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Research

A WRKY transcription factor from *Withania somnifera* regulates triterpenoid withanolide accumulation and biotic stress tolerance through modulation of phytosterol and defense pathways

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Summary

- *Withania somnifera* produces pharmacologically important triterpenoid withanolides that are derived via phytosterol pathway; however, their biosynthesis and regulation remain to be elucidated.
- A jasmonate- and salicin-inducible WRKY transcription factor from *W. somnifera* (*WsWRKY1*) exhibiting correlation with withaferin A accumulation was functionally characterized employing virus-induced gene silencing and overexpression studies combined with transcript and metabolite analyses, and chromatin immunoprecipitation assay.
- *WsWRKY1* silencing resulted in stunted plant growth, reduced transcripts of phytosterol pathway genes with corresponding reduction in phytosterols and withanolides in *W. somnifera*. Its overexpression elevated the biosynthesis of triterpenoids in *W. somnifera* (phytosterols and withanolides), as well as tobacco and tomato (phytosterols). Moreover, WsWRKY1 binds to W-box sequences in promoters of *W. somnifera* genes encoding squalene synthase and squalene epoxidase, indicating its direct regulation of triterpenoid pathway. Furthermore, while *WsWRKY1* silencing in *W. somnifera* compromised the tolerance to bacterial growth, fungal infection, and insect feeding, its overexpression in tobacco led to improved biotic stress tolerance.
- Together these findings demonstrate that *WsWRKY1* has a positive regulatory role on phytosterol and withanolides biosynthesis, and defense against biotic stress, highlighting its importance as a metabolic engineering tool for simultaneous improvement of triterpenoid biosynthesis and plant defense.

Introduction

Triterpenoids are ubiquitous in eukaryotes as primary sterol metabolites, which are important components of membranes and signaling (as steroidal hormones) (Moses *et al.*, 2013; Kemen *et al.*, 2014). Plants also synthesize specialized triterpenoids that are often accumulated as conjugates with macromolecules and serve as defense molecules against biotic and abiotic stresses (Augustin *et al.*, 2011; de Costa *et al.*, 2013). Triterpenoids also have a wide range of commercial applications because of their beneficial properties for humans. *Withania somnifera* (Ashwagandha, winter cherry or Indian ginseng), a solanaceous plant, accumulates pharmacologically important steroidal lactone triterpenoids called withanolides, which are termed 22-hydroxyergostan-26-oic acid-26,22-lactones (Dhar *et al.*, 2014). *W. somnifera* extracts are reported to possess various pharmacological activities, including antitumour, anti-inflammation, cardioprotective and neuroprotective properties (Mirjalili *et al.*, 2009; Sehgal *et al.*, 2012). So far > 40 withanolides have been isolated from this plant in which withaferin A and withanolide D have been reported to inhibit angiogenesis, Notch-1 and NFκB in cancer cells, and induce apoptosis in breast cancer cells (Kaileh *et al.*, 2007; Koduru *et al.*, 2010; Hahm *et al.*, 2011). Although extensive work has been carried out in *W. somnifera* with respect to metabolic profiling and pharmacological activities, there is a lack of information related to pathway steps in withanolide biosynthesis. Withanolides are reported to be formed via either the cytosolic mevalonate (MVA) pathway or the plastidial methylerythritol phosphate (MEP) pathway, which provide isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) for the synthesis of triterpenoid pathway intermediates (Sangwan *et al.*, 2008; Jadaun *et al.*, 2017). 'Head-to-tail' condensation of IPP and DMAPP forms C_{15} farnesyl diphosphate (FPP), which is further

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condensed by squalene synthase (SQS) to form the linear C_{30} triterpenoid precursor, squalene. Subsequently, squalene epoxidase (SQE) condenses squalene to form 2,3-oxidosqualene, which undergoes cyclization by specific oxidosqualene cyclases (OSCs) leading to tetra- or pentacyclic structures (Phillips *et al.*, 2006; Nes, 2011). Later, cycloartenol synthase (CAS) converts 2,3-oxidosqualene to cycloartenol, which acts as the committed precursor for plant sterols. It is believed that intermediates synthesized via the universal sterol pathway act as precursors for the biosynthesis of withanolides. These precursors are proposed to undergo various biochemical transformations like hydroxylation and glycosylation reactions, leading to the formation of various withanolides (Lockley *et al.*, 1976; Sangwan *et al.*, 2008; Singh *et al.*, 2014). As current knowledge of withanolides biosynthetic genes is lacking, use of transcription factors (TFs) that are often capable of coordinating transcription of multiple biosynthetic pathway genes holds great promise for improving the production of withanolides, either at the whole-plant level or in culture systems. By manipulating the expression of a single TF, it is possible to affect the expression of several coordinately regulated biosynthetic genes, leading to increased metabolite production (Grotewold, 2008).

WRKY TFs are generally known for their role in the regulation of stress tolerance in plants (Schluttenhofer & Yuan, 2015). However, accumulating evidence suggests that certain WRKYs regulate the production of secondary metabolites by regulating genes involved in few or many steps of a metabolic pathway. WRKYs regulating the biosynthesis of all three major classes of secondary metabolites (terpenes, phenylpropanoids and alkaloids) have been identified (Schluttenhofer & Yuan, 2015). For instance, WRKY TFs are reported to regulate the biosynthesis of specialized monoterpenes in tomato (Spyropoulou *et al.*, 2014), sesquiterpenes such as artemisinin in *Artemisia annua* (Ma *et al.*, 2009; Chen *et al.*, 2016), gossypol in *Gossypium arboreum* (Xu *et al.*, 2004) and capsidiol in tobacco (Ishihama *et al.*, 2011), diterpenes momilactone A in rice (Akagi *et al.*, 2014) and paclitaxel in *Taxus chinensis* (Li *et al.*, 2013), and polyterpenes in rubber tree (Zhang *et al.*, 2012; Wang *et al.*, 2013). In the case of triterpenoids, although *PqWRKY1* from *Panax quinquefolius* regulated triterpenoid pathway genes such as *FPPS*, *SQS* and *SQE* in *Arabidopsis*, its effect on triterpenoid metabolites was not studied (Sun *et al.*, 2013). In addition to WRKYs, TFs of basic helix–loop–helix (bHLH) and ethylene-responsive factor (ERF) class have been recently shown to regulate triterpenoid biosynthesis in *Medicago truncatula* and tomato, respectively (Thagun *et al.*, 2016; Cárdenas *et al.*, 2016; Mertens *et al.*, 2016).

It was reported that nitrogen starvation leads to drastic reduction of withanolides in *W. somnifera* (Devkar *et al.*, 2015). In our recent study, nitrogen treatment of *W. somnifera* enhanced the accumulation of phytosterols and withaferin A which correlated with higher expression of sterol pathway genes. Further, it was shown that, among different classes of TFs analyzed (WRKY, MYB, bHLH and bZIP), a particular TF WsWRKY1 (*W. somnifera* WRKY1) was highly induced in nitrogen-treated samples (Pal *et al.*, 2017). Here, we present the functional characterization of *WsWRKY1* which shows its important role in regulation of triterpenoid biosynthesis and defense in *W. somnifera*. Further, we demonstrate the positive regulatory role of *WsWRKY1* in phytosterol biosynthesis and defense in a heterologous host system. Our results show that the *WsWRKY1* regulates withanolide accumulation via transcriptional modulation of the phytosterol pathway and also controls defense response.

Materials and Methods

Plant material, phytohormone treatment and tissue collection

Withania somnifera (L.) Dunal cv Poshita (National Gene Bank, CSIR-CIMAP, India) seeds were germinated in plastic pots containing soil mixture and grown in a growth room at 22–25°C with a 16 : 8 h, light : dark photoperiod. For virus-induced gene silencing (VIGS) experiments, 3-wk-old four-leaf staged seedlings were used for agroinfiltration. For phytohormone treatments, third developmental leaves of *W. somnifera* were plucked from 10-wk-old plants kept in Petri dishes containing solutions of salicin (a derivative of salicylic acid, SA; 1.5 mM) or methyl jasmonate (MeJA; 500 μM) with respective controls. To achieve the required concentration of phytohormones, salicin was dissolved in deionized water, whereas MeJA was dissolved in dimethyl sulfoxide (DMSO; 22.8 μl corresponding to 4.3 M was added to 977.2 μl of DMSO) and diluted with deionized water. Samples were harvested at different time (h) intervals and stored at −80°C until further use. For tissue-specific expression analysis, roots, stems and leaves were collected separately from 6-wk-old plants, frozen in liquid nitrogen and stored at −80°C until further use. For generation of transgenic plants *Nicotiana tabacum* L. cv Xanthi was used.

Phylogenetic analysis

All WRKY sequences reported to be involved in secondary metabolism and some WRKYs that have been shown to be regulating stress tolerance were used for constructing the tree. A phylogenetic tree was constructed with the neighbor-joining method using default settings of MEGA v.6 (Tamura *et al.*, 2013). Bootstrap values were calculated from 1000 replicates.

Subcellular localization

The open reading frame (ORF) of *WsWRKY1* was amplified by PCR using leaf cDNA with gene-specific forward and reverse primers consisting of *Xba*I/*Bam*HI sites (Supporting Information Table S1). The resulting PCR product was cloned into pJET1.2/ vector for sequence confirmation followed by subcloning into the *Xba*I/*Bam*HI sites of p326–sGFP vector yielding the WsWRKY1-GFP construct (Fig. S1). Plasmids p326::*WsWRKY1-sGFP* and p326::*sGFP* were introduced into *Catharanthus roseus* protoplasts using PEG-mediated transformation as described previously (Yoo *et al.*, 2007; Nagegowda *et al.*, 2008). The transformed cells were then observed using a fluorescence microscope (DMI 3000B; Leica, Wetzlar, Germany).

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Construction of VIGS and overexpression vectors

Plasmids pTRV1 and pTRV2 (described in Liu *et al.*, 2002) procured from TAIR (www.arabidopsis.org) were used for generating the VIGS construct. A 540 bp *WsWRKY1* fragment was PCR-amplified from leaf cDNA using gene-specific primers (Table S1). The resulting PCR product was cloned into the pJET1.2/vector for sequence confirmation, followed by subcloning into the *Eco*RI site of the pTRV2 vector yielding pTRV2::*WsWRKY1* construct (Fig. S1). For generation of the overexpression construct, the ORF of *WsWRKY1* was PCR-amplified using leaf cDNA with specific forward and reverse primers (Table S1). The amplified fragment was cloned into the pJET1.2/vector and sequences were confirmed by nucleotide sequencing. Later, the *WsWRKY1* fragment was restriction-digested and subcloned into the *Xba*I and *Sac*I sites of the pBI121 binary vector under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV) to form the pBI121::*WsWRKY1* construct (Fig. S1). Generated VIGS and overexpression constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 by the freeze–thaw method.

VIGS and transient overexpression of *WsWRKY1*

Virus-induced gene silencing experiments were carried out by leaf infiltration according to Singh *et al.* (2015). As VIGS is prone to off targets, the sequence region unique to *WsWRKY1* was chosen for generating the VIGS construct. The specificity of *WsWRKY1* silencing was further confirmed by measuring the expression of two other similar *W. somnifera WRKY* genes that showed no effect on these genes (Fig. S2). Transient overexpression was performed in *W. somnifera* and tomato (*Solanum lycopersicum*) leaves according to Kumar *et al.* (2015). Briefly, overnight grown *Agrobacteria* cultures were pelleted and resuspended in infiltration buffer (50 mM MES, pH 5.6, 2 mM Na_3PO_4, 0.5% glucose, and 100 μM acetosyringone) to a final OD_{600} of 0.15–0.2. The suspension was further incubated at 28°C for 4 h before infiltration. *Agrobacteria* cultures were infiltrated into the first leaf pair and plants were maintained in the dark for 48 h. Leaves were harvested and stored at −80°C until further use for transcript and metabolite analyses.

Generation of transgenic tobacco plants

Plasmids pBI121::*WsWRKY1* and pBI121 were used to generate transgenic tobacco by the *Agrobacterium*-mediated leaf disc cocultivation method (Horsch *et al.*, 1985). After three rounds of selection on MS (Murashige & Skoog, 1962) medium supplemented with kanamycin (100 mg l^{-1}), regenerated shoots were excised and transferred to rooting media. Plantlets with well-established root systems were hardened for 2 wk in soilrite mix (Keltech Energies Ltd, Bengaluru, India) and then transferred to the glasshouse. T_1 seeds from individual transformants were selected on MS medium with kanamycin (50 mg l^{-1}), and the resistant seedlings were transferred to soil. Transformed plants were screened by PCR and leaves from positive lines were used for transcript and metabolite analyses, and bacterial growth assay.

RNA extraction and RT-qPCR analyses

Total RNA extraction, cDNA synthesis and quantitative reverse transcription-polymerase chain reaction (RT-qPCR) were carried out as described earlier (Rai *et al.*, 2013). *18S rRNA* for *W. somnifera*, and *EF-1α* for *N. tabacum* and tomato served as endogenous controls in RT-qPCR. For quantification of *WsWRKY1* transcripts, primers (Table S1) were designed outside the gene region cloned in pTRV2. RT-qPCR conditions were as follows: 94°C for 10 min for one cycle, followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. Fold-change differences in gene expression were analyzed using the comparative cycle threshold method.

Squalene and withanolide analysis

Squalene was extracted according to Qin *et al.* (2010) using 20 mg DW leaf tissue. For squalene analysis, the extracted residue was dissolved in 20 μl of chloroform/methanol (7 : 3, v/v) and was analyzed by high-performance liquid chromatography (HPLC, Model: SCL-10A*VP*; Shimadzu, Kyoto, Japan) following Lu *et al.* (2004). Authentic squalene standard (Sigma-Aldrich) was used for identification and quantification. Withanolides were also extracted according to Qin *et al.* (2010) and the residue was dissolved in methanol and analyzed by HPLC following Singh *et al.* (2015). All standards of withanolides were from Natural Remedies (Bangalore, India). The area of individual withanolides was determined after normalizing with the peak area of the internal standard, catharanthine (Sigma-Aldrich).

Sterol extraction and analysis

Total sterols were extracted and saponified according to Darnet & Rahier (2004). The extract was dried on Na_2SO_4 and evaporated to dryness and dissolved in 30 μl dichloromethane containing 120 ng of estrone (Sigma-Aldrich). Sample (2 μl) was injected into a GC 7890B apparatus (Agilent Technologies, Wilmington, DE, USA), which consisted of a 7693 automated sampler injection system, split/splitless injector, HP-5 fuse-silica capillary coated with 5% phenylmethyl siloxane column (30 m × 0.320 mm, 0.25 μm film thickness) and a flame ionization detection (FID) controlled by the Agilent OpenLAB CDS (EZchrom edition) v.A.04.07 software. GC conditions were followed as described previously (Singh *et al.*, 2015). Authentic cholesterol, campesterol, stigmasterol, β-sitosterol and cycloartenol were purchased from Sigma-Aldrich (Fig. S3). For quantification, a standard curve for individual authentic standard was generated and the relative response factor (RRF) for each standard was determined. Then the area of peaks corresponding to individual phytosterols was normalized with estrone. The RRF and concentration of estrone were used to determine the concentration of individual phytosterols using the respective normalized peak area. The concentrations of phytosterols were expressed as percentage content relative to control.

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Chromatin immunoprecipitation assay

For chromatin immunoprecipitation (ChIP) assays, 10 μg of p326::*WsWRKY1-sGFP* and p326::*sGFP* constructs were transfected into protoplasts prepared from *W. somnifera* leaves. Protoplasts were harvested after 16 h and ChIP assays were conducted as described previously (Bowler *et al.*, 2004; Saleh *et al.*, 2008). Protoplasts were fixed with formaldehyde and chromatin was isolated and sheared into fragments ranging from 300 to 400 bp by sonication. Anti-GFP antibody (Roche) was used for immunopurification of WsWRKY1-GFP–DNA and GFP–DNA complexes. ChIP-qPCR was performed using immunoprecipitated DNA and input DNA with primers flanking the W-box binding site of the *SQS* and *SQE* promoters. Primers detecting enrichment of both *SQS* and *SQE* promoters lacking W-box were used as negative controls. ChIP signals were compared between WsWRKY1-GFP–DNA and GFP–DNA, and the ChIP results are shown as percentage of input DNA.

Bacterial growth curve, fungal infection and insect feeding assays

For bacterial growth curve assay, avirulent and virulent (DC3000) strains of *Pseudomonas syringae* were cultured in 5 ml of nutrient broth medium in a shaking incubator at 28°C. Overnight-grown bacterial samples were centrifuged and bacterial pellets were homogenized in 10 mM $MgCl_2$ and cell density was calculated as 1×10^8 colony-forming units (CFU) ml^{-1}at $OD_{600} = 0.2$ (avirulent) and 1×10^6 CFU ml^{-1} at $OD_{600} = 0.002$ (virulent). Bacterial infiltration and cfu determination, and fungal infection and insect feeding assays were performed according to Singh *et al.* (2015).

Statistical analysis

Average mean, SE and number of replicates used for each experiment were employed for statistical evaluation using the GraphPad QuickCalc online software (http://www.graphpad.com/quickcalcs/ttest1.cfm). The statistical significance of differences between control and treated samples were tested by unpaired Student's *t*-test.

Results

Isolation and expression profiling of *W. somnifera WRKY1*

In our previous study, nitrogen treatment enhanced the accumulation of phytosterols and withanolides, which were correlated with induced expression of *WsWRKY1* (expressed sequence tag (EST) accession number GR923578), indicating the possible role of *WsWRKY1* in the regulation of triterpenoids (Pal *et al.*, 2017). Here, to further investigate the involvement of *WsWRKY1* in the regulation of triterpenoid withanolide biosynthesis, its expression was evaluated in response to MeJA and salicin that are known to induce withanolides accumulation in *W. somnifera* (Rana *et al.*, 2014). It was found that *WsWRKY1* transcripts were induced in response to both MeJA and salicin during different periods of treatment (Fig. 1a). Compared with the uninduced controls, MeJA treatment resulted in the gradual increase of *WsWRKY1* transcripts at 6 h and reached a maximum at 12 h (approximately fivefold): thereafter it decreased and remained constant (Fig. 1a). In contrast to this, under salicin treatment, *WsWRKY1* exhibited a gradual increase in its expression, peaking at 24 h (approximately fourfold) and was found slightly reduced at 48 h (Fig. 1a). In accordance with the induction of *WsWRKY1* expression, there was a correlation of withaferin A (major leaf withanolide) accumulation in response to both MeJA and salicin treatments (Fig. 1b). While MeJA treatment showed a gradual increase of withaferin A accumulation, reaching a maximum (2.8-fold) at 48 h, salicin treatment resulted in a gradual increase until 24 h (*c.* 4.8-fold) and reduced at 48 h (threefold). Between the two phytohormones, salicin treatment resulted in higher accumulation of withaferin A compared with MeJA treatment (Fig. 1b). Further tissue-specific expression analysis showed the highest expression of *WsWRKY1* in leaf (*c.* 5.5-fold) followed by stem (approximately twofold) with lowest expression in root tissue (Fig. 1c).

Sequence analysis and subcellular localization

As the EST sequence of *WsWRKY1* was lacking the 3′ region, full-length gene sequence was amplified using a forward primer (5′-ATGGAGAGAGTTAAAGGTTTGGAGAAAAAG-3′) and a reverse oligo $(dT)_{18}$ primer. Analysis of confirmed sequence revealed that *WsWRKY1* contained a 1023 bp ORF that encodes a protein of 341 amino acids (accession number MF092860) with a calculated molecular mass of 38.52 kDa. The WsWRKY1 protein contained one putative WRKY domain (WRKYGQK) together with one unique zinc finger-like motif in the C-terminal region (Fig. 2a), and belonged to the group III WRKY family of plant TFs as per the classification described by Eulgem *et al.* (2000). Generally WRKYs of group III contain a zinc-finger structure with the form of C–X_7–C–X_{23}-H–X–C, but WsWRKY1 has a unique C-X_7-C-X_{22}-H-X-C zing-finger motif. *In silico* analysis of WsWRKY1 sequence using prediction programs revealed that it has a nuclear localization signal (^{104}KKRK^{107}) (Fig. 2a), which is a general characteristic of TFs. To experimentally determine the subcellular localization, the coding region of *WsWRKY1* was fused to a GFP reporter gene, and the fusion construct was then transfected into *C. roseus* protoplasts. Analysis of fluorescence obtained from protoplasts expressing the WsWRKY1-GFP fusion protein clearly indicated a nuclear localization, whereas the fluorescence from the GFP control was distributed throughout the cell (Fig. 2b). Phylogenetic analysis of WsWRKY1 with characterized WRKY proteins involved in regulating plant specialized metabolism and stress tolerance showed its close similarity to group III WRKYs from *A. annua* (AaWRKY1), *M. truncatula* (MtWRKY109669), *Oryza sativa* (OsWRKY45 and OsWRKY89) and *C. roseus* (CrWRKY1) (Fig. 2c). Multiple sequence alignment of group III WRKYs revealed that WsWRKY1 shares highest sequence identity with AaWRKY1 (42%) followed by MtWRKY109669 (40%), OsWRKY, OsWRKY and CrWRKY1 (34%) (Fig. S4).

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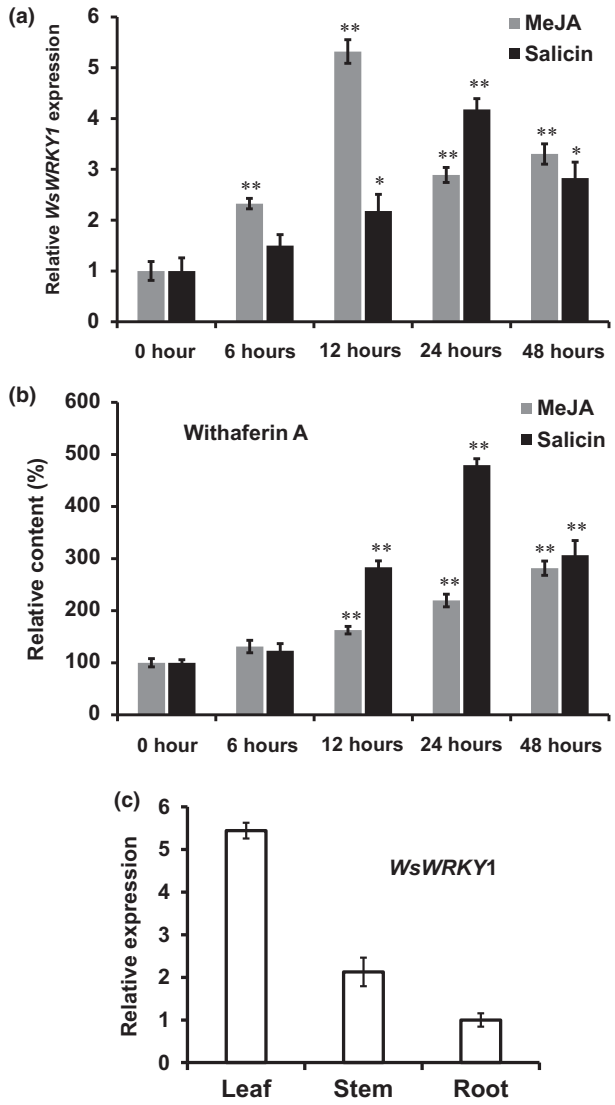

Fig. 1 Effect of methyl jasmonate (MeJA) and salicin on *Withania somnifera WRKY1* expression and withaferin A accumulation. (a, b) Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis of *WsWRKY1* expression (a) and high-performance liquid chromatography (HPLC) quantification of withaferin A (b) in *W. somnifera* leaves treated with 500 μM MeJA and 1.5 mM salicin at different time intervals. (c) Relative transcript abundances of *WsWRKY1* in different tissues of *W. somnifera*. Expression level is displayed as relative expression compared with untreated leaves. *18S rRNA* was used as an internal reference for normalization. The withaferin A level in the untreated control was set to 100% to determine its relative abundance in MeJA- and salicin-treated samples. The data shown are from three independent experiments. Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$. Error bars indicate mean ± SE.

Transcriptional down-regulation of *WsWRKY1* by VIGS leads to stunted growth and reduced withanolides

In order to test the *in planta* role in regulation of withanolide biosynthesis, *WsWRKY1* was subjected to VIGS based on the method used earlier in *W. somnifera* (Singh *et al.*, 2015). Surprisingly, VIGS of *WsWRKY1* caused stunted growth compared with empty vector (EV) control plants (Fig. 3a) and the degree of *WsWRKY1* silencing correlated with the stuntedness of the plant (Fig. 3a). Next, to determine the effect of *WsWRKY1* silencing on withanolides, leaves of similar developmental stages exhibiting the typical viral infection phenotype were collected for transcript and metabolite analyses. RT-qPCR analysis showed reduction of *c.* 75% of *WsWRKY1* expression in *WsWRKY1*-vigs leaves compared to the EV controls (Fig. 3b). Further, the effect of *WsWRKY1* silencing on withanolide accumulation was determined by subjecting the methanolic leaf extracts to HPLC, which revealed a drastic effect on withanolide accumulation, with a significant reduction in individual withanolides (Fig. 3c). Among the analyzed withanolides, all except 12-deoxywithastromonolide and withanolide A exhibited a significant decline in their accumulation in *WsWRKY1*-vigs leaves compared with EV controls (Fig. 3d). Withanoside IV and withanolide B exhibited the highest decline (*c.* 88%) followed by withaferin A (76%), and withanoside V (42%) (Fig. 3d).

Suppression of *WsWRKY1* by VIGS negatively affects sterol pathway genes resulting in reduced cycloartenol and phytosterols

Based on the observation that *WsWRKY1* silencing negatively affected plant growth and withanolide accumulation (Fig. 3), we hypothesized that *WsWRKY1* could be affecting the biosynthesis of phytosterols, which are required for normal growth and development of plants (Bach, 1995; Schaller, 2003) and as precursors for withanolide biosynthesis in *W. somnifera* (Lockley *et al.*, 1976; Sangwan *et al.*, 2008). This led us to look more closely at the expression of genes encoding enzymes involved in phytosterol biosynthesis. RT-qPCR analysis revealed that several genes of the upstream MVA pathway and downstream sterol pathway were significantly down-regulated, albeit at different levels in *WsWRKY1*-vigs tissues compared to control (Fig. 4a). Among the analyzed MVA pathway genes, transcripts for 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase, *HMGR1* and *HMGR2*) and HMG-CoA synthase (*HMGS*) displayed a reduction of *c.* 75%, whereas transcripts for mevalonate kinase (*MK*) exhibited *c.* 38% reduction (Fig. 4a). With respect to genes encoding enzymes involved in the synthesis of triterpenoid pathway intermediates, farnesyl diphosphate synthase (FPPS), SQS, and SQE displayed a reduction of *c.* 58%, 65% and 46%, respectively (Fig. 4a). While the gene encoding CAS (an oxidosqualene cyclase involved in providing flux towards sterol biosynthesis) was significantly down-regulated (*c.* 46%), genes for other oxidosqualene cyclases like β-amyrin synthase (BAS), lupeol synthase (LS) and lanosterol synthase (LAS) were not affected (Fig. 4a). Among the analyzed downstream sterol pathway genes, those encoding cycloeucalenol cycloisomerase (CEC1), Δ^{14}-sterol reductase (FK), C-7,8 sterol isomerase (HYD1), C-5 sterol desaturase (STE1), Δ^{24} sterol reductase (DWF1), sterol Δ^{7} reductase (DWF5), and C-22 sterol desaturase (CYP710A1) exhibited a reduction ranging from 30% to 67%, with

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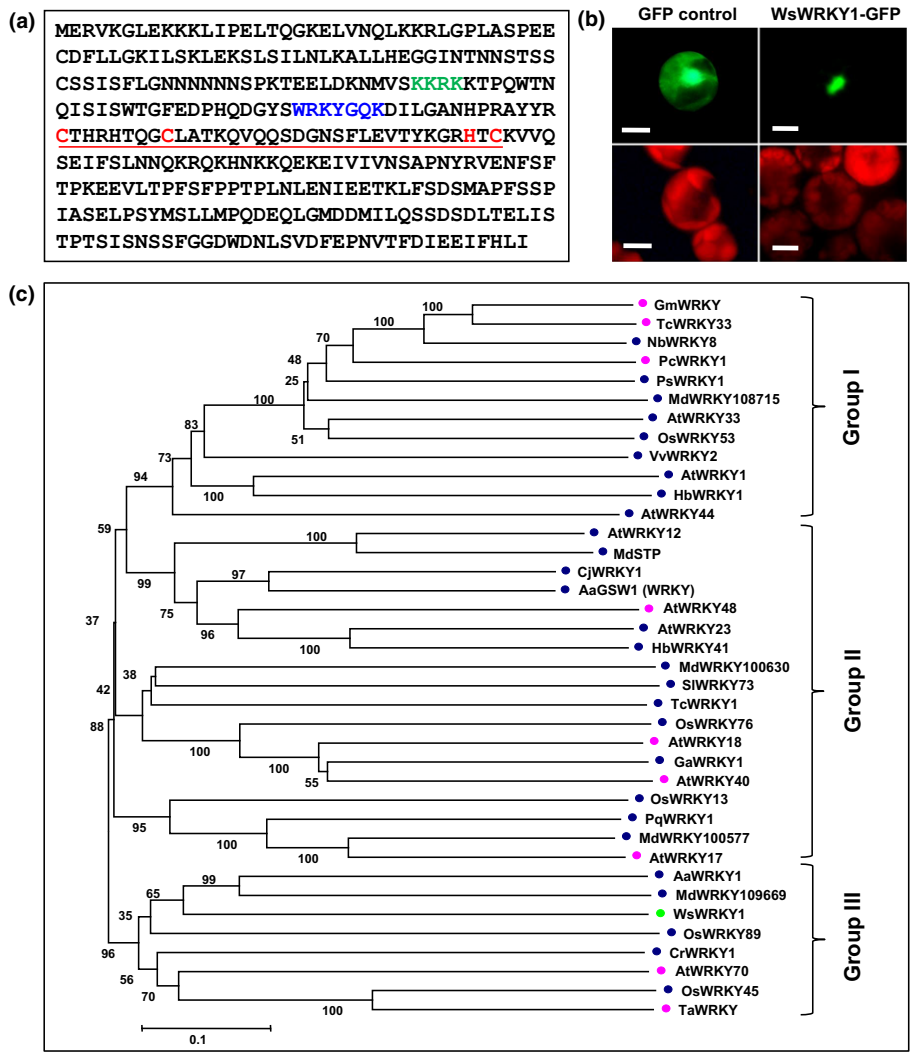

Fig. 2 Sequence analysis and subcellular localization of *Withania somnifera* WRKY1. (a) Amino acid sequence of WsWRKY1 showing the nuclear localization signal (in green), signature WRKY motif (in blue), and the characteristic zinc finger motif (underlined in red). (b) Subcellular localization of WsWRKY1 in *Catharanthus roseus* protoplasts. Green fluorescent protein (GFP) fluorescence (green) and Chl autofluorescence (red) observed in a fluorescence microscope are shown in upper and lower panels, respectively. Bars, 50 μm. (c) Phylogenetic relationship of WsWRKY1 with other plant WRKYs involved in the regulation of secondary metabolism and stress tolerance. The tree was constructed using MEGA v.6, and statistical reliability of individual nodes of the tree was assessed by bootstrap analyses with 1000 replicates. Names of sequences with blue and pink circles represent WRKYs involved in specialized metabolism and biotic/abiotic stress, respectively. WsWRKY1 characterized in this study is shown with a green circle. The WRKY proteins, their respective plant species, and GenBank accession numbers are as follows: AaWRKY1, *Artemisia annua* (FJ390842); AaGSW1 (WRKY), *A. annua* (KX465128); AtWRKY1, *Arabidopsis thaliana* (AF442389); AtWRKY12, *A. thaliana* (AF404857); AtWRKY17, *A. thaliana* (NP_565574.1); AtWRKY18, *A. thaliana* (NP_567882.1); AtWRKY23, *A. thaliana* (AY052647); AtWRKY33, *A. thaliana* (AK226301); AtWRKY40, *A. thaliana* (NP_178199.1); AtWRKY44, *A. thaliana* (NM_129282); AtWRKY48, *A. thaliana* (NP_191199.1); AtWRKY70, *A. thaliana* (NP_191199.1); CrWRKY1, *C. roseus* (HQ646368); CjWRKY1, *Coptis japonica* (AB267401); GaWRKY1, *Gossypium arboreum* (AY507929); GmWRKY, *Glycine max* (AJB84600.1); HbWRKY1, *Hevea brasiliensis* (JF742559); HbWRKY41, *H. brasiliensis* (GU372969); MdWRKY100577, *Medicago truncatula* (EU526033); MdWRKY100630, *M. truncatula* (EU526034); MdWRKY108715, *M. truncatula* (EU526035); MdWRKY109669, *M. truncatula* (EU526036); MdSTP, *M. truncatula* (HM622066); NbWRKY8, *Nicotiana benthamiana* (AB445392); OsWRKY13, *Oryza sativa* (EF143611); OsWRKY45, *O. sativa* (AK066255); OsWRKY53, *O. sativa* (AB190436); OsWRKY76, *O. sativa* (AK068337); OsWRKY89, *O. sativa* (AY781112); PqWRKY1, *Panax quinquefolius* (JF508376); PsWRKY1, *Papaver somniferum* (JQ775582); PcWRKY1, *Petroselinum crispum* (AAD55974.1); SlWRKY73, *Solanum lycopersicum* (NM_001247873); TcWRKY1, *Taxus chinensis* (JQ250831); TaWRKY, *Triticum aestivum* (APT42877); TcWRKY33, *Theobroma cacao* (EOY34631); VvWRKY2, *Vitis vinifera* (AY596466); WsWRKY1, *W. somnifera* (GR923578).

CYP710A1 and *DWF5* showing the least and highest down-regulation. However, transcripts for sterol methyltransferase (SMT1) and obtusifoliol 14-demethylase (OBT) were not significantly affected in *WsWRKY1*-silenced samples (Fig. 4a). The most striking down-regulation of genes as a result of *WsWRKY1* silencing was found among MVA pathway genes. As there was down-regulation of several sterol pathway genes including *SQS* and *CAS* in *WsWRKY1*-vigs leaves, we determined the effect of this down-regulation on cycloartenol and phytosterol accumulation. Quantification of results from GC

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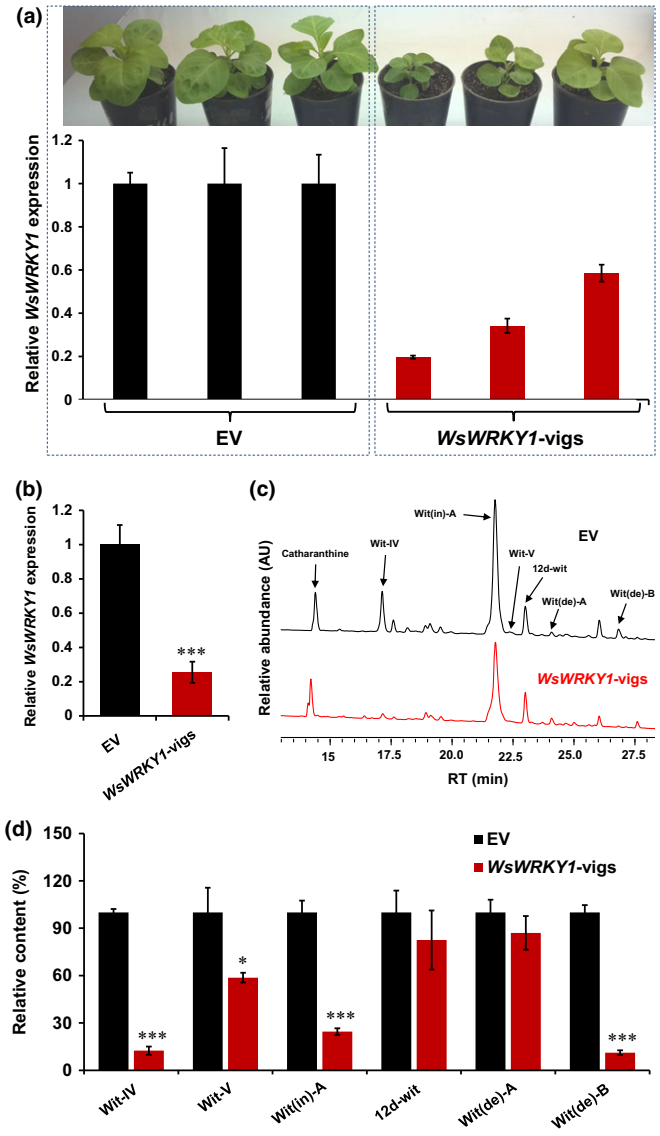

Fig. 3 *Tobacco rattle virus*-mediated silencing of *WsWRKY1* and its effect on plant growth and withanolide content in *Withania somnifera*. (a) Phenotype of *W. somnifera* plants exhibiting different degrees of *WsWRKY1* silencing. (b) Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of *WsWRKY1* expression; (c, d) high-performance liquid chromatography (HPLC) quantification of different withanolides in control and *WsWRKY1*-silenced leaves. Expression level of *WsWRKY1* was normalized to *18S rRNA* and set to 1 in an empty vector (EV) control to determine the relative reduction in *WsWRKY1*-silenced leaves. (c) Chromatograms showing relative withanolide content in *WsWRKY1*-silenced leaves compared with controls. Withanolides were extracted from dried leaves and quantified by HPLC analysis. (d) Levels of different withanolides are represented relative to EV. The area of individual withanolides was determined after normalizing with peak area of internal standard (catharanthine). Wit-IV, withanoside IV; Wit-V, withanoside V; Wit(de)-A, withanolide A; Wit(de)-B, withanolide B; Wit(in)-A, withaferin A; 12d-wit, 12-deoxywithastramonolide. The results shown are from (a) three, (b) five and (d) 10 independent experiments. Student's *t*-test: *, $P < 0.05$; ***, $P < 0.001$. Error bars indicate mean ± SE.

analysis revealed a significant reduction of all analyzed metabolites (Fig. 4b) in *WsWRKY1*-silenced leaves, with stigmasterol exhibiting the highest reduction (42%), followed by cycloartenol (44%), campesterol (39%), cholesterol (32%), and, with the lowest reduction, sitosterol (31%) (Fig. 4c).

Transient overexpression of *WsWRKY1* up-regulates phytosterol pathway genes resulting in elevated phytosterols and withanolides

Silencing of *WsWRKY1* resulted in negative regulation of sterol biosynthetic genes, leading to reduced accumulation of phytosterols and withanolides (Figs 3, 4). To investigate whether *WsWRKY1* overexpression positively regulates the expression of triterpenoid biosynthetic genes leading to increased phytosterol and withanolide content, a transient overexpression assay was carried out in *W. somnifera* leaves by agroinfiltration. Analysis of mRNA expression in *W. somnifera* leaves infiltrated with *A. tumefaciens* carrying the *WsWRKY1* gene under the control of the *CaMV 35S* promoter showed increased *WsWRKY1* transcripts (*c.* 3.4-fold) 2 d postinfiltration (Fig. 5a). Subsequent analysis of sterol biosynthetic genes showed up-regulation of all analyzed genes, with *HMGR2* and *CYP710A1* exhibiting the highest (*c.* 8.3-fold) and lowest (approximately twofold) values (Fig. 5a). The increased expression of sterol pathway genes resulted in enhanced accumulation of phytosterols (Fig. 5b). While campesterol showed the highest increase (700%) followed by cycloartenol (400%), cholesterol (340%) and stigmasterol (260%), β-sitosterol exhibited the smallest increase (200%) (Fig. 5b). Further analysis of withanolide contents in *WsWRKY1*-overexpressing leaves showed a significant increase in the accumulation of withaferin A (218%), withanolide A (207%), withanolide B (155%) and withanoside V (140%) (Figs 5c, S5). However, there was a significant reduction (30%) in 12-deoxywithastramonolide.

WsWRKY1 binds to promoters of *SQS* and *SQE*

Silencing and overexpression of *WsWRKY1* in *W. somnifera* down- and up-regulated the expression of *SQS* and *SQE*, respectively, suggesting the possible interaction of WsWRKY1 with promoters of these genes. The promoters of *W. somnifera SQS* and *SQE* have recently been found to contain the W-box core element TGAC at −373 and −288 positions, respectively (Fig. S6) (Bhat *et al.*, 2012; Razdan *et al.*, 2013). To confirm that the WsWRKY1 physically interacts with *SQS* and *SQE* promoters, a ChIP assay was carried out using *W. somnifera* protoplasts transformed with WsWRKY1–GFP and GFP constructs. Eluted ChIPed DNA was analyzed by RT-qPCR using primers corresponding to *SQS* and *SQE* promoter regions. While there was profound enrichment in both *SQS* and *SQE* promoters with primer pairs specific to W-box elements, there was no enrichment when other distant sequences in the *SQS* and *SQE* promoters were tested (Fig. 6a,b). This indicated the direct interaction of WsWRKY1 with *SQS* and *SQE* promoters, which supported the regulation of corresponding genes in VIGS and overexpression background. To further validate this, squalene was analyzed in leaves transiently overexpressing *WsWRKY1*. HPLC analysis and quantification showed a drastic increase of squalene (213%) compared with vector controls (Fig. 6c).

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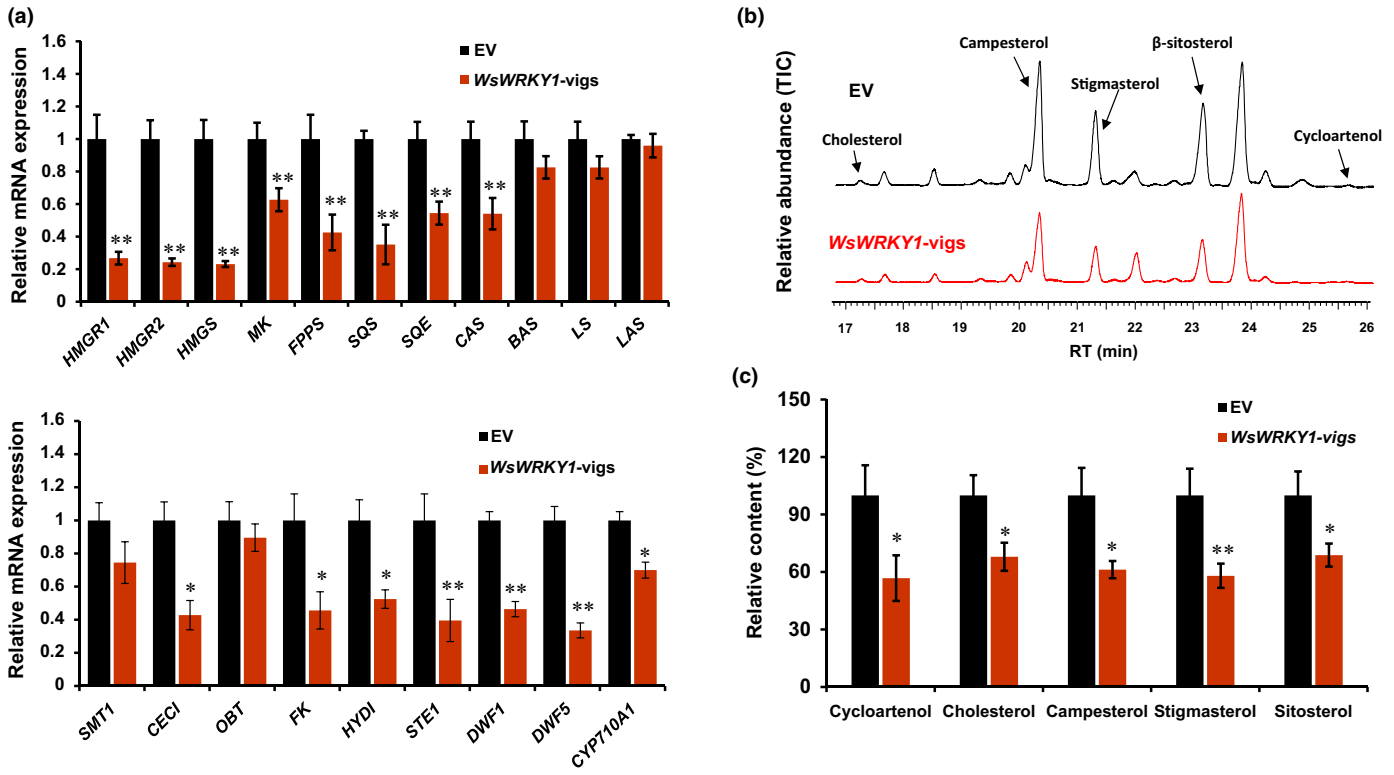

Fig. 4 Relative levels of mevalonate and sterol pathway gene expression, and phytosterols in control and *WsWRKY1*-silenced *Withania somnifera* leaves. (a) Expression levels of genes were normalized to *18S rRNA* and are represented as expression relative to an empty vector (EV) control that was set to 1. (b) Sterol content changes in *WsWRKY1*-silenced leaves compared with EV control. Total phytosterols were extracted from dried leaves and quantified by GC analysis. Total ion chromatograms (TIC) are shown. (c) Relative levels of phytosterols in *WsWRKY1*-silenced and control leaves. The peak area of individual phytosterols was normalized with the internal standard estrone, and the concentration of phytosterols in EV was set to 100 to determine the level in *WsWRKY1*-silenced leaves. The results shown are from three (a) and six (c) independent experiments. Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$. Error bars indicate mean ± SE.

WsWRKY1 silencing negatively affects the expression of defense genes, resulting in reduced tolerance to biotic stress

As WRKY proteins and phytosterols generally contribute to plant defense, the effect of *WsWRKY1* silencing on the expression of defense-related genes was determined by RT-qPCR. Among the genes encoding pathogenesis-related (PR) proteins, both SA-dependent *PR1* (62%) and *PR5* (74%), and jasmonic acid (JA)-dependent *PR3* (58%) exhibited a drastic reduction in their transcript abundances in *WsWRKY1*-vigs samples compared with controls (Fig. 7a). It has been reported that expression of *PR* is dependent on nonexpressor of PR (NPR) (Fan & Dong, 2002), as well as brassinolide (Divi *et al.*, 2010). Hence, we analyzed the effect of *WsWRKY1* silencing on the expression of genes encoding NPR1 and BR6OX2 (which catalyzes the conversion of castasterone to brassinolide) and observed a significant reduction of *NPR1* (44%) and *BR6OX2* (67%) transcripts (Fig. 7a), indicative of a link between the concentrations of endogenous sterols and brassinolide and the level of NPR-dependent *PR* gene expression. The reduced gene expression of *PR*, *NPR* and *BR6OX2* in *WsWRKY1*-silenced leaves was correlated with reduced tolerance to biotic stresses (Fig. 7). A bacterial growth assay using extracts isolated from *P. syringae*-infiltrated leaves showed that *WsWRKY1*-silenced leaves supported a significantly higher multiplication of both avirulent and virulent *P. syringae* strains than extracts from EV-infiltrated leaves (Fig. 7b). In fungal infection assays with the necrotrophic pathogen *Botrytis cinerea*, *WsWRKY1*-vigs leaves developed severe disease symptoms and sustained more tissue damage than control leaves at 4 d postinoculation (Fig. 7c). *WsWRKY1*-vigs leaves were chlorotic or macerated, whereas leaves from control plants remained green with necrotic spots restricted to the initial culture droplet area, without much spreading of infection (Fig. 7c). *WsWRKY1*-vigs leaves exhibited reduced tolerance to *B. cinerea* infection, which was evident by a significant increase (*c.* 2.71-fold) in the average diameter of lesions compared with that of EV leaves (Fig. 7d). Further, insect feeding assay using tobacco cutworms (*Spodoptera litura*) showed that the larvae preferred eating *WsWRKY1*-silenced leaves than control leaves (Fig. 7e). Also, preference index (PI) determination showed a higher preference of tobacco cutworm larvae for *WsWRKY1*-vigs leaves (PI = 1.58) compared with the EV control (PI = 1.0) (Fig. 7f).

Overexpression of *WsWRKY1* in tobacco positively regulates phytosterol biosynthesis and biotic stress

To check whether *WsWRKY1* positively affects phytosterol and defense response in a heterologous host system, transgenic tobacco plants overexpressing *WsWRKY1* (OE-*WsWRKY1*) were

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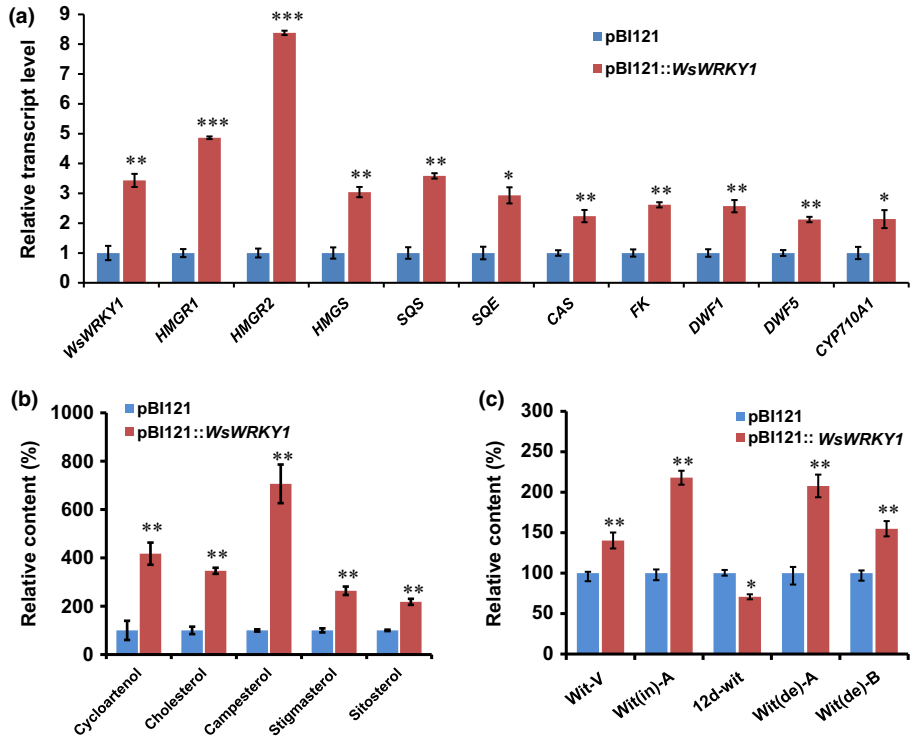

Fig. 5 Effect of transient overexpression of *WsWRKY1* on mevalonate and phytosterol pathway genes, phytosterols, and withanolides in *Withania somnifera*. (a) Relative levels of *WsWRKY1*, mevalonate and sterol pathway genes in leaves infiltrated with *Agrobacterium* harboring pBI121 control and pBI121::*WsWRKY1* overexpression constructs. Expression levels of genes were normalized to *18S rRNA* and are represented as expression relative to a pBI121 control that was set to 1. (b) Phytosterols were extracted from dried leaves and quantified by GC analysis. The peak area of individual phytosterols was normalized with the internal standard estrone and expressed as relative percentage to the pBI121 control. (c) Levels of individual withanolides are represented relative to the pBI121 control. The area of individual withanolides was determined after normalizing with the peak area of the internal standard. Wit-V, withanoside V; Wit(de)-A, withanolide A; Wit(de)-B, withanolide B; Wit(in)-A, withaferin A; 12d-wit, 12-deoxywithastramonolide. The results shown are from three to four (a), four (b) and six (c) biological replicates. Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate mean ± SE.

generated. The expression levels of three independent lines were determined by RT-qPCR. Plants transformed with the empty pBI121 vector served as controls. Transgenic OE-*WsWRKY1* lines showed expression ranging from 40- to 85-fold in analyzed lines (Fig. 8a). To investigate if the overexpression of *WsWRKY1* has any effect on the transcript abundance of sterol pathway genes, the mRNA expression of *HMGR1*, *FPPS*, *SQS*, *SQE*, *CAS*, *OBT* and *SMT1* in OE-*WsWRKY1* lines was analyzed. Results indicated that all selected pathway genes were up-regulated, albeit at different levels, in all OE-*WsWRKY1* lines as compared with the pBI121 control (Fig. 8b). The up-regulation of sterol pathway genes in OE-*WsWRKY1* could have a positive regulatory effect on phytosterol accumulation similar to the results obtained in *W. somnifera* (Fig. 5a,b). Hence, phytosterols were analyzed using leaves of OE-*WsWRKY1* plants and control tobacco plants, which showed a significant increase in sterol intermediates squalene and cycloartenol (Fig. 8c,d). With respect to phytosterols, except for β-sitosterol, which showed a significant reduction in all OE-*WsWRKY1* lines, all other phytosterols exhibited a significantly increased accumulation (Fig. 8d). To determine the effect of *WsWRKY1* overexpression on biotic stress, a bacterial growth assay was performed, which revealed a significant tolerance of OE-*WsWRKY1* samples to *P. syringae* (strain DC3000) growth compared with controls (Fig. 8e).

Discussion

In this study, we set out to investigate the transcriptional regulation of triterpenoid withanolide biosynthesis in *W. somnifera*. Our previous study found that a particular TF belonging to the WRKY class (*WsWRKY1*) was highly induced in response to nitrogen treatment, with a corresponding increase in withanolides (withaferin A) as well as phytosterols (Pal *et al.*, 2017). Here, a series of investigations was carried out to further determine the functional role of *WsWRKY1*. Phytohormones have been shown to up-regulate the expression of pathway genes and WRKY TFs involved in secondary metabolism with a corresponding increase in target secondary metabolites (Ma *et al.*, 2009; Suttipanta *et al.*, 2011; Chen *et al.*, 2016). In *W. somnifera*, the expression of *SQS*, *SQE* and *CAS*, involved in phytosterol biosynthesis, was highly induced by MeJA and SA, with a corresponding increase in withanolides (Bhat *et al.*, 2012; Dhar *et al.*, 2014). Moreover, RNAi and homologous overexpression of *CAS* resulted in a respective reduction and enhancement of withanolide accumulation in *W. somnifera* plants (Mishra *et al.*, 2016). In this study, *WsWRKY1* exhibited significant up-regulation in response to MeJA and salicin (Fig. 1a), and its expression was correlated with accumulation of withaferin A, the major withanolide in leaves (Fig. 1b). *WsWRKY1* up-regulation in response to

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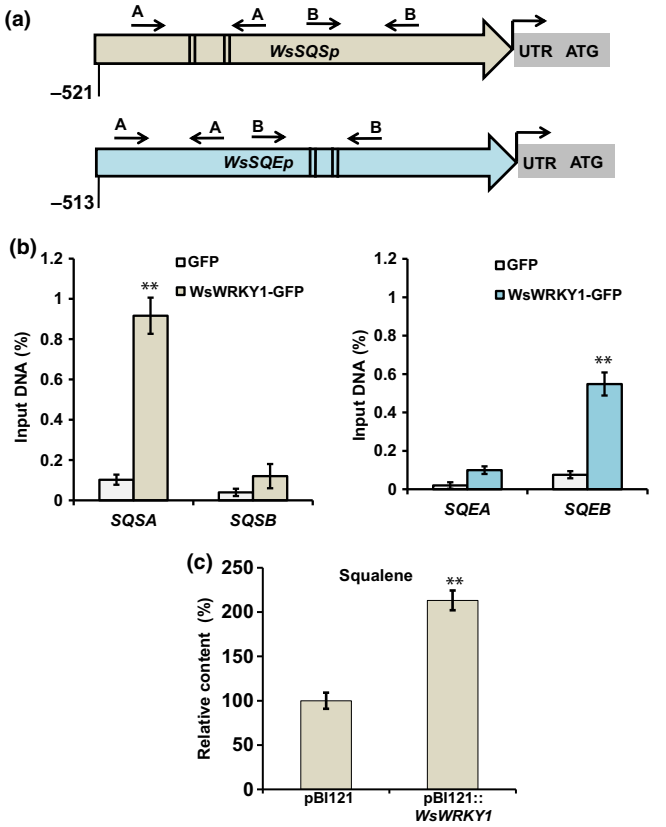

Fig. 6 Binding of WsWRKY1 to *WsSQS* and *WsSQE* promoters, and effect of *WsWRKY1* overexpression on squalene accumulation. (a) Schematic diagram showing the location of W-box core elements (indicated as vertical lines) in promoter sequences of *WsSQS* and *WsSQE*. A and B indicate the location of primers corresponding to promoter regions of *WsSQS* and *WsSQE* used in chromatin immunoprecipitation (ChIP) coupled with quantitative PCR (ChIP-qPCR) analysis. (b) ChIP assay of WsWRKY1. *Withania somnifera* leaf protoplasts were transfected with WsWRKY1-GFP and GFP constructs. WsWRKY1-GFP and GFP chromatin complexes were immunoprecipitated using anti-GFP antibody. ChIP and input DNA samples were quantified by qPCR using specific primers A and B. The ChIP results are shown as a percentage of input DNA. Data represent three independent experiments. Student's *t*-test: **, $P < 0.01$. (c) *Withania somnifera* leaves were infiltrated with *Agrobacterium* harboring pBI121 control and pBI121::*WsWRKY1* overexpression constructs, and tissue was collected after 48 h. Squalene was extracted from dried leaves and analyzed by high-performance liquid chromatography and represented as relative levels. The data shown are from three independent experiments. Student's *t*-test: **, $P < 0.01$. Error bars indicate mean ± SE.

phytohormones and its high abundance in leaves indicated its possible role in regulation of withanolide biosynthesis in *W. somnifera* (Fig. 1c).

To determine the involvement of *WsWRKY1* in withanolide biosynthesis, a reverse-genetics VIGS approach was used to down-regulate *WsWRKY1* in *W. somnifera* leaves. Silencing of *WsWRKY1* resulted in stunted growth of the plant with a significant decrease in withanolide content (Fig. 3). The morphogenetic inhibition and a reduced withanolide content caused by *WsWRKY1* silencing in *W. somnifera* were similar to previous reports in which down-regulation/knockout of sterol pathway genes such as *HMGR* (Suzuki *et al.*, 2004), *CAS1* (Gas-Pascual *et al.*, 2014), *SMT* (Choe *et al.*, 1999), *FK* (Schrick *et al.*, 2000) and *OBT* (Burger *et al.*, 2003) led to reduced phytosterols and severely stunted growth in different plants. Hence, we hypothesized that the effect of *WsWRKY1* silencing on both withanolides and plant growth in *W. somnifera* could be a result of the depletion of sterol precursors, which are required for withanolide formation (Singh *et al.*, 2014) as well as for normal growth and development of the plant (Bach, 1995; Schaller, 2003). Indeed, *WsWRKY1*-silenced leaves exhibited significant down-regulation of several genes of the phytosterol pathway, including genes of the upstream MVA pathway, and the first committed step of triterpene formation (SQS) (Wu *et al.*, 2012) (Fig. 4a). Interestingly, among the genes for branch point oxidosqualene cyclases, only *CAS* exhibited significant down-regulation similar to downstream sterol pathway genes, whereas *BAS*, *LAS* and *LS* were unaffected by *WsWRKY1* silencing (Fig. 4a). This indicated the specific regulation of sterol branch of the triterpenoid pathway by *WsWRKY1*. Such differential regulation between distinct parts of the triterpenoid pathway has recently been reported in *M. truncatula* by bHLH family TFs (TRITERPENE SAPONIN BIOSYNTHESIS ACTIVATING REGULATOR, TSAR1 and TSAR2) (Mertens *et al.*, 2016), and in tomato by the APETALA2/ERF GLYCOALKALOID METABOLISM 9 (*GAME9*) (Cárdenas *et al.*, 2016) and a jasmonate-responsive ERF (JRE) (Thagun *et al.*, 2016) (Fig. S7). Transcript reduction of sterol pathway genes by *WsWRKY1* silencing was accompanied by a significant reduction in the concentrations of cycloartenol and phytosterols (Fig. 4c). This suggested that *WsWRKY1* controls the flux towards sterol and withanolide biosynthesis through the regulation of several pathway steps. Recently, determination of the relative importance of transcriptional control at each individual step of isoprenoid precursor biosynthesis showed that the 'flux control' in the sterol pathway is shared by several enzymes (Lange *et al.*, 2015). Specialized metabolites are generally formed from primary metabolites (Laursen *et al.*, 2015); hence reduced withanolide (specialized metabolite) accumulation in *WsWRKY1*-silenced plants could be a result of reduced phytosterols (primary metabolites). Alternatively, reduced withanolides might be a result of down-regulation of yet to be identified genes of the withanolide pathway by *WsWRKY1* silencing, which is corroborated by the more drastic reduction of withanolides than of phytosterols (Figs 3, 4). This was supported by the fact that the expression of genes encoding CYP450 enzymes (WsCYP93Id, WsCYP76A and WsCYP98A) and GTs (WsSGT1, WsSGT2 and WsSGT4), which are indicated to be involved in withanolide formation (Rana *et al.*, 2014; Srivastava *et al.*, 2015; Singh *et al.*, 2016), was drastically reduced in *WsWRKY1*-silenced *W. somnifera* leaves (Fig. S8).

In contrast to *WsWRKY1*-vigs, transient overexpression of *WsWRKY1* in *W. somnifera* leaves up-regulated phytosterol pathway genes such as *HMGS*, *HMGR*, *SQS*, *SQE*, *FK*, *DWF1* and *DWF5* (Fig. 5), and withanolide pathway genes such as *WsCYP93Id*, *WsCYP76A*, *WsCYP98A*, *WsSGT1*, *WsSGT2* and *WsSGT4* (Fig. S8), thus leading to enhanced phytosterol and withanolide content (Fig. 5). This supported the observation

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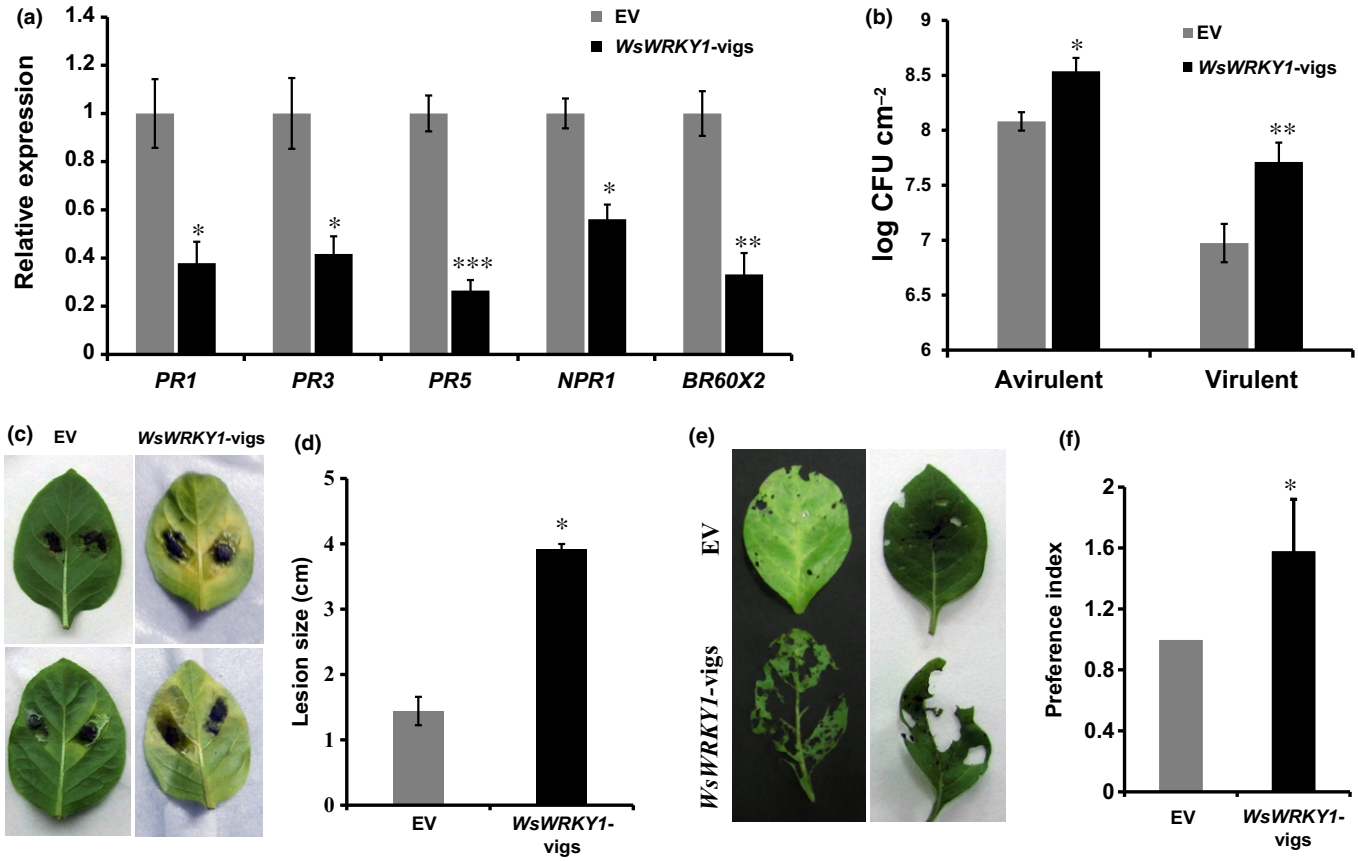

Fig. 7 Effect of *WsWRKY1* silencing on defense-related genes and biotic stress in *Withania somnifera*. (a) Expression levels of genes were normalized to *18S rRNA* and are represented as expression relative to the empty vector (EV) control, which was set to 1. (b) Bacterial growth assay with *Pseudomonas syringae*. The first pair of EV and *WsWRKY1*-vigs leaves at 30 d postinfiltration (dpi) displaying a viral infection phenotype was infiltrated with avirulent and virulent (DC3000) strains of *P. syringae*. Bacterial growth at 4 dpi was obtained by plating serial dilutions of leaf extracts from EV and *WsWRKY1*-vigs. (c) Phenotype of *Botrytis cinerea* infection 4 d postinoculation on detached leaves of EV control and *WsWRKY1*-vigs leaves. The first pair of EV and *WsWRKY1*-vigs leaves at 30 dpi having viral infection phenotype was inoculated with a 20 µl drop of *Botrytis cinerea* spore suspension (*c.* 10^5 spores ml^{-1}) at the center of the abaxial side and kept at 23°C with high humidity. (d) Statistical data of lesion size at 4 dpi. (e) Insect feeding assay of EV and *WsWRKY1*-vigs leaves using tobacco cutworm (*Spodoptera litura*). Equal numbers of second-instar cutworms (six each) were placed on EV and *WsWRKY1*-vigs leaves, and the area fed was recorded after 12 h. (f) Feeding preference index (PI) of tobacco cutworms. The eaten area of leaf was measured 12 h after the start of the experiment to calculate the PI. The presented data are from three independent experiments. Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate mean ± SE.

made in VIGS studies that *WsWRKY1* regulates phytosterol and withanolide biosynthesis, suggesting the possible *in planta* interaction of WsWRKY1with promoters of genes involved in phytosterol and withanolide biosynthesis. As WRKY proteins bind to W-box elements (Eulgem *et al.*, 2000) to regulate the gene expression, it is possible that W-boxes (TTGACC/T) could be present in promoters of MVA and sterol pathway genes in *W. somnifera*. Indeed, promoter regions of *SQS* and *SQE* contain W-box in *W. somnifera* (Bhat *et al.*, 2012; Razdan *et al.*, 2013). The ChIP-qPCR assay showed that WsWRKY1 binds to promoters of *SQS* and *SQE* (Fig. 6b), suggesting their direct regulation by the WsWRKY1. This was further supported by increased accumulation of squalene when *WsWRKY1* was transiently overexpressed in *W. somnifera* leaves (Fig. 6c). Interestingly, although the promoter of *CAS* lacks a W-box (Dhar *et al.*, 2014), its expression was down- and up-regulated in *WsWRKY1* silencing and overexpression background, respectively, which may be a result of the indirect effect of reduced and increased transcripts of several genes upstream of CAS (Figs 4a, 5a). It was reported earlier that overexpression of *HMGS* (Wang *et al.*, 2012) and silencing of *SQS* (Singh *et al.*, 2015) result in corresponding up- and down-regulation of other sterol pathway genes, indicating a tight regulation of sterol pathway genes. However, at this point, the possibility of direct binding of WsWRKY1 to promoters of phytosterol pathway genes other than *SQS* and *SQE* cannot be ruled out in *W. somnifera*. Moreover, ectopic expression of *WsWRKY1* in tobacco not only up-regulated sterol pathway genes but also increased the phytosterol content (Fig. 8), demonstrating that *WsWRKY1* is also capable of regulating sterol pathway in other plant species. Also, *WsWRKY1* overexpression in tobacco led to faster growth and improved plant phenotype in terms of plant height (Fig. S9), which was in contrast to the negative effect of *WsWRKY1*-vigs on *W. somnifera* growth. Further, a transient overexpression of *WsWRKY1* in the crop plant, tomato, resulted

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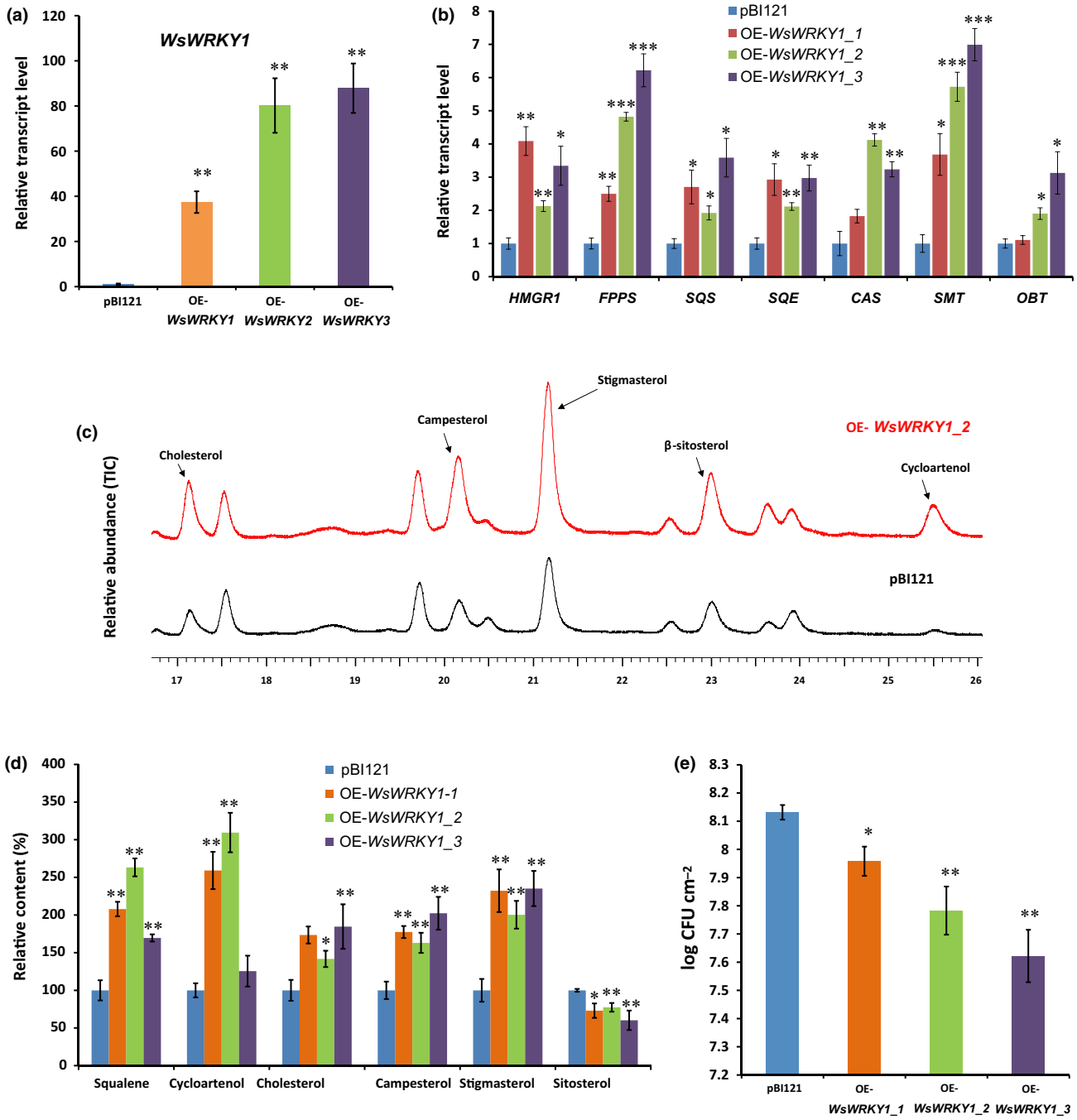

Fig. 8 Analysis of phytosterol pathway gene expression, triterpenoid content, and bacterial growth in transgenic tobacco overexpressing *WsWRKY1*. Expression of *WsWRKY1* (a) and sterol (b) pathway genes in transgenic tobacco (*Nicotiana tabacum*) lines. Expression levels of genes were normalized to *EF-1α* and are represented as expression relative to the pBI121 control, which was set to 1. (c) Sterol content changes in OE-*WsWRKY1* leaves compared with control. Phytosterols were extracted from dried leaves and quantified by GC analysis. Total ion chromatograms are shown. (d) Relative concentrations of phytosterols in OE-*WsWRKY1* and control leaves. The peak area of individual phytosterols was normalized with the internal standard estrone and expressed as relative percentage to the pBI121 control. (e) Bacterial growth assay was performed by infiltrating leaves of pBI121 and OE-*WsWRKY1* with the virulent *P. syringae* (DC3000) strain. Bacterial growth at 3 d post infiltration (dpi) was obtained by plating serial dilutions. The data presented here are from three (a, b), four (d), and three (e) independent experiments. Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate mean ± SE.

in improved formation of squalene and phytosterols (Fig. S10), indicating that *WsWRKY1* can be a promising candidate for enhancing nutraceutically important phytosterols in tomato fruits. These results comprehensively prove that *WsWRKY1* is involved in regulation of phytosterol biosynthesis, which in turn controls withanolide accumulation in *W. somnifera*. Given that TFs are often capable of coordinating the transcription of multiple biosynthetic pathway genes (Grotewold, 2008), it is not surprising that *WsWRKY1* affects several genes of the MVA and phytosterol pathways. Similar coordinated up-regulation of

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biosynthetic genes of secondary metabolism by WRKYs has been observed in other plant species. For example, overexpression of a trichome-specific WRKY in *A. annua* (AaGSW1) up-regulated the genes of the artemisinin pathway, resulting in improved accumulation of artemisinin (Chen *et al.*, 2016). Similarly, *CrWRKY1* in *C. roseus* positively regulated the expression of several terpene indole alkaloid (TIA) pathway genes, thereby improving the biosynthesis of TIAs (Suttipanta *et al.*, 2011). Moreover, analysis of recently released genome sequence of Solanaceae plants such as tomato and potato revealed the presence of W-box sequences in MVA and sterol pathway genes (Table S2), suggesting a similar scenario in *W. somnifera.*

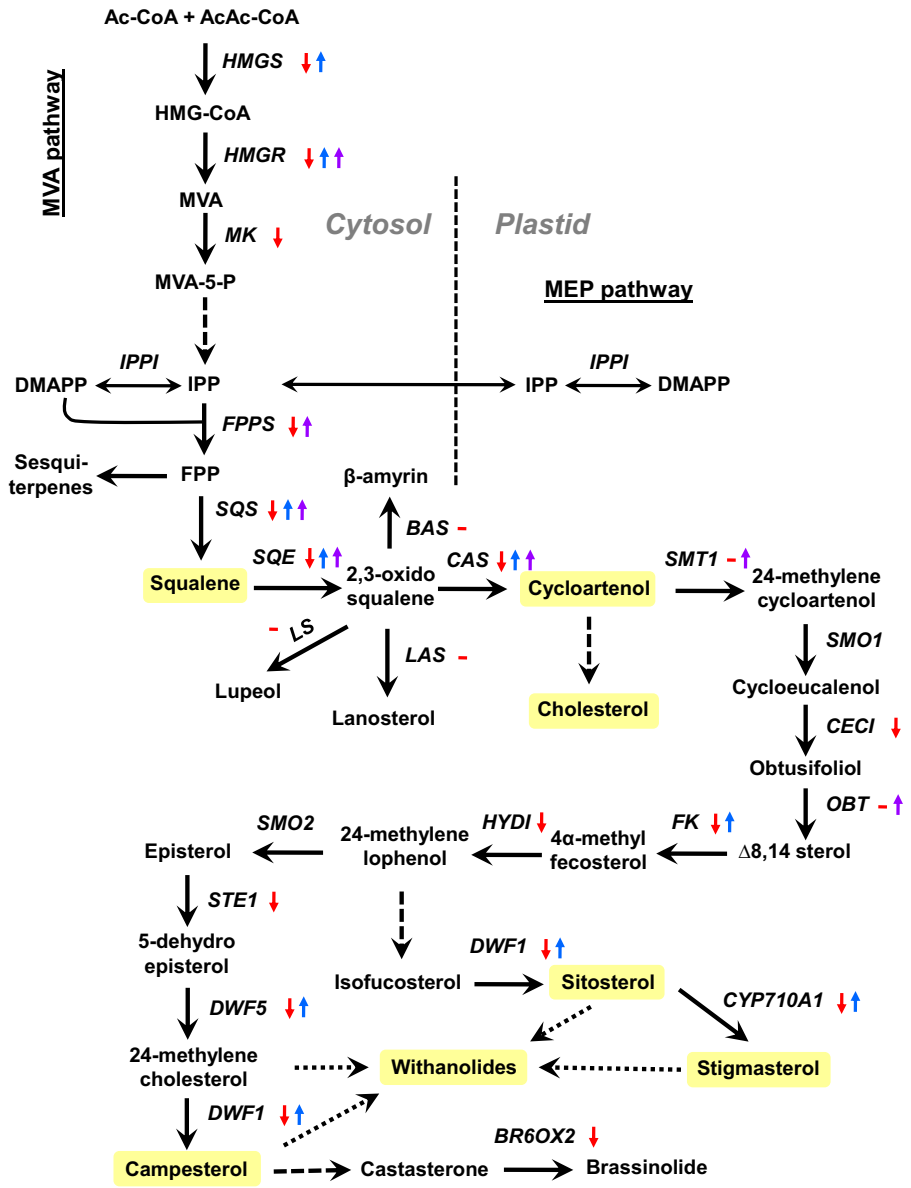

Fig. 9 Simplified view of isoprenoid biosynthesis in plants. Solid black arrows indicate single-step reactions, dashed arrows denote several steps, dotted arrows represent unidentified steps and the double arrow between cytosolic and plastid compartments indicates metabolic crosstalk between them. Down-regulation of genes as a result of *WsWRKY1* silencing in *Withania somnifera* is indicated by downward red arrows, and up-regulation of genes in *W. somnifera* and tobacco overexpressing *WsWRKY1* is shown as upward blue and purple arrows, respectively. Red hyphens indicate no change in expression level as a result of *WsWRKY1* silencing in *W. somnifera.* Metabolites that are quantified in this study are shown in yellow. Ac-CoA, acetyl CoA; AcAc-CoA, acetoacetyl CoA; *BAS*, β-amyrin synthase; *BR6OX2*, brassinosteroid-6-oxidase 2; *CAS*, cycloartenol synthase; *CECI*, cycloeucalenol cycloisomerase; *CYP710A1*, C-22 sterol desaturase; DMAPP, dimethylallyl diphosphate; *DWF1*, Δ^{24} sterol reductase; *DWF5*, sterol Δ^{7} reductase; *FK*, Δ^{14}-sterol reductase; FPP, farnesyl diphosphate; *FPPS*, FPP synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; *HMGR*, HMG-CoA reductase; *HMGS*, HMG-CoA synthase; *HYDI*, C-7,8 sterol isomerase; IPP, isopentenyl diphosphate; *IPPI*, isopentenyl diphosphate isomerase; *LAS*, lanosterol synthase; *LS*, lupeol synthase; MEP, 2-*C*-methyl-D-erythritol 4-phospahte; MVA, mevalonate; MVA-5-P, 5-phosphomevalonate; *MK*, mevalonate kinase; *OBT*, obtusifoliol 14-demethylase; *SMO1*, sterol-4α-methyl oxidase 1; *SMO2*, sterol-4α-methyl oxidase 2; *SMT1*, sterol methyltransferase 1; *SQE*, squalene monooxygenase/epoxidase; *SQS*, squalene synthase; *STE1*, C-5 sterol desaturase.

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WRKY TFs are generally known to be responsive to pathogen infection and phytohormone treatment, and to act as positive or negative regulators of plant defense responses (Schluttenhofer & Yuan, 2015). Suppression of *WsWRKY1* in *W. somnifera* negatively affected defense responsive genes (Fig. 7a), which was similar to the effect of *WsSQS* silencing (Singh *et al.*, 2015). Consistent with the MeJA- and SA-induced expression of *WsWRKY1* in *W. somnifera* (Fig. 1a), both SA-dependent *PR1* and *PR5* (thaumatin) and JA-dependent *PR3* (chitinase) expression were down-regulated in *WsWRKY1*-silenced plants (Fig. 7a). *WsWRKY1*-vigs plants also showed drastic reductions of *BR6OX2* mRNA levels (Fig. 7a), suggesting possible reduction in endogenous brassinolide concentrations. Moreover, *NPR1*, a master regulator of *PR* genes (Fan & Dong, 2002), was also significantly reduced in *WsWRKY1*-vigs plants (Fig. 7a). Reduction of defense-related genes (*PR*) and their regulators (*NPR1*, *BR60X2*) could be a result of low transcript abundance of *WsWRKY1*, thereby resulting in reduced expression of defense-related genes and their regulators, which are known to be a direct target of WRKY TFs, owing to the presence of WRKY binding site(s) (W-box) in their promoters (Asai *et al.*, 2000; Yu *et al.*, 2001). These observations were further supported by reduced tolerance of *WsWRKY1*-vigs plants to bacteria *P. syringae* and fungus *B. cinerea* infection, and tobacco cutworm feeding (Fig. 7). Specialized metabolites regulated by WRKY TFs contribute to overall fitness of the plant by enhancing tolerance to various stresses (Schluttenhofer & Yuan, 2015). Withanolides have been shown to possess antibacterial and antifungal properties (Choudhary *et al.*, 1995), and antifeedant properties against herbivorous insects (Waiss *et al.*, 1993). Hence, it seems likely that reduced concentrations of withanolides could have compromised the tolerance of *WsWRKY1*-vigs leaves to bacterial and fungal infection, and to insect feeding. Furthermore, reduced *NPR1* expression may also have contributed to the reduced tolerance of *WsWRKY1*-vigs leaves to cutworm feeding. It was shown that *NPR1*-silenced *Nicotiana attenuata* plants were more susceptible to *Spodoptera exigua* larvae, suggesting NPR1's role in mediating herbivore resistance in plants (Rayapuram & Baldwin, 2007). In contrast to the negative effect of *WsWRKY1* silencing on bacterial stress in *W. somnifera* (Fig. 7b), transgenic tobacco overexpressing *WsWRKY1* displayed an enhanced tolerance to *P. syringae* (Fig. 8e), confirming the involvement of *WsWRKY1* in biotic stress tolerance.

Overall, *WsWRKY1* regulated the expression of MVA pathway genes and downstream sterol pathway genes, resulting in corresponding regulation of phytosterols and withanolides (Fig. 9). Studies on TFs such as *M. truncatula* TSARs (Mertens *et al.*, 2016), tomato GAME9 and JRE (Thagun *et al.*, 2016; Cárdenas *et al.*, 2016) along with studies on the involvement of *W. somnifera* WRKY1 (this work) in triterpenoid pathway regulation, indicate that various branches of the pathway are differentially regulated by TFs belonging to divergent classes, further highlighting the complex regulation of terpenoid biosynthesis in plants. It is possible that these TFs interact in combination to bring about the required accumulation of target metabolites, as has been reported for terpene indole alkaloid biosynthesis in *C. roseus* (Paul *et al.*, 2016). The presence of WsWRKY1 homologs in other solanaceae plants (such as tomato, potato and capsicum; Fig. S11) further suggests that these homologs might be functioning in a similar fashion to regulate the phytosterol branch of the triterpenoid pathway in these species as well. Overall, these findings demonstrate the importance of *WsWRKY1* in the regulation of *W. somnifera* phytosterol and withanolide biosynthesis in particular, and plant phytosterol biosynthesis and defense in general. Hence, *WsWRKY1* could be useful for engineering enhanced withanolide biosynthesis in *W. somnifera* and improved phytosterol and biotic stress tolerance in solanaceous plants.

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Author contributions

A. K. Singh., S.R.K., V.D., A.R. and S.P. performed the experiments. A. K. Singh, S.R.K., V.D., A.R., S.P., A. K. Shasany and D.A.N. analyzed the data. D.A.N. conceived and coordinated the research, and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 p326-sGFP, pTRV2 and pBI121 derived vector maps used for subcellular localization, VIGS and overexpression studies, respectively.

Fig. S2 Expression analysis of *WsWRKY1* and two other similar *WRKY* genes in *WsWRKY1*-vigs leaves.

Fig. S3 GC chromatogram showing authentic sterol standards.

Fig. S4 Multiple sequence alignment of WsWRKY1 with other group III WRKYs involved in secondary metabolism.

Fig. S5 HPLC chromatograms showing relative abundance of withanolides in *W. somnifera* infiltrated with pBI121 and pBI121::*WsWRKY1* constructs.

Fig. S6 Promoter sequences of genes encoding SQS, SQE and CAS in *W. somnifera*, indicating the presence or absence of conserved W-box elements.

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Fig. S7 Differential regulation of distinct parts of the triterpenoid pathway in *M. truncatula*, tomato, *W. somnifera* and tobacco by different transcription factors.

Fig. S8 Effect of silencing and transient overexpression of *WsWRKY1* on genes encoding CYP450s and GTs indicated to be involved in withanolide biosynthesis in *W. somnifera*.

Fig. S9 Representative images showing the effect of WsWRKY1 overexpression in tobacco seedlings, young plants and mature plants.

Fig. S10 Effect of transient overexpression of *WsWRKY1* on squalene and phytosterols in tomato leaves.

Fig. S11 Phylogenetic tree showing the relationship between WsWRKY1 and putative WRKY sequences from other solanaceous plants.

Table S1 List of oligonucleotide primers used in this study

Table S2 Position of W-box in promoters of selected MVA and sterol pathway genes of *Nicotiana tabacum* and tomato

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