Plant Cell Rep (2016) 35:195–211
DOI 10.1007/s00299-015-1879-5

ORIGINAL ARTICLE

Ectopic overexpression of *WsSGTL1*, a sterol glucosyltransferase gene in *Withania somnifera*, promotes growth, enhances glycowithanolide and provides tolerance to abiotic and biotic stresses

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Received: 18 June 2015 / Revised: 25 August 2015 / Accepted: 5 October 2015 / Published online: 30 October 2015

Abstract

Key message **Overexpression of sterol glycosyltransferase (SGTL1) gene of *Withania somnifera* showing its involvement in glycosylation of withanolide that leads to enhanced growth and tolerance to biotic and abiotic stresses.**

Abstract *Withania somnifera* is widely used in Ayurvedic medicines for over 3000 years due to its therapeutic properties. It contains a variety of glycosylated steroids called withanosides that possess neuroregenerative, adaptogenic, anticonvulsant, immunomodulatory and antioxidant activities. The *WsSGTL1* gene specific for 3β-hydroxy position has a catalytic specificity to glycosylate withanolide and sterols. Glycosylation not only stabilizes the products but also alters their physiological activities and governs intracellular distribution. To understand the functional significance and potential of *WsSGTL1* gene, transgenics of *W. somnifera* were generated using *Agrobacterium tumefaciens*-mediated transformation. Stable integration and overexpression of *WsSGTL1* gene were confirmed by Southern blot analysis followed by quantitative real-time PCR. The *WsGTL1* transgenic plants displayed number of alterations at phenotypic and metabolic level in comparison to wild-type plants which include: (1) early and enhanced growth with leaf expansion and increase in number of stomata; (2) increased production of glycowithanolide (majorly withanoside V) and campesterol, stigmasterol and sitosterol in glycosylated forms with reduced accumulation of withanolides (withaferin A, withanolide A and withanone); (3) tolerance towards biotic stress (100 % mortality of *Spodoptera litura*), improved survival capacity under abiotic stress (cold stress) and; (4) enhanced recovery capacity after cold stress, as indicated by better photosynthesis performance, chlorophyll, anthocyanin content and better quenching regulation of PSI and PSII. Our data demonstrate overexpression of *WsSGTL1* gene which is responsible for increase in glycosylated withanolide and sterols, and confers better growth and tolerance to both biotic and abiotic stresses.

Keywords Abiotic and biotic stress tolerance · Glycosylation · Growth · Transgenic *Withania somnifera* · Withanolides · *WsSGTL*1

Communicated by H. S. Judelson.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-015-1879-5) contains supplementary material, which is available to authorized users.

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Introduction

Withania somnifera Dunal (L), commonly known as Ashwagandha, is widely used in Ayurvedic medicines due to immense therapeutic properties of its different parts (Kumar et al. 2007; Sharma et al. 2011). This plant 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 as well as withanosides (Chatterjee et al. 2010; Chaturvedi et al. 2012; Chen et al. 2011; Jayaprakasam and Nair 2003; Sharma et al. 2011). Withanosides have been reported to possess neuroregenerative, adaptogenic, anticonvulsant, immunomodulatory and antioxidant activities (Bhattacharya et al. 1997; Jayaprakasam and Nair 2003; Matsuda et al. 2001; Zhao et al. 2002).

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. 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* (WsHMGR) 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), a key enzyme in the pathway of biosynthesis ofisoprenoids, from W. somnifera encodes a polypeptide of 343 amino acids, its amino acid sequence-homology and phylogenetic analysis suggest that W.somnifera FPPS has a close similarity to itscounterparts from tomato (SlFPPS) and capsicum (CaaFPPS) and its expression is significantly elevated inresponse 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) encode polypeptides of 717 and 475 amino acids residues, respectively. Its expression analysis suggested that *WsDXS* and *WsDXR* 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 *WsCPR1* and *WsCPR2* 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* (*WsSGTL1*) 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 transcriptional level. Empirical evidence suggested that repression of competitive branch OSCs like WsOSC/BS and WsOSC/LS possibly leads to diversion of substrate pool towards WsOSC/CS for increased withanolide production (Dhar et al. 2014; Singh et al. 2015a).

WsSGTL1 specific for 3β-hydroxy position has a catalytic specificity to glycosylate withanolide and sterols (Madina et al. 2007a). 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 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).

Considering the significance and potential of *WsSGTL1* gene, it was tempting to demonstrate the effects of overexpression of this gene on glycosylation of withanolide biosynthesis as no work on this has been reported till date. Overexpression of key genes of withanolide biosynthetic pathway would be helpful in understanding their synthesis and will provide wealth information at molecular level for

metabolic engineering in this medicinal plant (Singh et al. 2015a). In the present study, for functional analysis of *WsSGTL1* gene, transgenic lines of *W. somnifera* overexpressing this gene were developed through *Agrobacterium tumefaciens*-mediated transformation. The study was aimed to understand the changes in qualitative and quantitative patterns of withanolides, glycowithanolide (majorly withanoside V), their accumulation in leaf and root tissues along with glycosylation of sterols to provide an insight into the biosynthesis of metabolites that will be valuable to human and for other important cellular functions. 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 and Khetagoudar 2012). Also, it is active throughout the year and fed a total of 112 cultivated crop plants in tropical and temperate zones (Sharma and Seth 2005) and 40 species of cultivated and 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).

Results

A. tumefaciens-mediated transformation and confirmation of integration of WsSGTL1 gene in overexpression lines of *W. somnifera*

To analyze the function of *WsSGTL1* gene, we have generated transgenic lines of *W. somnifera* overexpressing *WsSGTL1* gene. Nine transgenic lines were obtained which were *NPT11* positive in T_0 generation, out of this five transgenic lines of T_1 generation were raised as shown in Fig. 1a–g. T_1 transgenics were confirmed by PCR, RT-PCR and real-time PCR analysis (Supplementary Fig. S1a–c). On the basis of RT-PCR expression analysis, three best lines were selected for further analysis. Southern blot analysis confirmed the insertion of single copy of *NPTII* gene in L1 and L3 lines, while two copies were inserted in L6 line and no *NPTII* gene in WT (Supplementary Fig. S1d). L6 line hereinafter was named as L2 line.

Effect of overexpression of WsSGTL1 gene on plant growth and development

Transgenic *W. somnifera* plants overexpressing *WsSGTL1* grew vigorously than WT (Supplementary Fig. S2a–c) plants. The transgenic lines were taller and stronger than WT with higher number of nodes (Fig. 2a–c). The total leaf area and biomass were observed to be four times higher than WT plants (Fig. 2d, 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. 2e, 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. 2h, i). The transgenics showed comparatively low seed yield without any significant difference in fruit weight and seed weight (Fig. 2j–l).

Overexpression of WsSGTL1 reduces withanolide content and directs withanoside V accumulation

To investigate the *WsSGTL1* overexpression effect on withanolides, major withanolides were analyzed 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. 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 threefold) and withanone (approximately sevenfold, Fig. 3a–c). Further, to validate the results obtained in overexpressed lines, transgenic lines and WT leaves were subjected to analyze glycowithanolide, i.e., withanoside V content. It was observed that transgenics exhibited drastically higher amount of withanoside V (approximately 10-fold, Fig. 3d). Demonstrating the effectiveness of overexpression of *WsSGTL1* gene, root samples were also analyzed for withanolide content. Considerable decrease in withanolide A (approximately 7.3-fold), withanone (approximately 10-fold) and withaferin A content (approximately sixfold, Fig. 3e–g) was observed. For further confirmation of results, accumulation of withanoside V was investigated which was observed higher (approximately 10-fold, Fig. 3h). The reduction of withanolides and increase in withanoside V in root samples of transgenic lines were similar to leaf samples of transgenic lines, demonstrating the effectiveness of *WsSGTL1* gene involved in biosynthesis of withanolide thereby regulating glycosylation of withanolides.

Synthesis of glycosylated sterols in transgenic plants

To determine the effect of *WsSGTL1* gene on sterol biosynthesis, the overexpressed *WsSGTL1* 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

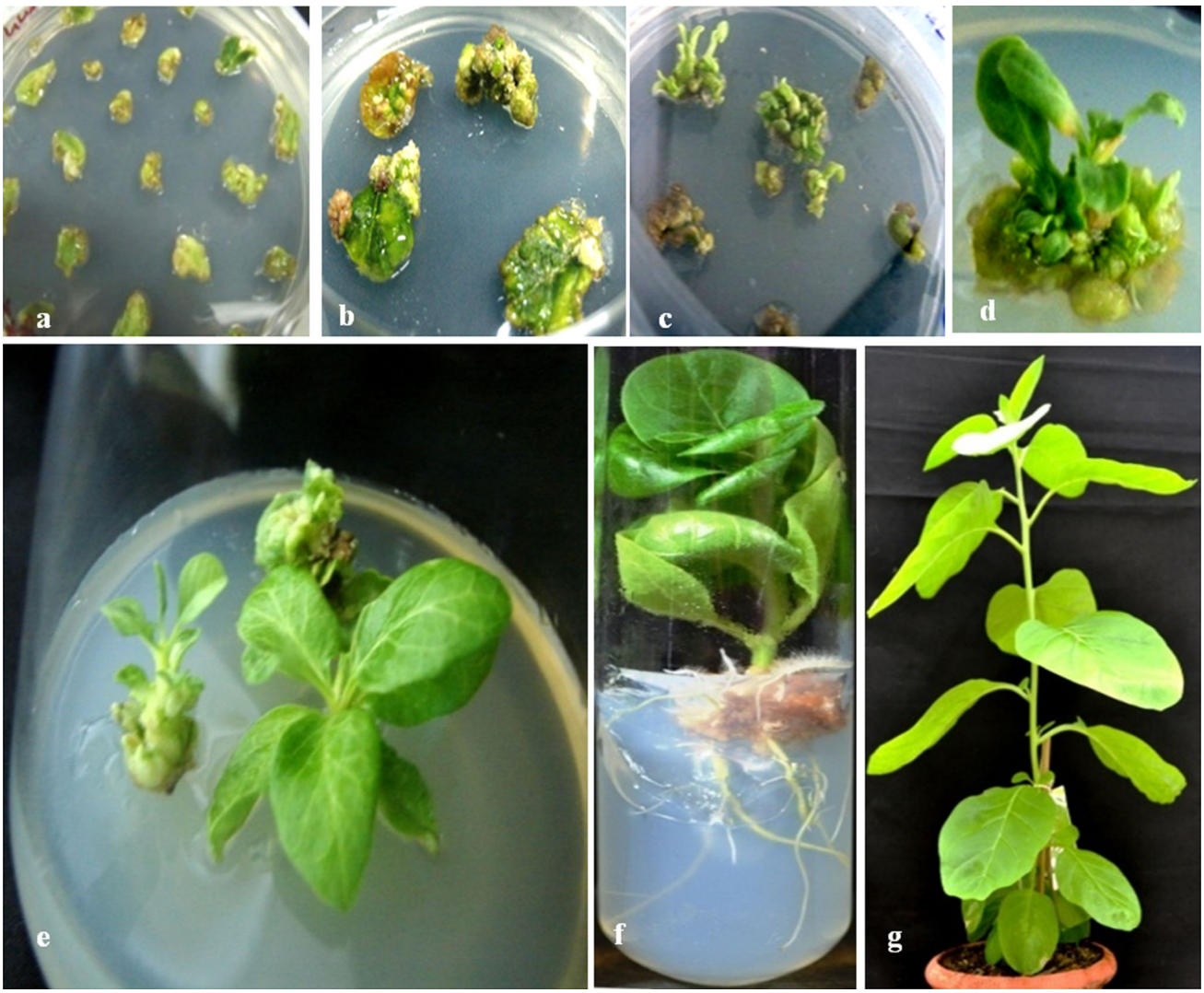

Fig. 1 *Agrobacterium tumefaciens*-mediated transformation of *W. somnifera*. **a** Cotyledonary leaf explants. **b–d** Shoot induction on selection media. **e** Plantlet formation. **f** Putative transgenic plant under in vitro condition. **g** Putative transgenic plant in pot

campesterol was approximately fivefold (Fig. 4a), glycosylated β-sitosterol was approximately 10-fold (Fig. 4b) and glycosylated stigmasterol was approximately 13.5-fold (Fig. 4c) which were obtained in transgenic lines as compared to WT plants.

Physiological activity of transgenic plants under cold stress

Chlorophyll fluorescence image analysis

Chlorophyll fluorescence imaging for maximum photochemical quantum yield (Fv/Fm) is shown in Supplementary Fig. S3. 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 10–20 % reduction. Supplementary Fig. S3e shows minimum heterogeneity due to maximum leaf area damaged, while Supplementary Fig. S3f–h shows 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 (Supplementary Fig. S3i–l).

Photosynthesis rate (A), transpiration rate (E) and stomatal conductance (gs)

Photosynthesis rate (*A*) showed significant reduction during cold stress condition (Fig. 5a). WT plants reduced *A* by 81 %, while in transgenic lines (L1, L2, L3) it was reduced

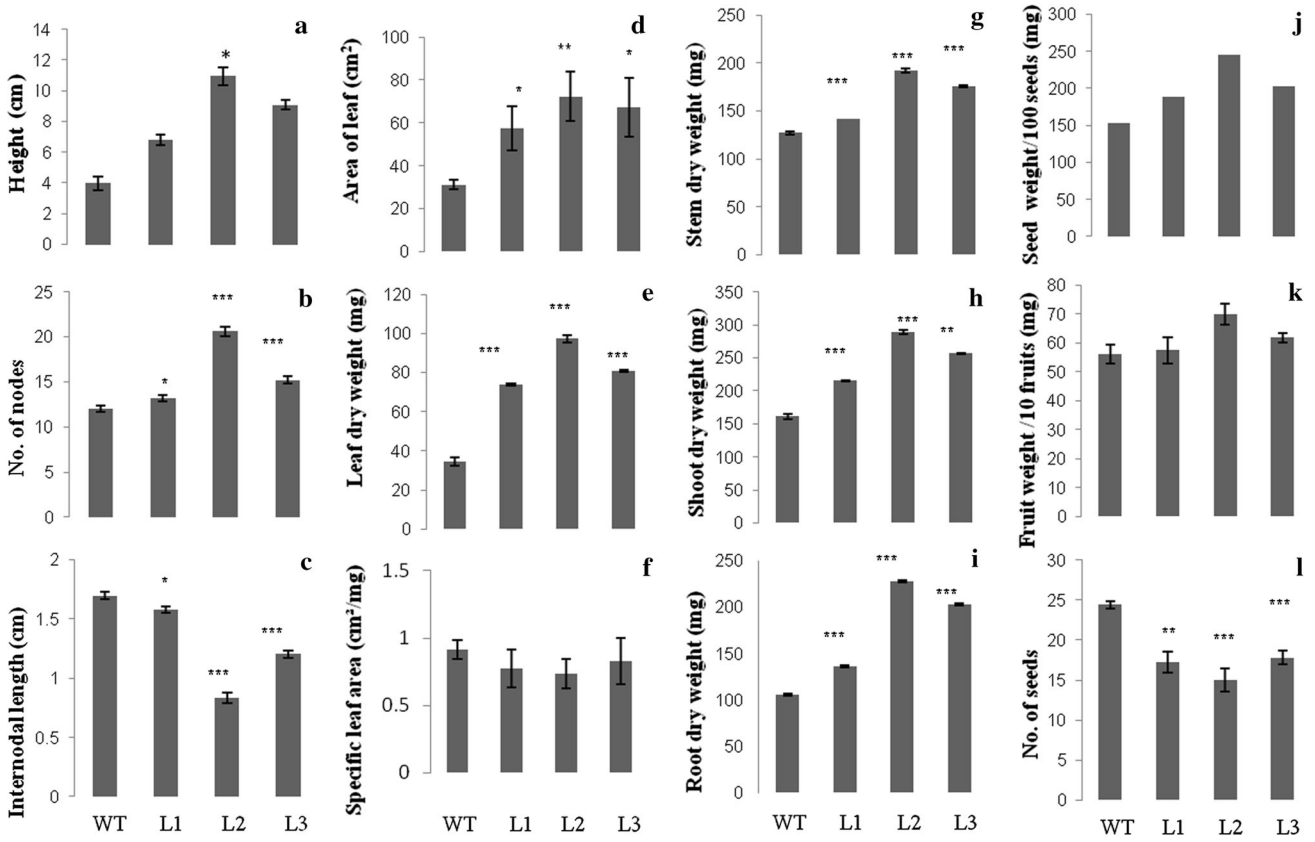

Fig. 2 Growth analysis of WT and transgenic plants. Plants were harvested after 14 weeks of growth. Results are mean ± SE of five different plants. *Asterisks* indicate that mean values are significantly different between WT and transgenic plants (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

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. 5b, 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. 5d).

Analysis of chlorophyll, carotenoid, anthocyanin, water potential, electrolyte leakage and stomatal density

Since *A* depends on chlorophyll content of the leaf, 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. 6a, b). Anthocyanin content increased by 2-, 4-, 7-, 3-fold in WT, L1, L2, L3 lines, respectively, under cold condition and did not decrease again under recovery condition (Fig. 6c). Water potential (WP) was increased by 2.2- and 1.7-fold in L2 and L3 lines, respectively, under cold stress; however, in WT and L1 lines, it did not increase much. Under recovery condition, WP reached at their control level in all the four lines (Fig. 6d). 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. 7a), 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

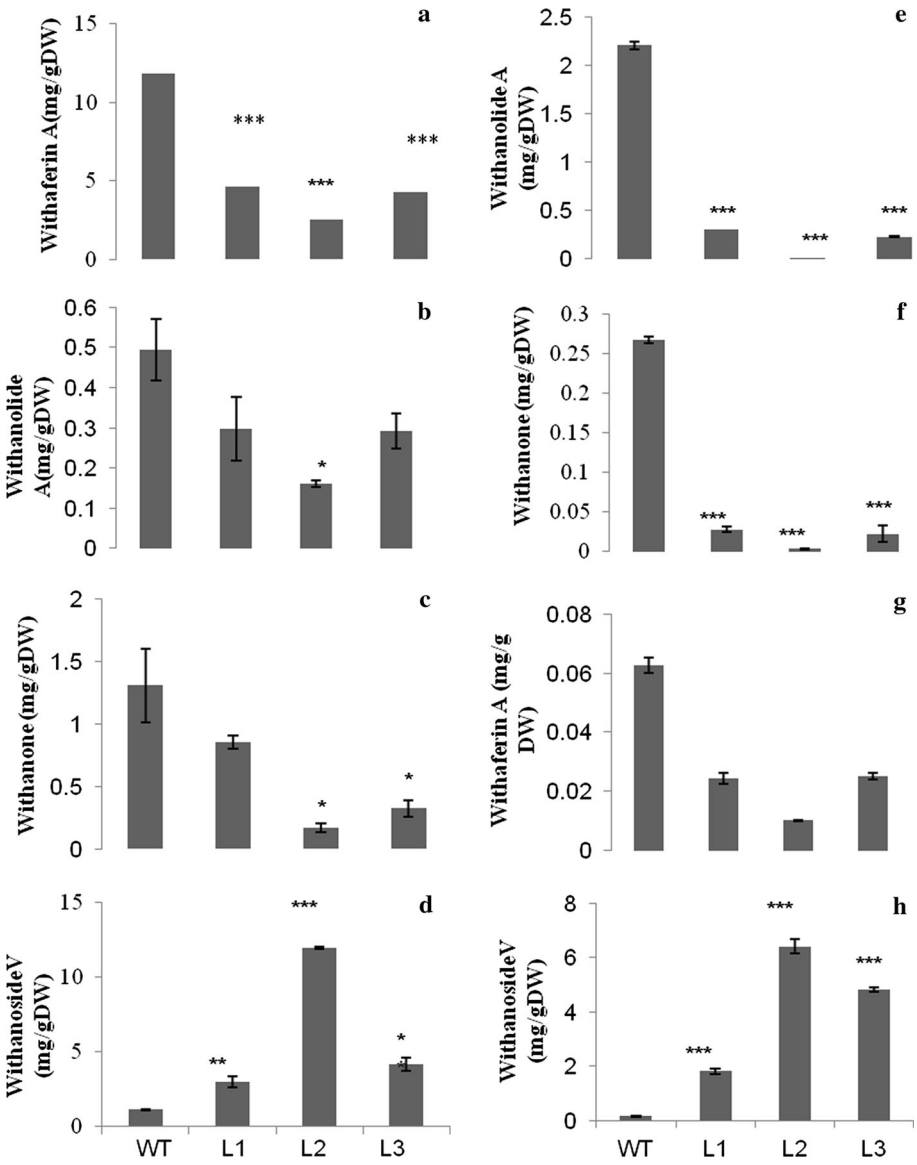

Fig. 3 Withanolides in WT and transgenic plants (L1, L2 and L3). **a**–**d** From samples of leaf extract. **e**–**h** From samples of root extract. Results are mean ± SE of three independent experiments. *Asterisks* indicate that mean values are significantly different between WT and transgenic plants (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

increase in the stomatal density was observed in all the transgenic lines (Fig. 7b). 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.

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. 8, Supplementary Figs.S4, S5). 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. 8a, 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. 8m, 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. 8b). Y(NO) increased and Y(NPQ) decreased in WT plants (Fig. 8g) and these changes could not recover much during recovery condition (Fig. 8f, 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

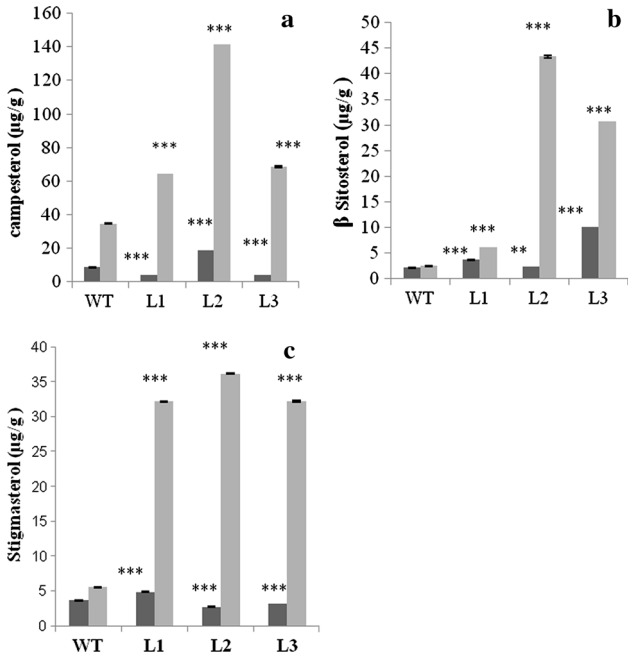

Fig. 4 Quantitative estimation of glycosylation of sterols due to enzymatic activity of *WsSGTL1*. Free sterols were measured by breaking bond between sterols and sugar moiety after acid hydrolysis of extract and compared with free sterols before hydrolysis (*black bar* represents sterols before hydrolysis and *gray 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 (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$)

L2 line while remained constant in WT plants under stress condition (Fig. 8q). 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. 8s–u). Electron transfer rate through PSI and PSII decreased in WT plants under stress condition (Fig. 8j–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. 8j, l, v, x). L1 and L3 lines also behaved in the same manner as L2 line (Supplementary Fig. S4, S5).

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 2–4 days resulted in 90–100 % mortality (Fig. 9a). The percent mortality on WT plant's leaves was 3.3–6.6 % after 2 and 4 days, respectively (Fig. 9b). All the transgenic leaves showed higher resistance to larvae of *S. litura* than WT leaves.

Discussion

The present study is the first report of development of transgenic plants of *W. somnifera* overexpressing a desired gene (*WsSGTL1)* using *A. tumefaciens* as transformation vehicle. In this study, we have functionally characterized *WsSGTL1* by its expression in *W. somnifera*, and analyzed various parameters including phenotypic, physiological, chemical profiling through HPLC analysis and responses of transgenic lines against biotic and abiotic stresses.

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

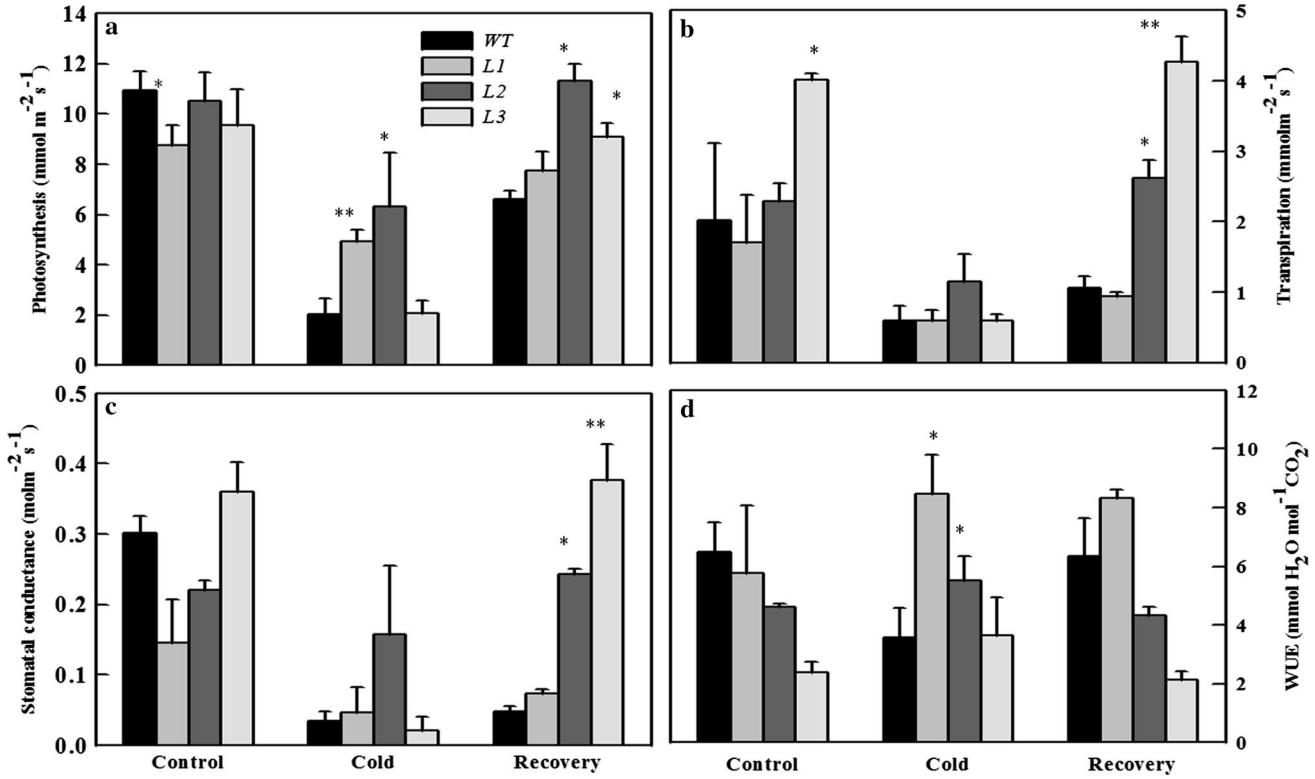

Fig. 5 Physiological parameters measured in WT and transgenic lines (L1, L2 and L3) of *W. somnifera*. **a** Photosynthesis rate. **b** Stomatal conductance. **c** Transpiration. **d** Intrinsic water use efficiency was monitored in plants before cold stress, immediate after 1 h of cold treatment (0 °C) and on 10th day of recovery. Data represent the mean ± SD of five separate measurements. *Asterisks* indicate that mean values are significantly different between WT and transgenic plants (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

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 lead 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. Downregulation 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 was 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 up to 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 18–38 % 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 42–56 % 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.

Glycosyltransferases 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;

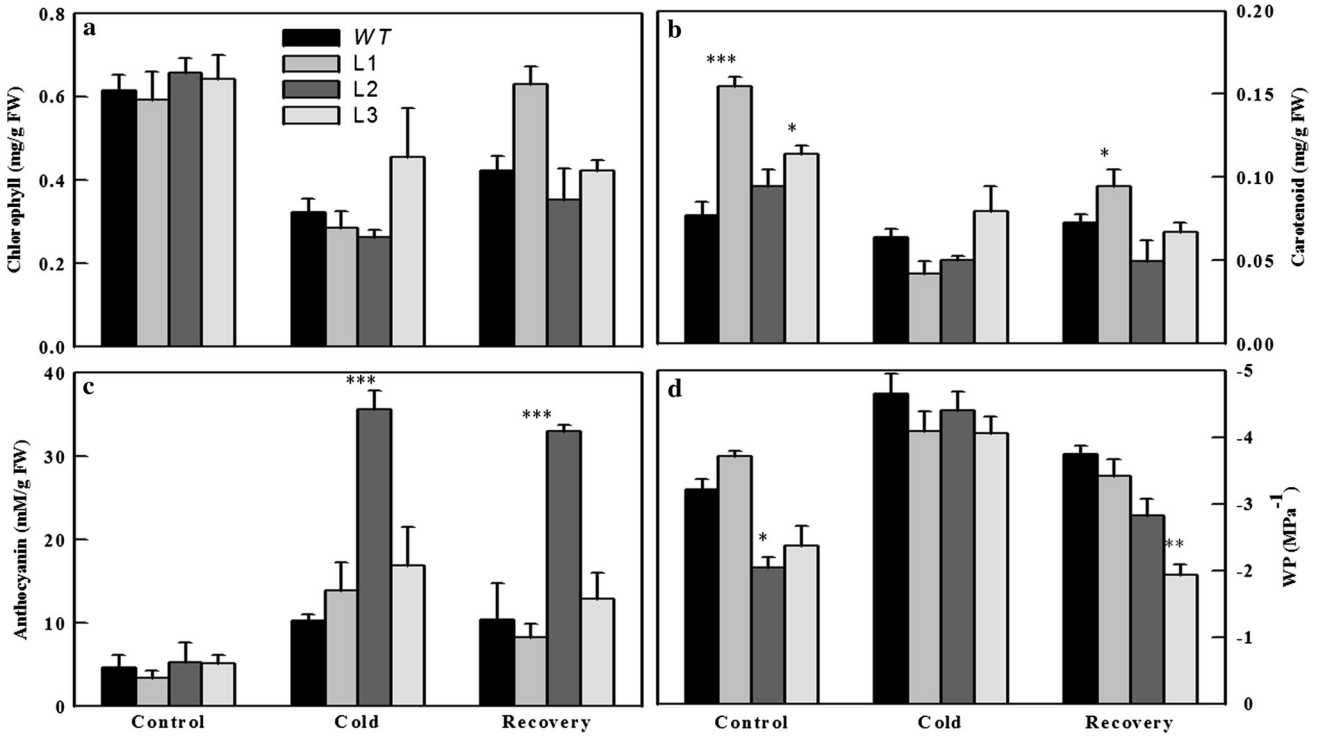

Fig. 6 Pigment concentrations in WT and transgenic lines (L1, L2 and L3) of *W. somnifera*. **a** Chlorophyll. **b** Carotenoids. **c** Anthocyanin. **d** Water potential before cold stress, immediate after 1 h of cold treatment (0 °C) and on 10th day of recovery. Data represent the mean ± SD of five separate measurements. *Asterisks* indicate that mean values are significantly different between WT and transgenic plants (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

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 et al. 2010). Enhanced expression of *WsSGTL1* was reported to provide tolerance towards different kinds 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 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. 8h). 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 *S. litura* larvae for the leaves of *W. somnifera* have been reported by Ascher et al. (1984) and also Gaur and Kumar (2010) suggesting that *W.*

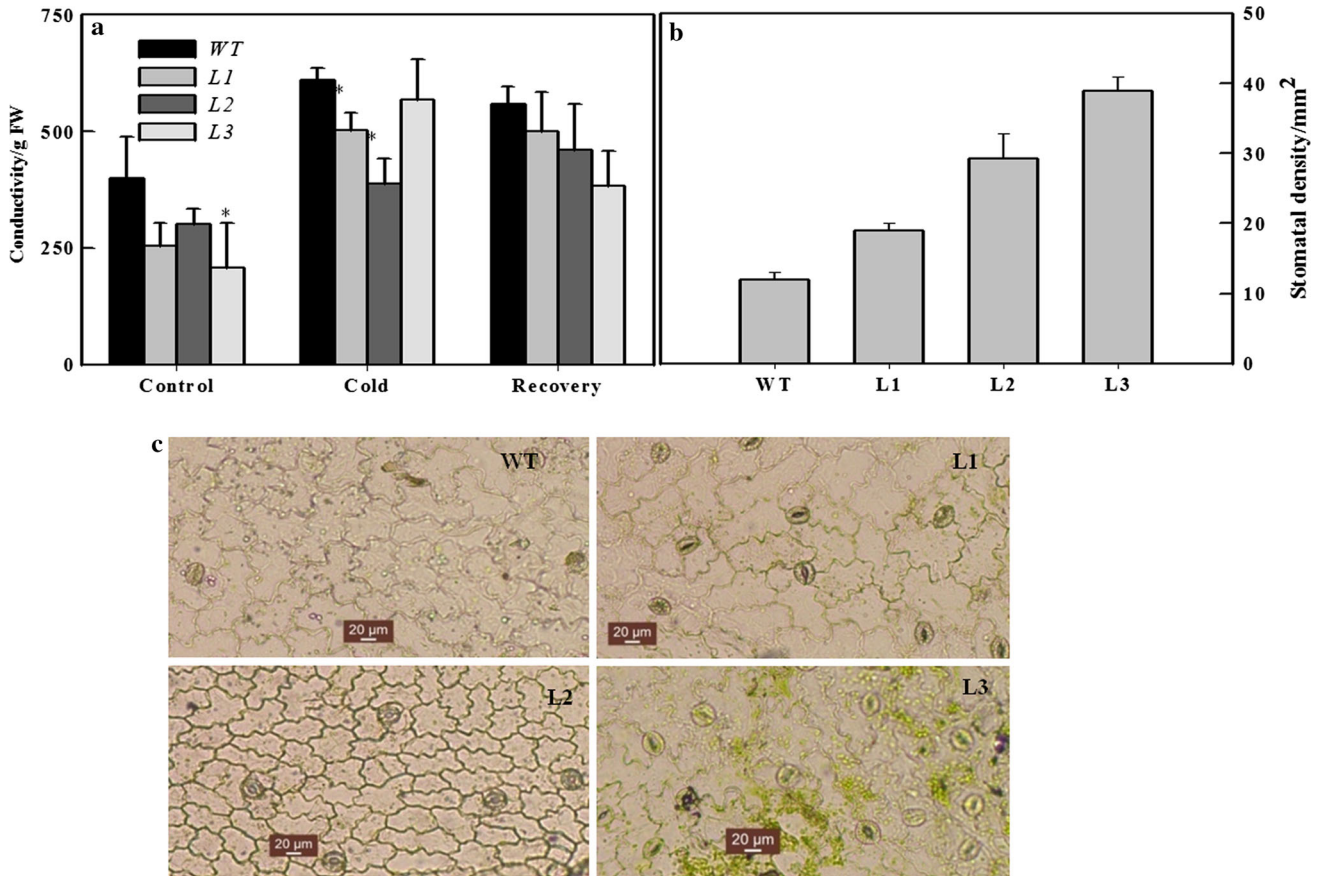

Fig. 7 **a** Electrolyte leakage in terms of % conductivity for WT and transgenic lines before cold stress, immediate after 1 h of cold treatment (0 °C) and on 10th day of recovery. **b** Stomatal density of the upper leaf epidermis of WT and transgenic lines L1, L2 and L3. Values represent an average stomatal density ± SD in an area of 1 mm^2 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 (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

somnifera acts as an insect growth regulator causing disruption of the endocrine mechanism regulating molting and metamorphosis. In the present study, overexpression of *WsSGTL1* gene has provided the plant resistance against *S. litura*. 100 % mortality of larvae that reared on the leaves of transgenic lines was observed. This might be due to the overexpression of *WsSGTL1* gene that increased glycowithanolides. This finding is similar as reported by Pandey et al. (2014), where the expression of *WsSGTL1* gene in transgenic tobacco modulates the glycosylation profile and provided resistance towards *S. litura*. The apparent similarity in resistance against *S. litura* may be attributed to the effect of *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 *Arabidopsis* improved the resistance against fungal toxins (Poppenberger et al. 2003).

WsSGTL1 gene has been proposed to be an important member of the *GT's* gene family of *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, since overexpression of this gene increases glycosylation of withanolides and other sterols which provides the resistance to the transgenic plants against *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 *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 *WsSGTL1* gene in *W. somnifera.*

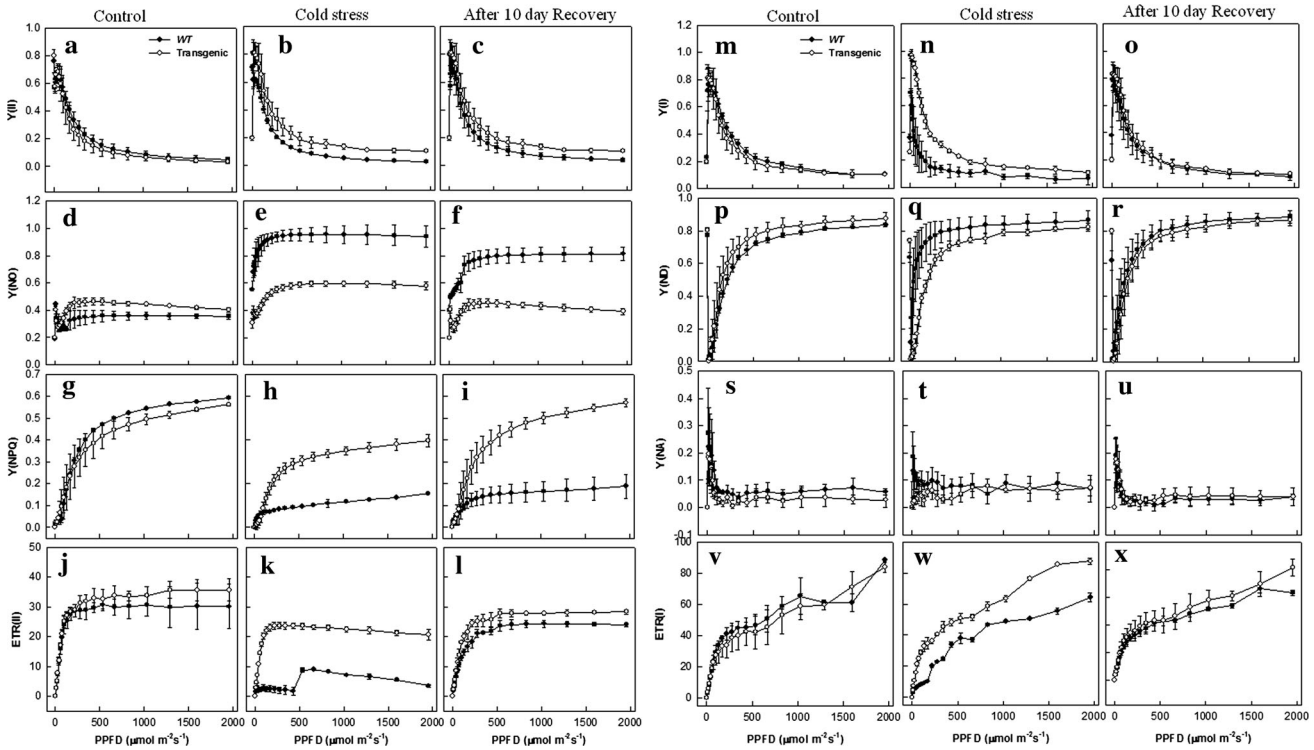

Fig. 8 Light response curves for energy fluxes of PSII and PSI for WT and L2 before cold stress, immediate after 1 h of cold treatment (0 °C) and on 10th day of recovery. **a–c** Photochemical quantum yield for PSII Y(II). **d–f** Quantum yield of non-light-induced non-photochemical fluorescence quenching 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

Materials and methods

Plant material and culture conditions

The seeds of *W. 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 and 3–4 thorough washings with RO water and kept overnight in 0.1 N 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 rinses 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 (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 °C. Segments of cotyledonary leaves were used as explants for transformation. For cold stress, the plants were shifted from 25 °C in green house to illuminated growth chambers at 4 °C for 24 h followed by recovery in glass house.

Gene cloning and construct preparation

Cloning of *WsSGTL1* (2.1 kb) gene and construct preparation were carried out as reported earlier (Mishra et al. 2013). The resultant pBI121 harboring *WsSGTL1* gene in sense orientation was mobilized into LBA4404 strain of *Agrobacterium tumefaciens* by electroporation. *Agrobacterium* cells harboring pBI121-*WsSGTL1* gene containing neomycin phosphotransferase *(NPT11)* gene as marker were selected on Luria Broth medium containing 50 mg/l kanamycin.

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-*WsSGTL1* gene. The explants were infected with resuspended *A. tumefaciens* cultures ($OD_{600} = 0.4$) for 20 min under continuous shaking at room temperature and sonicated in a Branson sonifier (Bransonic-Ultrasonic Cleaner, USA, Model No. 3210EMTH) with three pulses of 4 s duration at the

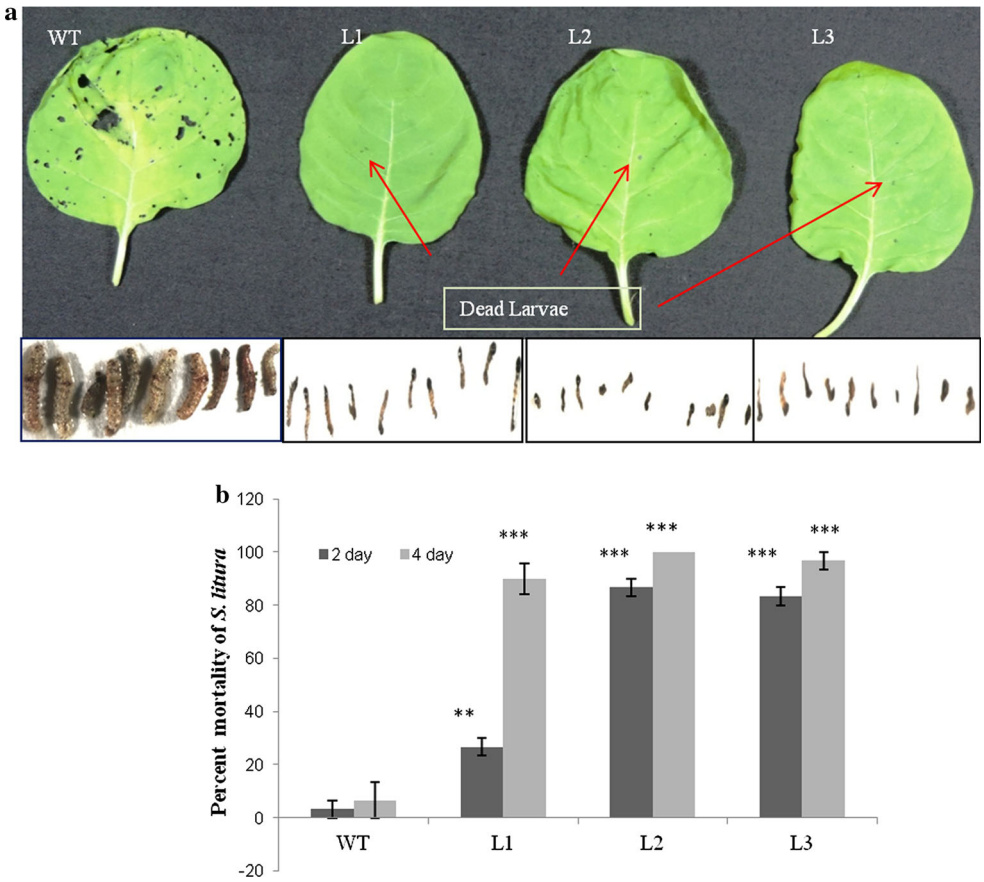

Fig. 9 Evaluation of transgenic plants against neonate larvae of *Spodoptera litura*. **a** Detached leaves of *W. somnifera*. **b** Percent mortality of *S litura*. *Asterisks* indicate that mean values are significantly different between WT and transgenic plants (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

maximum output power (14 W). Thereafter, transferred on to 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 cefotaxime 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 days 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.

Molecular confirmation of transgenics

PCR confirmation was done in the 9 putative transgenic (T_0) plants for the presence of *NPT11* gene. Five transgenic lines were fertile and produced seeds. Seeds 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 T_1 generation and WT using DNeasy Plant Minikit, Qiagen. PCR was conducted using *NPTI1* gene primers (Supplementary Table S1) 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 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min as final extension step. The amplified products were analyzed by 1 % agarose gel electrophoresis.

RNA isolation, RT-PCR and relative expression

RNA was isolated 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 T_1 transgenics and WT, semiquantitative RT-PCR and real-time PCR was done. The primers have been listed in Supplementary Table S1. 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 using Delta–Delta ct method. Three independent experiments were conducted using three biological replications.

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 *ECoR1* 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 radiolabelled probe was prepared using 550 bp fragment of *NPTII* gene (primer given in Supplementary table S1) and overnight hybridization was performed (Sambrook et al. 2001). After hybridization, autoradiography was done and analyzed on Molecular Imager FX (Bio-Rad).

Phenotypic analysis

T_1 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 days) and final dry weight was determined.

Extraction and HPLC analysis of secondary plant metabolites

Withanolides and Withanoside V: 500 mg of leaves and roots of transgenic lines and WT were crushed and dried into liquid nitrogen. The chemical extraction and HPLC analysis have been performed according to Ganzera et al. (2003).

Sterols

Dried leaf samples (100 mg) were homogenized in 1 ml of methanol (80 % v/v) followed by incubation at 40 °C for 2 h. Glycosylated and nonglycosylated sterols were analyzed quantitatively from acid-hydrolysed or non-hydrolysed extracts of transgenic lines and WT plants, respectively, as reported by Pandey et al. (2014).

All 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.

Physiological studies

For each experiment, T_1 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_2 levels inside the leaf cuvette were maintained at 400 ppm, photosynthetic photon flux density (PPFD) was 400 µmol m^{-2} s^{-1}, 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^{-2} s^{-1} and measurements of steady-state A, gs, E, internal CO_2 concentration (Ci), and quantum yield of PSII in the light (ϕ) 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 vapor pressure deficit (VPD) ranging between 0.5 and 1 kPa, leaf temperature of 25 °C, and CO_2 concentration of 400 µmol mol^{-1}, and Fv/Fm measurements were made using a light pulse of 2000 µmol photons m^{-2} s^{-1}.

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., 1200–1500 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) (Klughammer and Schreiber 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 were 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, $AA = A_{530} - (0.288 \times A_{650})$, 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 30,000 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 (three 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.

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.

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-month-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.

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 using ANOVA-INDOSTAT software.

Acknowledgments We are grateful to the Director, CSIR-National Botanical Research Institute, Lucknow, for the facilities provided. SS is thankful to CSIR for the award of Senior Research Fellowship. PM is thankful to the Department of Biotechnology, New Delhi, for the financial support provided through the project No. GAP 231225.

Compliance with ethical standards

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

Author contribution statement Conceived and designed the experiments: SS PM LR, Corrected the manuscript: PM SS, Performed the experiments: SS RS, Analyzed the data: SS LR AN IZA. Wrote the paper: SS.

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