Analysis of the mechanisms regulating the expression of isoprenoid biosynthesis genes in hydroponically-grown *Nicotiana benthamiana* plants using virus-induced gene silencing

Go Atsumi1, Uiko Kagaya2, Noriko Tabayashi2 & Takeshi Matsumura1

Secondary metabolites in plants play important roles in defence against biotic and abiotic stresses. Although the biosynthesis pathways of secondary metabolites have been extensively studied, the regulatory mechanism of gene expression involved in these pathways remains poorly understood. In this study, we develop a virus-induced gene silencing (VIGS) system that enables a rapid analysis of the regulatory mechanism of genes involved in the biosynthesis of isoprenoids, one of the largest groups in secondary metabolites, using hydroponically-grown *Nicotiana benthamiana*. Using VIGS, we successfully reduced the transcript levels of 3-hydroxy-3-methylglutaryl-CoA reductase 1 (*HMGR1*), cycloartenol synthase 1 (*CAS1*), sterol side chain reductase 2 (*SSR2*) and S-adenosyl-L-Met-dependent C-24 sterol methyltransferase 1 (*SMT1*) in leaf, stem and root tissues in approximately 2 weeks. We identified novel feedback and feed-forward regulation of isoprenoid biosynthesis genes when *CAS1*, which encodes a key enzyme involved in the biosynthesis of sterols and steroidal glycoalkaloids, was down-regulated. Furthermore, the regulation of these genes differed among different tissues. These results demonstrate that our system can rapidly analyse the regulatory mechanisms involved in the biosynthesis of secondary metabolites.
the physiology of differentiated cells. By contrast, virus-induced gene silencing (VIGS) technology can be used to manipulate transient gene expression in intact tissues. In VIGS, target transcripts are transiently degraded in a homology-dependent manner using a virus vector carrying a partial fragment of the target gene. VIGS does not require the generation of transgenic plants and has been used to suppress the expression of target genes in many plant species, including herbs and woody plants.\(^{15}\) VIGS has been successfully used to study the function of genes involved in the biosynthesis of flavonoids in soybean (\textit{Glycine max})\(^{16,17}\) and steroidal glycoalkaloids in tomato (\textit{Solanum lycopersicum})\(^{18-20}\).

Isoprenoids constitute one of the largest groups of secondary metabolites; more than 50,000 isoprenoids have been identified until now\(^{21}\). In this study, we developed tobacco rattle virus (TRV)-based VIGS system to analyse the regulatory mechanisms of genes involved in the isoprenoid biosynthesis pathways in tobacco (\textit{Nicotiana benthamiana}). We have selected a hydroponic culture system of \textit{N. benthamiana}, which is suitable for the analysis of intact underground tissues and facilitates the control of factors affecting metabolite accumulation, such as the nutritional status. We demonstrated the successful down-regulation of genes including 3-hydroxy-3-methylglutaryl-CoA reductase 1 (HMGR1), cycloartenol synthase 1 (CAS1), sterol side chain reductase 2 (SSR2) and S-adenosyl-L-Met-dependent C-24 sterol methyltransferase 1 (SMT1) in leaf, stem and root tissues. Using this experimental system, we identified novel feedback and feed-forward regulation of isoprenoid biosynthesis genes, and the differential regulation of these genes in different tissues.

Results

Comparison of VIGS efficiency between soil and hydroponic culture. Since growth conditions, such as nutrient levels, affect the efficiency of RNA silencing\(^{22,23}\), we compared the efficiency of VIGS between hydroponically- and soil-grown plants of \textit{N. benthamiana} using the \textit{phytoene desaturase} (\textit{PDS}) gene; this gene is widely used for the evaluation of the VIGS efficiency, as its knockdown results in the bleaching of plant tissues, such as leaves, which is easy to recognize visually\(^{24,25}\). For comparison, we cloned a 400-nt or 200-nt antisense sequence of the \textit{PDS} coding region into the TRV vector (TRV/\textit{asNbPDS400}, as\textit{NbPDS200}). No striking differences were observed between hydroponically- and soil-grown plants, regardless of whether they were healthy or virus-infected (Fig. 1). As reported in previous studies\(^{26,27}\), plants inoculated with TRV/as\textit{NbPDS400} and TRV/as\textit{NbPDS200} began to show bleaching of leaves within 7 days post-infection (dpi), and extensive bleaching was observed within 14 dpi in soil-grown plants (Fig. 1). No marked differences were detected in the efficiency of VIGS of \textit{PDS} between soil- and hydroponically-grown plants. These results suggest that VIGS is effectively induced in our hydroponic system.

Cloning of \textit{N. benthamiana} isoprenoid biosynthesis genes. We cloned six isoprenoid biosynthesis genes including, 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS), HMGR1, mevalonate kinase (MVK), CAS1, SSR2 and SMT2 using genome sequence draft of \textit{N. benthamiana} (Sol Genomics Network database, \url{https://solgenomics.net/}) (Fig. 2). We identified two closely-related sequences of each gene, which is not consistent with allotetraploidy of \textit{N. benthamiana}\(^{22}\). To differentiate between the two copies of a gene, we used ‘\textit{a}’ and ‘\textit{b}’ as suffixes (see Supplementary Table S1). The lengths of open reading frames (ORFs) of each gene, and nucleotide identities between the two copies of a gene are summarised in Supplementary Tables S1 and S2, respectively.

BLAST search identified two \textit{HMGS} candidates, Niben101Scf01111g01003 (\textit{NhHMGSa}) and Niben101Scf01729g01015 (\textit{NhHMGSb}), which were similar to the tomato orphan, \textit{SthMGS}, as indicated by phylogenetic analysis (see Supplementary Fig. S1)\(^{28}\). BLAST search using the \textit{N. tabacum} orthologs of \textit{HMGR}, \textit{NhHMGR1} and \textit{NhHMGR2}\(^{29}\), revealed several candidate clones including truncated sequences. We determined three candidates, Niben101Scf09686g00013 (\textit{NhHMGR1a}), Niben101Scf13180g01003 (\textit{NhHMGR1b}) and Niben101Scf02023g05002 (\textit{NhHMGR2a}). Phylogenetic analysis revealed that \textit{NhHMGR1a} and \textit{NhHMGR1b} were more closely related to \textit{NhHMGR10} and \textit{SthHMGR1}\(^{30}\), respectively, than to \textit{NhHMGR2a} and \textit{NhHMGR2b}, which were close to \textit{NhHMGR2a} (see Supplementary Fig. S1). BLAST search using \textit{Arabidopsis thaliana} \textit{AtMVK}\(^{31}\) and keyword search identified two genes, Niben101Scf25893g00005 (\textit{NbMVKa}) and Niben101Scf00370g03023 (\textit{NbMVKb}) (see Supplementary Fig. S1). A partial sequence of \textit{NhCAS1} (Niben101Scf16532g01001; former ID was \textit{NhS00021029g0013}), which we refer to as \textit{NhCAS1a}, has been isolated previously\(^{29}\). In addition to \textit{NhCAS1a}, we identified a novel \textit{CAS1} allele, Niben101Scf08080g00009 (\textit{NhCAS1b}), and determined the sequences of both ORFs. Phylogenetic analysis suggested that \textit{NhCAS1a} and \textit{NhCAS1b} were orthologous to \textit{NicASM}\(^{31}\) (see Supplementary Fig. S1). BLAST search using \textit{SthSSR2}\(^{30}\) identified four closely-related sequences, Niben101Scf03969g04003 (\textit{NhSSR2a}), Niben101Scf00271g04029 (\textit{NhSSR2b}), Niben101Scf02156g03023 and Niben101Scf04964g02005; sequences of these genes had nucleotide identities, 87.4%, 86.9%, 79.9% and 77.2% with \textit{SthSSR2}, respectively. Phylogenetic analysis suggested that \textit{NhSSR2a} and \textit{NhSSR2b} were more closely related to \textit{SthSSR2} and \textit{SthSSR2}, respectively, than to \textit{NhSSR1} and \textit{SthSSR1} (see Supplementary Fig. S1). BLAST search using \textit{NhSMT2}\(^{30,36}\) revealed two candidate genes, Niben101Scf13874g01006 (\textit{NhSMT1a}) and Niben101Scf03085g05002 (\textit{NhSMT1b}), which were closely related to \textit{NhSMT1-1} (U81312) and \textit{NhSMT1-2} (AF053766), respectively (see Supplementary Fig. S1).

Knockdown of isoprenoid biosynthesis genes using VIGS. We constructed TRV vectors to down-regulate \textit{NhHMGR1}, \textit{NhCAS1}, \textit{NhSSR2} and \textit{NhSMT1}. We cloned 400 nt antisense fragments of these genes in the TRV vectors to target both transcript copies (\textit{a} and \textit{b}) of each gene (see Methods). The knockdown of \textit{NhHMGR1}, \textit{NhSSR2} and \textit{NhSMT1} did not markedly affect plant growth in comparison to the vector control expressing antisense partial GFP fragment (\textit{asGFP}) plants. By contrast, knockdown of \textit{NhCAS1} expression gradually induced cell death along veins in leaf tissues within 7 dpi (see Supplementary Fig. S2).

Scientific Reports | (2018) 8:14804 | DOI:10.1038/s41598-018-32901-5
Because secondary metabolites are often synthesised in a tissue-specific manner\(^3,37\), we analysed the knockdown efficiency of various isoprenoid biosynthesis genes in leaves, stems and roots via real-time PCR. Both transcripts of each gene (a and b) were detected simultaneously in these tissues, except for \(NbSMT1\). As shown in Fig. 1, VIGS against \(PDS\) gene was extensively and uniformly induced in leaves at 14 dpi. Therefore, we investigated the level of each transcript by real-time PCR at 15 dpi.

We compared the basal level of each gene among leaf, stem and root tissues in healthy plants used for negative control. Real-time PCR analysis showed that \(NbHMGR1\) expression in leaves and stems was significantly lower than that in roots, respectively (Fig. 3a). The expression of \(NbCAS1\) and \(NbSSR2\) in leaves was higher than that in stems and roots (Fig. 3a). Whereas no significant difference was observed in \(NbSMT1a\) expression, \(NbSMT1b\) expression in leaves and stems was >250-fold lower than that in roots (Fig. 3a).

Compared with the control, transcript levels of \(NbHMGR1\), \(NbCAS1\), \(NbSSR2\) and \(NbSMT1a\) was reduced to approximately 23%, 12%, 9% and 11%, respectively, in leaf tissues (Fig. 3b), 26%, 17%, 17% and 11%, respectively, in stem tissues (Fig. 3c) and 31%, 22%, 14% and 6%, respectively, in root tissues by VIGS (Fig. 3d). The transcript level of \(NbSMT1b\) was reduced to approximately 40% in root tissues, although no significant decrease was observed in leaf and stem tissues (Fig. 3b–d), which might be due to low expression levels of \(NbSMT1b\) in leaf and stem tissues, as stated above (Fig. 3a). These results demonstrate that the TRV vectors effectively down-regulate isoprenoid biosynthesis genes in leaf, stem and root tissues of \(N.\ benthamiana\).

To investigate whether the metabolite composition was changed in \(NbHMGR1\), \(NbCAS1\), \(NbSSR2\) and \(NbSMT1\)-silenced plants, leaf extracts were prepared from each plant inoculated with TRV/\(asNbHMGR1\),
cycloartanol by SSR2 or SMT1, respectively. (Fig. 2). We showed that reported in mutant, and reduced the levels of campesterol and stigmasterol (see Supplementary A. thaliana smt1 potato and tomato34 (see Supplementary Fig. S3).

We analysed feedback and feed-forward regulation of isoprenoid biosynthesis genes using VIGS. The expression of NbHMGR1, NbCAS1, NbSMT1a and NbSMT1b was transiently suppressed by VIGS, and their effect on expression levels of NbHMGS (NbHMGSb detected), NbHMGR, NbMVK (both NbMVKa and NbMVKb), NbCAS1, NbSRR2 and NbSMT1 was analysed in leaf tissues. The suppression of NbCAS1 significantly altered the expression of genes both upstream and downstream of NbCAS1 in the isoprenoid biosynthesis pathway. For upstream genes, NbCAS1 knockdown significantly increased NbHMGR1 expression in leaf tissues; however, the expression of NbHMGS and NbMVK was not affected (Fig. 4a). For downstream genes, NbCAS1 knockdown significantly decreased the expression of NbSRR2a and NbSMT1a to approximately 5% and <60%, respectively (Fig. 4a). By contrast, the expression of NbSMT1b was significantly increased (>600-fold) in leaves of NbCAS1 knockdown plants (Fig. 4a), suggesting that NbSMT1a and NbSMT1b are regulated antagonistically (Fig. 4b). In stem tissues of NbCAS1 knockdown plants, the expression of NbHMGR1, NbSMT1b, NbHMGS and NbMVK was up-regulated, whereas that of NbSRR2 and NbSMT1a was down-regulated (Fig. 5a, Supplementary Fig. S4). In root tissues of NbCAS1 knockdown plants, the expression of NbHMGS and NbSMT1b was up-regulated; however, no significant differences were observed in the expression of other genes (Fig. 5b, Supplementary Fig. S4). In the case of NbSRR2, no difference in its transcript level in roots of NbCAS1 knockdown plants may be explained by a lower basal level of expression of NbSRR2 in roots compared with leaves and stems (Figs 3a and 5b). These results suggest that the down-regulation of NbCAS1 expression enhances the upstream pathway and weakens the downstream pathway at the transcriptional level in leaf and stem tissues.

CAS1 converts 2,3-oxidosqualene to cycloartenol, which is then converted into cycloartenol or 24-methylene cycloartenol by SSR2 or SMT1, respectively. (Fig. 2). We showed that NbCAS1 knockdown up-regulated NbHMGR1 expression; however, knockdown of NbSMT1 or NbSRR2 did not increase NbHMGR1 expression (Fig. 4a). We investigated whether simultaneous down-regulation of NbSRR2 and NbSMT1 up-regulated NbHMGR1 expression. We constructed a TRV vector carrying 200-nt antisense sequences of NbSSR2 and NbSMT1 (TRV/asNbSRR2 + asNbSMT1), as 200-nt antisense sequences were shown sufficient for the induction of VIGS against PDS gene (Fig. 1). In leaves infected with TRV/asNbSRR2 + asNbSMT1, the expression of NbSRR2 and NbSMT1a was reduced to approximately 9.8% (NbSRR2a and 7.3% (NbSMT1a); these levels were comparable with those in single asNbSRR2 knockdown plants (decreased to 8.1%) or asNbSMT1 (decreased to 5.9%), respectively (Fig. 6). Expression analyses indicated that leaves infected with TRV/asNbSRR2 + asNbSMT1 did not show up-regulation of NbHMGR1 expression (Fig. 6).
Discussion

We could successfully down-regulated the expression of isoprenoid biosynthesis genes (Fig. 3), and present evidence for some transcriptional feedback and feed-forward regulation using VIGS in hydroponically-grown *N. benthamiana*. Each partial antisense sequence of *NbHMGR1* (as*NbHMGR1*), *NbCAS1* (as*NbCAS1*), *NbSSR2* (as*NbSSR2*), *NbSMT1* (as*NbSMT1*) or GFP (asGFP, vector control) was expressed in TRV vector. Total RNA was extracted from leaf (a,b), stem (a,c) or root (a,d) tissues at 15 dpi and used to synthesise cDNAs for real-time PCR analysis. The expression level of each gene was normalised relative to that of *NbEF1α*. Fold changes in expression level are indicated relative to the vector control (asGFP). Error bars indicate standard deviation of four biological replicates. Statistical analyses were conducted using the Tukey–Kramer test. Different letters above bars indicate statistically significant differences ($P < 0.05$).

Figure 3. Expression level of each isoprenoid biosynthesis gene targeted by VIGS in hydroponically-grown *N. benthamiana*. Each partial antisense sequence of *NbHMGR1* (as*NbHMGR1*), *NbCAS1* (as*NbCAS1*), *NbSSR2* (as*NbSSR2*), *NbSMT1* (as*NbSMT1*) or GFP (asGFP, vector control) was expressed in TRV vector. Total RNA was extracted from leaf (a,b), stem (a,c) or root (a,d) tissues at 15 dpi and used to synthesise cDNAs for real-time PCR analysis. The expression level of each gene was normalised relative to that of *NbEF1α*. Fold changes in expression level are indicated relative to the vector control (asGFP). Error bars indicate standard deviation of four biological replicates. Statistical analyses were conducted using the Tukey–Kramer test. Different letters above bars indicate statistically significant differences ($P < 0.05$).
and increases HMGR1 expression. However, another recent study has shown that the basal level of cycloartenol accumulation is often below the limit of detection in N. benthamiana. Therefore, a decrease in cycloartenol itself might not trigger the feedback regulation. GC/MS analysis indicated that 2,3-oxidosqualene was extensively accumulated in NbCAS1-knock-down plants but not in control plants (see Supplementary Fig. S3). Therefore, the feed-back regulation on HMGR1 might be triggered to metabolize the over-accumulated 2,3-oxidosqualene. Another possibility is that a transient decrease in NbSSR2 and NbSMT1 expression may insufficiently decrease the level of downstream metabolites required for feedback regulation, although NbCAS1 knockdown rapidly decreases the level of cycloartenol and downstream metabolites that use cycloartenol as a substrate. Up-regulated expression of NbHMGR1 in leaves and stems of NbCAS1 knockdown plants of N. benthamiana plants is inconsistent with a previous study using tobacco BY2 cells showing unchanged NhHMGR1 transcript level and reduction in NhHMGR2 transcripts in CAS1 knockdown BY2 cells. These contradictory results might originate from differences between tissue types (leaf and stem) and cell culture or the degree of knockdown; CAS1 expression decreased to 17% in leaves and 22% in stems in this study (Figs 4a and 5a) but to 65% in BY2 cell cultures. Feedback regulation of HMGR is also observed in pharmacological inhibition of enzymes or knockdown of genes regulating squalene metabolism. Treatment with either squalestatin [a squalene synthase (SQS) inhibitor] or terbinafine [a squalene epoxidase (SQE) inhibitor] increases the enzymatic activity of HMGR in tobacco BY2 cell cultures and A. thaliana. By contrast, there are controversies over the transcriptional regulation.

Figure 4. Feedback and feed-forward regulation in isoprenoid biosynthesis genes in NbCAS1 knockdown leaves. (a) Real-time PCR analysis of isoprenoid biosynthesis genes in leaf tissues at 16 dpi with each TRV vector. The expression level of various genes was normalised relative to that of NbEF1α. Fold change is presented relative to the vector control (asGFP). Error bars indicate standard deviation of four biological replicates. Statistical analyses were conducted using the Dunnett method. Data from vector control were used as a control for statistical analysis. *P < 0.05; **P < 0.01. (b) Schematic representation of fold changes in transcript levels of genes relative to the vector control (asGFP) in the NbCAS1 knockdown leaves in isoprenoid biosynthesis pathway. Abbreviations of genes are described in the legend of Fig. 2. N.S. = not significant.
Wentzinger et al. have shown that treatment with squalestatin, but not terbinafine, increases the mRNA level of *NtHMGR*. Kobayashi et al. have shown that the expression of *AtHMGR1* is up-regulated by squalestatin and down-regulated by terbinafine treatment in *A. thaliana* carrying 35S::ADS. By contrast, Nieto et al. have shown that neither squalestatin nor terbinafine treatment changes the mRNA level of *AtHMGR1* or *AtHMGR2* in *A. thaliana*. These contradictory results may be due to differences in experimental conditions and/or due to lack of coordination between gene expression level and enzyme activity. Singh and colleagues have shown that knockdown of *SQS* via VIGS up-regulates the expression of upstream genes, including *HMGR*, and suppresses the expression of downstream genes in *Withania somnifera*. Under biotic stress condition, Chappell et al. have shown that pathogen elicitor upregulates HMGRI activity and downregulates SQS activity. Collectively, these reports indicate that SQS is a major control point in isoprenoid pathway. As knockdown of *CAS1* triggered feed-back regulatory response on *HMGR1* expression, *CAS1* might also be an important control point, although it is unknown that the feed-back regulation also occurred on HMGRI enzymatic activity.

We showed that *NbSSR2* expression was significantly decreased in leaves and stems of *NbCAS1* knockdown plants (Figs 4a and 5a). The GAME9 protein, an APETALA2/Ethylene Response Factor, regulates the expression of both...
SSR2 and CAS1 in tomato and potato leaves. Co-expression analysis indicates that SlSSR2 is co-expressed with 13 sterol metabolism-related genes, including SlCAS1 in tomato. It is possible that NbCAS1 and NbSSR2 are also transcriptionally co-regulated in N. benthamiana. Elucidation of the mechanism that explains the reduction in NbSSR2 expression in NbCAS1 knockdown plants will provide new insights into the transcriptional regulation of NbSSR2.

Additionally, we showed that NbSMT1a and NbSMT1b were under antagonistic regulation; NbSMT1a was down-regulated, whereas NbSMT1b was significantly up-regulated in NbCAS1 knockdown plants (Figs 4a and 5a). The transcript level of NbSMT1a was not significantly different among leaf, stem and root tissues, whereas that of NbSMT1b was significantly lower in leaves and stems than in roots (Fig. 4), indicating their differential transcriptional regulation. Because N. benthamiana is an allotetraploid, NbSMT1a and NbSMT1b may be homoeologs. Although models for regulatory relationships among homoeologous genes have been proposed, the information available is insufficient. N. benthamiana will serve as an excellent model for elucidating regulatory differences between homoeologs.

In summary, we could successfully down-regulate the isoprenoid biosynthesis genes, and could reveal novel feed-back and feed-forward regulations using hydroponically-grown N. benthamiana in approximately two weeks. Our VIGS system will be a powerful tool to elucidate gene function and regulatory gene networks involved in the biosynthesis of secondary metabolites, especially if knockdown of the target gene has deleterious effects on plant growth.

Methods

Plant growth conditions. N. benthamiana was grown hydroponically in a nutrient solution (Otsuka hydroponic composition, Otsuka Chemical Co., Ltd., Osaka, Japan) or in soil at 24°C and 16 h light/8 h dark. The nutrient solution (pH 6.0) used for hydroponic culture contained N (175.1 ppm), P2O5 (80 ppm), K2O (272.7 ppm), CaO (153.3 ppm), MgO (40 ppm), MnO (1.6 ppm), B2O3 (1.6 ppm), Fe (3.51 ppm), Cu (0.032 ppm), Zn (0.084 ppm) and Mo (0.0329 ppm).

Isolation of genes encoding biosynthesis enzymes of isoprenoids. N. benthamiana tissues were homogenised in liquid nitrogen. Total RNA was isolated using the acid guanidinium thiocyanate–phenol–chloroform (AGPC) extraction method, followed by purification with a FARB minicolumn (Favorgen Biotech Corp.)
Ping-Tung, Taiwan). Total RNA was digested with Turbo DNase (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed using random hexamer or oligo-dT by PrimeScript reverse transcriptase (TaKaRa Bio, Kusatsu, Japan), according to the manufacturer’s instructions.

Primers for the isolation of putative ORFs of *N. benthamiana* were designed using sequence information of *N. benthamiana* draft genome v1.0.1 available for predicted cDNAs at Sol Genomics Network (https://solgenomics.net/) by conducting BLAST and keyword searches (see Supplementary Table S3). Each amplified fragment was cloned into the pGEM-T Easy vector (Promega, Fitchburg, WI, USA) or pCR4Blunt-TOPO vector (Thermo Fisher Scientific). Each consensus sequence was determined from at least three plasmid clones. We also conducted 5’ rapid amplification of cDNA ends (RACE) using a GeneRacer Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions, and determined the putative start codon of each fragment.

**Phylogenetic analysis.** Sequence alignments were conducted by using MUSCLE, and a maximum likelihood tree was inferred using the MEGA7 package. Models for nucleotide substitution and rates among sites were determined using MEGA7. For all genes, the Tamura 3-parameter model was used as the nucleotide substitution model, and a discrete gamma distribution was used to model evolutionary rate differences among sites. Only for *CAS1*, the rate variation model allowed some sites to be evolutionarily invariable. The significance of the nodes was estimated with 1,000 bootstrap replicates.

**Preparation of plasmid constructs.** pTRV1 (stock# CD3-1039) and pTRV2-MCS (stock# CD3-1040) were obtained from the Arabidopsis Biological Resource Center (ABRC), Ohio, USA. VIGS constructs were designed using SGN VIGS Tool (vigs.solgenomics.net) with modifications. Each fragment was PCR amplified using primers described in Supplementary Table S4, followed by digestion with EcoRI and BamHI. The digested fragments were cloned into pTRV2-MCS digested with EcoRI and BamHI.

**Virus inoculation.** *Agrobacterium tumefaciens* LBA4404 cells transformed with each construct were suspended in MES buffer [10 mM 2-(N-morpholino)ethane-sulfonic acid, 10 mM MgCl₂ (pH 5.7)], and cell suspensions were adjusted to an optical density at 600 nm (OD₆₀₀) of 0.5. Acetosyringone was added to the suspensions at a final concentration of 200 μM, followed by incubation at room temperature for 2–4 h. Suspensions of *Agrobacterium* carrying pTRV1 and pTRV2 carrying the target constructs were mixed in a 1:1 (v/v) ratio and infiltrated into *N. benthamiana* leaves using a needleless syringe.

**Real-time RT-PCR.** The leaf, stem and root tissues of *N. benthamiana* were homogenised in liquid nitrogen. Total RNA was isolated from these tissue samples using the AGPC extraction method and then purified with a FARB minicolumn (Favorgen Biotech Corp.). Total RNA was digested with Turbo DNase (Thermo Fisher Scientific) and reverse transcribed using random hexamer by PrimeScript II reverse transcriptase (TaKaRa), according to the manufacturer’s instructions. Real-time PCR was performed using LightCycler 96 system (Roche Diagnostics, Basel, Switzerland). The reaction mixture (10 μl) contained FastStart Essential DNA Probes Master (Roche Diagnostics), 0.5 μM each of forward and reverse primers, 0.2 μM Universal ProbeLibrary Probe (Roche Diagnostics) and cDNA obtained by reverse transcribing 5–10 ng of total RNA. Samples were incubated for 10 min at 95°C, followed by 45 cycles of 95°C for 10 s and 60°C for 30 s. Transcript levels of each gene were normalised to those of *NbEF1α* (GenBank accession number AY206004). Primers and probes were designed using Universal ProbeLibrary Assay Design Center (https://qpcr.probefinder.com/organism.jsp) and are listed in Supplementary Table S5.

**GC/MS analysis.** Leaf extracts were prepared according to Itkin et al. Freeze-dried *N. benthamiana* leaves were disrupted into a fine powder by the use of the Multi-beads shocker MB701 (Yasui Kikai, Osaka, Japan). The extraction was conducted at 75°C for 1 h with 20 ml of chloroform-methanol (2:1) for 1 h in 20 ml 6% KOH in methanol. After cooling of samples to RT, 12 ml of n-hexane and 12 ml of water were added, and the mixture was shaken for 30 s. After centrifugation, the n-hexane phase was collected. The aqueous phase was re-extracted twice with n-hexane. The collected n-hexane phases were evaporated and the residues re-suspended in 0.5 ml n-hexane. 5β-Cholest-3α-ol (Sigma-Aldrich, MO, USA) was used as an internal standard, and N-Methyl-N-trimethylsilyltrifluoroacetamide (Tokyo Chemical Industry, Tokyo, Japan) was used for trimethylsilylation. Samples mixed with N-Methyl-N-trimethylsilyltrifluoroacetamide were incubated at RT for 30 min, and used for GC/MS analysis by a GC-QP2020 system with GC-2010plus (Shimazu, Kyoto, Japan). GC analysis was performed with Rxi-5SilMS (30 m × 0.25 mm, 0.25 μm film thickness) column (Shimazu). Helium (99.9999%) was used as carrier gas at a flow of 1.2 ml/min with constant linear velocity mode, 40 cm/s. The injection volume was 2.0 μl with splitless mode, and injector temperature was set at 250°C. The column temperature was programmed as follows, 60°C for 3 min, an increase of 30°C/min to 220°C, followed by an increase of 2°C/min to 300°C, ending with a hold at 300°C for 10 min. The ion source temperature was kept at 230°C, and the interface temperature at 320°C. Mass spectra were taken at 70 eV, with a scan interval of 0.3 s and ions were monitored from 40 to 550 m/z.

**Nucleotide sequence accession number.** The GenBank/ENA/DBJ accession numbers for putative full-length ORF sequence of *HMGS, HMGR*, MVK, *CAS1*, SSR2 and SMT1 are LC382272 (NbHMGSa), LC382273 (NbHMGSb), LC382274 (NbHMGR1a), LC382275 (NbHMGR1b), LC382276 (NbHMGR2a), LC382277 (NbMVKa), LC382278 (NbMVkb), LC382279 (NbCAS1a), LC382280 (NbCAS1b), LC382281 (NbSSR2a), LC382282 (NbSSR2b), LC382283 (NbSMT1a) and LC382284 (NbSMT1b).
Data Availability
The GenBank/ENA/DDBJ accession numbers for genes isolated in this study were described in Methods section. The data supporting the findings of this study are available within the manuscript or upon request.

References
1. Wink, M. Introduction. In Functions and Biotechnology of Plant Secondary Metabolites. Annual Plant Reviews 39; Wink, M., Ed.; Wiley-Blackwell: London, UK, pp. 1–20 (2010).
2. Facchinini, P. J. & De Luca, V. Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. Plant J. 54, 763–784 (2008).
3. Li, D., Heiling, S., Baldwin, I. T. & Gaquerel, E. Illuminating a plant’s tissue-specific metabolic diversity using computational metabolomics and information theory. Proc. Natl. Acad. Sci. USA 113, E7616–E7618 (2016).
4. Yamamoto, K. et al. Cell-specific localization of alkaloids in Catharanthus roseus stem tissue measured with Imaging MS and Single-cellMS. Proc. Natl. Acad. Sci. USA 113, 3891–3896 (2016).
5. Patra, B., Schluittenhofer, C., Wu, Y., Pattanaik, S. & Yuan, L. Transcriptional regulation of secondary metabolite biosynthesis in plants. Biochem. Biophys. Acta 1829, 1236–1247 (2013).
6. Holmberg, N. et al. Sterol C-24 methyltransferase type 1 controls the flux of carbon into sterol biosynthesis in tobacco seed. Plant Physiol. 130, 303–311 (2002).
7. Wentzinger, L. F., Bach, T. J. & Hartmann, M.-A. Inhibition of squalene synthase and squalene epoxidase in tobacco cells triggers an up-regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Plant Physiol. 130, 334–346 (2002).
8. Liao, P. et al. Transgenic tobacco overexpressing Brassica juncea HMG-CoA synthase 1 shows increased plant growth, pod size and seed yield. PLoS ONE 9, e8264 (2014).
9. Gas-Pascual, E., Simonovik, B., Schaller, H. & Bach, T. J. Inhibition of cycloartenol synthase (CAS) function in tobacco BY-2 cells. Lipids 50, 1–12 (2015).
10. Singh, A. K. et al. Virus-induced gene silencing of Withania somnifera squalene synthase negatively regulates sterol and defence-related genes resulting in reduced withanolides and biotic stress tolerance. Plant Biotechnol. J. 13, 1287–1299 (2015).
11. Liao, P., Chen, X., Wang, M., Bach, T. J. & Chye, M.-L. Improved fruit α-tocopherol, carotenoid, squalene and phytosterol contents through manipulation of Brassica juncea 3-Hydroxy-3-Methylglutaryl-CoA Synthase1 in transgenic tomato. Plant Biotechnol. J. 16, 784–796 (2018).
12. Kobayashi, K. et al. Platform for ‘Chemical Metabolic Switching’ to increase sesquiterpene content in plants. Plant Biotechnol. J. 34, 65–69 (2017).
13. Babichuk, E. et al. Allelic mutant series reveal distinct functions for Arabidopsis cycloartenol synthase 1 in cell viability and plastid biogenesis. Proc. Natl. Acad. Sci. USA 105, 3163–3168 (2008).
14. Diener, A. C. et al. Sterol methyltransferase 1 controls the level of cholesterol in plants. Plant Cell 12, 853–870 (2000).
15. Lange, M., Yellina, A. L., Orashakova, S. & Becker, A. Virus-induced gene silencing (VIGS) in plants: an overview of target species and the virus-derived vector systems. Methods Mol. Biol. 975, 1–14 (2013).
16. Nagamatsu, A. et al. Functional analysis of soybean genes involved in flavonoid biosynthesis by virus-induced gene silencing. Plant Biotechnol. J. 5, 778–790 (2007).
17. Nagamatsu, A. et al. Down-regulation of flavonoid 3′-hydroxylase gene expression by virus-induced gene silencing in soybean reveals the presence of a threshold mRNA level associated with pigmentation in pubescence. J. Plant Physiol. 166, 32–39 (2009).
18. Itkin, M. et al. Bioynthesis of antinutritional alkaloids in solanaceous crops is mediated by clustered genes. Science 341, 175–179 (2013).
19. Cárdenas, P. D. et al. GAME9 regulates the biosynthesis of steroidal alkaloids and upstream isoprenoids in the plant mevalonate pathway. Nat. Commun. 7, 10654 (2016).
20. Sonawane, P. D. et al. Plant cholesterol biosynthetic pathway overlaps with phytosterol metabolism. Nat. Plants 3, 16205 (2016).
21. Chang, M. C. Y. & Keasling, J. D. Production of isoprenoid pharmaceuticals by engineered microbes. Nat. Chem. Biol. 2, 674–681 (2006).
22. Hosokawa, M. et al. Phosphorus starvation induces post-transcriptional CHS gene silencing in Petunia corolla. Plant Cell Rep. 32, 601–609 (2013).
23. Sett, S. et al. Post-translational regulation of the dicing activities of Arabidopsis DICER-LIKE 3 and 4 by inorganic phosphate and the redox state. Plant Cell Physiol. 58, 485–497 (2017).
24. Komagai, M. H. et al. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derivedRNA. Proc. Natl. Acad. Sci. USA 92, 1679–1683 (1995).
25. Ruiz, M., Voinnet, O. & Baulcombe, D. Initiation and maintenance of virus-induced gene silencing. Plant Cell 10, 937–946 (1998).
26. Liu, Y. et al. Tobacco Yar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J. 30, 415–429 (2002).
27. Ratcliffe, F., Martin Hernandez, A. & Baulcombe, D. Technical advance: tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J. 25, 237–245 (2001).
28. Bombarely, A. et al. A draft genome sequence of Nicotiana benthamiana to enhance molecular plant-microbe biology research. Mol. Plant Microbe Interact. 25, 1523–1530 (2012).
29. Besser, K. et al. Divergent regulation of terpenoid metabolism in the trichomes of wild and cultivated tomato species. Plant Physiol. 149, 499–514 (2009).
30. Merret, R., Carion, J.-R., Bach, T. J. & Hemmerlin, A. A serine involved in actin-dependent subcellular localization of a stress-induced tobacco BY-2 hydroxymethylglutaryl-CoA reductase isoform. FEBS Lett. 581, 5295–5299 (2007).
31. Choi, D., Ward, B. L. & Bostock, R. M. Differential induction and suppression of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes in response to Phytophthora infestans and to its elicitor arachidonic acid. Plant Cell 4, 1333–1344 (1992).
32. Riou, C., Tourte, Y., Lacroute, F. & Karst, F. Isolation and characterization of a cDNA encoding Arabidopsis thaliana mevalonate kinase by genetic complementation in yeast. Gene 148, 285–297 (1994).
33. Gas-Pascual, E., Benna, A., Bach, T. J. & Schaller, H. Plant oxidosqualene metabolism: cycloartenol synthase–dependent sterol biosynthesis in Nicotiana benthamiana. PLoS ONE 9, e919156 (2014).
34. Sawai, S. et al. Sterol side chain reductase 2 is a key enzyme in the biosynthesis of cholesterol, the common precursor of toxic steroidal glycoalkaloids in potato. Plant Cell 26, 3763–3774 (2014).
35. Bouvier-Navé, P., Hesselstein, T., Desprez, T. & Benveniste, P. Identification of cDNAs encoding sterol methyltransferases involved in the second methylation step of plant sterol biosynthesis. Eur. J. Biochem. 246, 518–529 (1997).
36. Bouvier-Navé, P., Hesseltein, T. & Benveniste, P. Two families of sterol methyltransferases are involved in the first and the second methylation steps of plant sterol biosynthesis. Eur. J. Biochem. 256, 88–96 (1998).
37. Wink, M. Introduction: biochemistry, physiology and ecological functions of secondary metabolites. In Biochemistry of Plant Secondary Metabolism. Annual Plant Reviews 49; Wink, M., Ed.; Wiley-Blackwell: London, UK, pp. 1–19 (2010).
38. Corey, E. J., Matsuda, S. P. & Bartel, B. Isolation of an Arabidopsis thaliana gene encoding cycloartenol synthase by functional expression in a yeast mutant lacking lanosterol synthase by the use of a chromatographic screen. Proc. Natl. Acad. Sci. USA 90, 11628–11632 (1993).
39. Nieto, B., Forés, O., Arró, M. & Ferrer, A. Arabidopsis 3-hydroxy-3-methylglutaryl-CoA reductase is regulated at the post-translational level in response to alterations of the sphingolipid and the sterol biosynthetic pathways. *Phytochemistry* **70**, 53–59 (2009).

40. Chappell, J., Vonlanken, C. & Vogeli, U. Elicitor-inducible 3-hydroxy-3-methylglutaryl coenzyme a reductase activity is required for sesquiterpene accumulation in tobacco cell suspension cultures. *Plant Physiol.* **97**, 693–698 (1991).

41. Glover, N. M., Redestig, H. & Dessimon, C. Homoeologs: what are they and how do we infer them? *Trends Plant Sci.* **21**, 609–621 (2016).

42. Chen, Z. J. & Ni, Z. Mechanisms of genomic rearrangements and gene expression changes in plant polyploids. *Bioessays* **28**, 240–252 (2006).

43. Chomczynski, P. & Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159 (1987).

44. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **32**, 1792–1797 (2004).

45. Kumar, S., Stecher, G. & Tamura, K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**, 1870–1874 (2016).

46. Tamura, K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol. Biol. Evol.* **9**, 678–687 (1992).

47. Senthil-Kumar, M. & Mysore, K. S. Tobacco rattle virus–based virus-induced gene silencing in *Nicotiana benthamiana*. *Nat. Protoc.* **9**, 1549–1562 (2014).

48. Atsumi, G. *et al*. P3N-PIPO, a frameshift product from the P3 gene, pleiotropically determines the virulence of clover yellow vein virus in both resistant and susceptible peas. *J. Virol.* **90**, 7388–7404 (2016).

49. Itkin, M. *et al*. Glycoalkaloid Metabolism Is Required for Steroidal Alkaloid Glycosylation and Prevention of Phytotoxicity in Tomato. *Plant Cell* **23**, 4507–4525 (2012).

50. Kramer, C. Y. Extension of multiple range tests to group means with unequal numbers of replications. *Biometrics* **12**, 309–310 (1956).

**Acknowledgements**

We thank Chikako Ukaji and Misaki Ito for technical assistance. This work was supported by Smart Cell Project from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

**Author Contributions**

G.A. and T.M. designed the research. G.A. and U.K. conducted the experiment and the data analysis. G.A., N.T. and T.M. discussed the results and wrote the manuscript.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-32901-5.

**Competing Interests:** The authors declare no competing interests.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

---

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018