L2 line. Electrolyte leakage decreased under recovery condition in all the lines but not up to control level. Stomatal density in the control and the transgenic lines was calculated by counting the stomata in leaves of the WT, L1, L2 and L3 plants (three leaves/line at three different locations in all three transgenic lines).
A considerable increase in the stomatal density was observed in all the transgenic lines (Fig. 3.12 B). This represented an increase of 18–38% in the total adaxial stomatal density as compared to WT plants. There was, however, no change in the stomatal size from that in the WT plants.

**Figure 3.11** Pigment concentrations in WT and transgenic lines (L1, L2 and L3) of *W.somnifera*. A Chlorophyll. B Carotenoids. C Anthocyanin. D Water potential before cold stress, immediate after 1h of cold treatment (0 °C) and on 10th day of recovery. Data represent the means ±SD of five separate measurements. Asterisks indicate that mean values are significantly different between WT and transgenic plants (*, P < 0.05; **, P < 0.01; ***, P <0.001).
Figure 3.12 A Electrolyte leakage in terms of % conductivity for WT and transgenic lines before cold stress, immediate after 1h of cold treatment (0 °C) and on 10th day of recovery. B Stomatal density of the upper leaf epidermis of WT and transgenic lines L1, L2 and L3. Values represent an average stomatal density ± SD in an area of 1 mm² of three independent leaves. C Leaf adaxial surface showing stomatal density in WT and transgenic overexpressing (WsSGTL1) W.somnifera plants of three independent lines, L1, L2 and L3. The small black bar at the base of each picture on the left hand side represents a length of 20 µm. Asterisks indicate that mean values are significantly different between WT and transgenic plants (*, P < 0.05; **, P < 0.01; ***, P <0.001).

3.4.5.4 Energy distribution between PSI and PSII: Light saturation curves were performed for WT and transgenic lines in control, cold stress and recovery condition to understand activity of photosystem I (PSI) and photosystem II (PSII) along with flux distribution between quenching and photosynthesis (Fig.3.13,3.14,3.15). Under control condition, no significant differences were observed in quantum yields of PSII (Y(II)),
non-photochemical quenching due to non regulated energy dissipation (Y(NO)), non 
photochemical quenching due to regulated energy dissipation (Y(NPQ)) (Fig. 3.13 
A,D,G) quantum yield of PSI (Y(I)) yield of non-photochemical energy dissipation due 
to donor-side limitation (Y(ND)) and acceptor side (Y(NA)) (Fig. 3.13 M,P,S) and 
electron transport rate ETR (I) and (II) in L2 line. Both WT and L2 line behaved in 
similar manner at different light intensity during the light response curve. However 
under cold stress condition, Y(II) was high for L2 line (Fig. 3.13 B). Y(NO) increased 
and Y(NPQ) decreased in WT plants (Fig. 9G) and these changes could not recover 
much during recovery condition (Fig. 3.13 F,I). In L2 line Y(NO) and Y(NPQ) did not 
change under cold and recovery stage. It maintained at steady phase and kept high the 
photochemical quantum yield of PSII. In WT plants, Y(I) also decreased during cold 
stress. Limitation at donor side Y(ND) decreased in L2 line while remained constant in 
WT plants under stress condition (Fig. 3.13 Q). Y(NA) did not show any change in WT 
and L2 line under control, stress and recovery condition. It remained at constant level of 
0.08 (Fig. 3.13 S, T, U). Electron transfer rate through PSI and PSII decreased in WT 
plants under stress condition (Fig. 3.13 J-L, V-X). However in L2 line, it reduced 
slightly. In control and recovery condition at low light there was no difference in L2 and 
WT line in reference to ETR (I) and (II). At high light there were slight differences (Fig. 
3.13 J, L, V, X). L1 and L3 lines also behaved in the same manner as L2 line (Fig. 3.14, 
3.15)
Figure 3.13 Light response curves for energy fluxes of PSII and PSI for WT and L2 before cold stress, immediate after 1h of cold treatment (0 °C) and on 10th day of recovery. A-C Photochemical quantum yield for PSII Y(II). D-F Quantum yield of non-light-induced non-photochemical fluorescence quenching for PSII, Y(NO). G-I Quantum yield of light-induced non-photochemical fluorescence quenching for PSII, Y(NPQ). J-L ETR of PSII (ETRII). M-O Photochemical quantum yield for PSI Y(I). P-R Quantum yield of non-photochemical energy dissipation in PSI due to donor side limitation, Y(ND). S-U Quantum yield of non-photochemical energy dissipation in PSI due to acceptor side limitation Y(NA). V-X ETR of PSI (ETRI). Values are average ± SEs of three to five replicates.
Figure 3.14 Light response curves for energy fluxes of PSII and PSI for WT and L1 before cold stress, immediate after 1h of cold treatment (0 ºC) and on 10th day of recovery. A-C Photochemical quantum yield for PSII Y(II). D-F Quantum yield of non-light-induced non-photochemical fluorescence quenching for PSII, Y(NO). G-I Quantum yield of light-induced non-photochemical fluorescence quenching for PSII, Y(NPQ). J-L ETR of PSII (ETRII). M-O Photochemical quantum yield for PSI Y(I). P-R Quantum yield of non-photochemical energy dissipation in PSI due to donor side limitation, Y(ND). S-U Quantum yield of non-photochemical energy dissipation in PSI due to acceptor side limitation Y(NA). V-X ETR of PSI (ETRI). Values are average ± SEs of three to five replicates.

3.4.6 Evaluation of transgenic plants for S. litura resistance

The larvae consumed good amount of leaf of WT and developed normally, however, negligible amount of different transgenic leaves were fed by larvae. Feeding of S. litura larvae on transgenic leaves for 2 to 4 days resulted in 90% to 100% mortality (Fig. 3.16). The percent mortality on WT plant’s leaves was 3.3% to 6.6% after 2 and 4 days,
respectively (Fig. 3.16B). All the transgenic leaves showed higher resistance to larvae of *S. litura* than WT leaves.

**Figure 3.15** Light response curves for energy fluxes of PSII and PSI for WT and L3 before cold stress, immediate after 1h of cold treatment (0 °C) and on 10\textsuperscript{th} day of recovery. A-C Photochemical quantum yield for PSII Y(II). D-F Quantum yield of non-light-induced non-photochemical fluorescence quenching for PSII, Y(NO). G-I Quantum yield of light-induced non-photochemical fluorescence quenching for PSII, Y(NPQ). J-L ETR of PSII (ETRII). M-O Photochemical quantum yield for PSI Y(I). P-R Quantum yield of non-photochemical energy dissipation in PSI due to donor side limitation, Y(ND). S-U Quantum yield of non-photochemical energy dissipation in PSI due to acceptor side limitation Y(NA). V-X ETR of PSI (ETRI). Values are average ± SEs of three to five replicates.
3.5 DISCUSSION

The present study reports the development of transgenic plants of *W. somnifera* overexpressing a desired gene (*WsSGTL1*) using *A. tumefaciens* as transformation vehicle and characterization of *WsSGTL1* gene has been done, viz., molecular, morphological, physiological, chemical and responses of transgenic lines against biotic and abiotic stresses.

![Figure 3.16](image_url)

**Figure 3.16** Evaluation of transgenic plants against neonate larvae of *Spodoptera litura*. A Detached leaves of *W.somnifera*. B Percent mortality of *S.litura*. Asterisks indicate that mean values are significantly different between WT and transgenic plants (*, P < 0.05; **, P < 0.01; ***, P <0.001).
Glycosylation, being a very important process for the normal growth and development of the plant may be considered as the key factor for the observed changes in phenotypes. These changes were likely to be associated with increase in glycosylation and can be documented by enhanced glycosylated products (withanoside V, glycosylated campesterol, glycosylated stigmasterol and glycosylated β sitosterol) in transgenic lines of *W. somnifera*. Woo et al. (1999) hypothesized that UDP-glycosyltransferase regulates activity of a ligand (s) needed for cell division in pea and alfalfa where *PsUGT1* expression was required for normal plant growth and development. Lerouxel et al. (2005) have shown the confirmed role of DGL1 (defective glycosylation 1-1) in N-linked glycosylation, cell growth and differentiation in plants. Qin et al. (2013) demonstrated that a leaky rice mutant for *OsDGL1* locus resulted in an N-glycosylation defect and thereby disrupted the synthesis of matrix polysaccharides in the cell wall and caused cell death in rice. It has been reported by Chaturvedi et al. (2011) that SGTs glycosylate steroidal hormones, such as brassinosteroids, function as growth and development regulators in plants. Brassinosteroids have been shown to regulate gene expression, stimulate cell division, cell elongation, vascular differentiation, photomorphogenesis and modulate reproductive biology (Fujioka and Yokota, 2003; Yamamoto et al. 2007). Differential pattern of withanolide contents obtained in transgenic lines could have brought the changes in phenotype as has been reported by Sangwan et al. (2008). They have reported that withanolides might act as growth regulators per se or may manifest growth modulatory effect by virtue of their strong sharing of the metabolic pathway of biosynthetic origin with brassinosteroids. We have observed that overexpression of *WsSGTL1* gene in *W. somnifera* with increased glycosylation causes early and higher growth, which opens new vistas for further study of the function of *WsSGTL1* gene in the control of plant growth and
development. Biosynthesis of withanolides, the signature secondary metabolites of *W. somnifera* (Sangwan et al. 2004), takes place from triterpenoid through metabolic divergence from the sterol pathway at the level of 24-methylene cholesterol (Sangwan et al. 2008). In the present study, phytochemical analysis of transgenic leaves and roots revealed the presence of secondary metabolites differently as present in WT. The observed significant decrease in withanolide content in transgenic lines indicated that these compounds have been glycosylated by overexpression of *WsSGTL1* gene and leads to more production of glycosylated withanolide i.e. withanoside V. Our results are similar to the reports of Lim and Bowles (2004) who reported that overexpression of glycosyltransferase genes led to a significant increase in their respective glucosides.

Due to overexpression of *WsSGTL1* gene, the production of a glycosylated product withanoside V has been increased in transgenic lines. In *W. somnifera*, withanosides and sitoindosides are the glycosylated forms of steroidal lactones synthesized through the action of GTs (Chaturvedi et al. 2012; Gupta et al. 2013; Lairson et al. 2008; Mizutani and Ohta, 2010). Our results were in accordance to these reported findings. Down regulation of TOGT, a tobacco glycosyltransferase recognizing multiple phenolic substrates in vitro, led to a decreased accumulation of scopoletin glucoside in planta (Chong et al. 2002; Lim and Bowles 2004). Jones et al. (2003) reported that in the biosynthesis of flavonol glycoside in *A.thaliana*, UGT73C6 and UGT78D1 glycosyltransferases were involved. In addition to this, glycosylated sterols, viz., campesterol, stigmasterol, and sitosterol were quantified by HPLC analysis to know the influence of *WsSGTL1* gene. Glycosylated sterols were increased upto 13.5 fold however in *N. tabacum* 2.5 fold was observed (Pandey et al. 2014) further confirming the role of *WsSGTL1* in glycosylation of sterols.
One of the major effects of overexpression of \( WsSGTL1 \) gene of \( W.somnifera \) was the increase of 18% to 38% in stomatal density (but without a change in size) and increased size of subsidiary cells in progeny of several independent transgenic lines (Fig. 7C). This was accompanied with a drastic effect on specific photosynthesis parameters that include 42% to 56% increase in stomatal conductance and transpiration, but not affected the rate of photosynthesis, electron transport, and quantum yield and decreased water use efficiency. Similarly, Miyazawa et al. (2006) in poplar showed that stomatal density of upper leaves was affected by conductance of lower leaves but was independent of photosynthesis.

Glycosyltransferase are supposed to be involved in the tolerance of plants to biotic and abiotic stresses (Zhang et al. 2014). For example, UDP-glycosyltransferase is involved in resistance against biotic and abiotic stresses through the activities of glycosylated hormones and secondary metabolites in plants (O’Donnell et al. 1998; Roberts et al., 1999). Expression of UGT74E2 (UDP-glucosyltransferase) in \( A. thaliana \) under drought stress improved the rooting capacity and altered anthotaxy traits by regulating IBA and NAA activities, thereby improving resistance against drought and salt stress (Tognetti 2008). Enhanced expression of \( WsSGTL1 \) was reported to provide tolerance towards different kind of environmental stresses (Chaturvedi et al. 2012; Mishra et al. 2013; Pandey et al. 2014; Sharma et al. 2007). Chaturvedi et al. (2012) has reported 30 fold increase in \( WsSGTL1 \) expression after cold stress, however, no enhanced expression of \( WsSGTL1 \) gene could be observed after heat stress. Therefore, in the present study, experiments were performed only on cold stress. Under cold stress condition, \( A, E \) and \( gs \) decreased in WT and transgenic plants but percent reduction was less in L2 line. Similarly, electron transport rates as well as the quantum yields of PSII and PSI were lower in WT plants under cold stress condition but in transgenic lines
these changes were less. The defect actually lies on the PSII side, leading to a reduction in the flow of electrons from PSII to PSI in WT plants under stress condition. The very low PSII ETRs seem to result from considerably reduced levels of NPQ (Fig. 8h), acting as a protective mechanism against the environmental stress, was not working and created high excitation pressure due to low stomatal conductance. On the other hand, in transgenic lines increase in the levels of NPQ is a response to avoid damage to the photosynthetic apparatus from high excitation pressure usually was in response to highlight conditions (Adams and Adams 1996; Oquist and Huner 2003). But it may also result from an increase in conductance. In L2 line, the fraction of energy that is passively dissipated in the form of heat and fluorescence \([Y(NO)]\) was maintained at the level of 0.3 under cold stress, which is the baseline value under low light, indicating that there was no excess excitation pressure in PSII reaction centers under the cold stress (Huang et al. 2012). Anthocyanin content was also increased in L2 plants which protected them against cold stress. Similarly, Nicotra et al. (2003) showed increased anthocyanin content in evergreen leaves in response to freezing stress. It is a well known pigment which protects leaf from different stresses like drought (Singh et al. 2013b) and high light (Ranjan et al. 2014) and it accelerated the regulated energy dissipation as NPQ increased (Fig. 3.13H). In WT plants, NPQ was reduced under cold stress due to less enhancement of anthocyanin content. Under stress condition in PSI, quantum yield and limitation at donor side decreased.

The antifeedant properties for \textit{S.littora} larvae for the leaves of \textit{W. somnifera} has been reported by Ascher et al. (1984) and also Gaur et al. (2010) suggesting that \textit{W. somnifera} acts as an insect growth regulator causing disruption of the endocrine mechanism regulating molting and metamorphosis. In the present study, overexpression of \textit{WsSGTL1} gene has provided the plant resistance against \textit{S. litura}. 100% mortality of
larvae that reared on the leaves of transgenic lines was observed. This might be due to the overexpression of \textit{WsSGTL1} gene that increased glycowithanolides. This finding is similar as reported by Pandey et al. (2014), where the expression of \textit{WsSGTL1} gene in transgenic tobacco modulates the glycosylation profile and provided resistance towards \textit{S. litura}. The apparent similarity in resistance against \textit{S. litura} may be attributed to the effect of \textit{WsSGTL1} gene in both the plants that causes increase in glycosylated products.

Flanders et al. (1992) reported association of steroidal glycoalkaloid tomatine in Colorado potato with field resistance. It has also been observed that overexpression of UGT73C5 in transgenic \textit{Arabidopsis} improved the resistance against fungal toxins (Poppenberger et al. 2003).

\textit{WsSGTL1} gene has been proposed to be an important member of the \textit{GT’s} gene family of \textit{W. somnifera}, which has the capacity to glycosylate withanolides and sterols thereby, regulates the growth and development of plants as well as directs the increase in number of stomata. Further, overexpression of this gene increases glycosylation of withanolides and other sterols which provide the resistance to the transgenic plants against \textit{S. litura} causing 100% mortality of the larvae. It may be a significant link in the interface between abiotic (cold stress) and biotic stress pathways. Further, it will be interesting to determine the effects of silencing \textit{WsSGTL1} gene that may function well in a network of complementary activities which can be warranted in future to fully establish the multifaceted roles played by \textit{WsSGTL1} gene in \textit{W. somnifera}. 