Terpene synthases in cucumber (*Cucumis sativus*) and their contribution to herbivore-induced volatile terpenoid emission

Jun He1,2, Francel Verstappen1, Ao Jiao1, Marcel Dicke3, Harro J. Bouwmeester1,4 and Iris F. Kappers1

1Laboratory of Plant Physiology, Plant Sciences Group, Wageningen University & Research, 6700AA, Wageningen, the Netherlands; 2Citrus Research Institute, Southwest University, 400712, Chongqing, China; 3Laboratory of Entomology, Plant Sciences Group, Wageningen University & Research, 6700AA, Wageningen, the Netherlands; 4Plant Hormone Biology Group, Swammerdam Institute for Life Sciences, University of Amsterdam, 1000BE, Amsterdam, the Netherlands

**Summary**

- Terpenoids play important roles in flavour, pollinator attraction and defence of plants. In cucumber (*Cucumis sativus*) they are important components of the herbivore-induced plant volatile blend that attracts natural enemies of herbivores.
- We annotated the cucumber *TERPENE SYNTHASE* gene (*CsTPS*) family and characterized their involvement in the response towards herbivores with different feeding guilds using a combined molecular and biochemical approach.
- Transcripts of multiple *CsTPS* genes were upregulated in leaves upon herbivory and the products generated by the expressed proteins match the terpenoids recorded in the volatile blend released by herbivore-damaged leaves. Spatial and temporal analysis of the promoter activity of *CsTPS* genes showed that cell content-feeding spider mites (*Tetranychus urticae*) and thrips (*Frankliniella occidentalis*) induced promoter activity of *CsTPS9* and *CsTPS19* within hours after initiation of infestation, while phloem-feeding aphids (*Myzus persicae*) induced *CsTPS2* promoter activity.
- Our findings offer detailed insights into the involvement of the *TPS* gene family in the dynamics and fine-tuning of the emission of herbivore-induced plant volatiles in cucumber, and open a new avenue to understand molecular mechanisms that affect plant–herbivore interactions.

**Introduction**

Specialized metabolites modulate interactions of plants with their biotic environment. Numerous endogenous compounds function in direct defence as toxins and repellents towards herbivores and pathogens (Schoonhoven et al., 2005; Hopkins et al., 2009). Volatile compounds have additional functions as attractants for pollinators and carnivorous enemies of herbivores, as well as in inter- and intra-plant communication (Pichersky & Gershenzon, 2002; Degenhardt et al., 2003; Kappers et al., 2005; Dudareva & Pichersky, 2020). Upon herbivory, the plant’s specialized metabolome changes depending on the feeding habit of the infesting herbivore. For example, chewing caterpillars inflict significant damage, while aphids cause only little tissue damage, manoeuvring their flexible stylet intercellularly through the epidermis and mesophyll to reach the phloem (Kloth et al., 2016). Other herbivores inflict moderate damage, including spider mite and thrips that pierce mesophyll plant cells and feed on their contents. In addition to the mechanical wounding inflicted, cues in the herbivore’s oral secretion trigger a cascade of reactions including early Ca2+ signalling and a burst of reactive oxygen species (Maffei et al., 2007), followed by changes in the concentrations of phytohormones (Wu & Baldwin, 2010). The synthesis, perception and crosstalk of these hormones, the transcription factors involved and their target genes together constitute a complicated signal-transduction network through which the plant metabolome and therefore the defensive state of the plant is rearranged.

Terpenoids represent the most diverse group of plant specialized metabolites (Aharoni et al., 2005) and many have roles in the interaction between plants and their environment. Terpenoids are the main constituents of the blend of leaf-emitted volatiles after oviposition, herbivory and wounding that induce endogenous jasmonic acid (JA) (Bohlmann et al., 2000; Herde et al., 2008; Cao et al., 2010; Hilker & Fatouros, 2015), and nonvolatile terpenoids increase in plant organs upon exposure to (a)biotic stresses (Bohlmann et al., 2000; Balkema-Boomstra et al., 2003; Nagegowda, 2010).

Terpenoids are composed of isoprenoid units originating from either the mevalonate (MVA) or the 2-C-methylerythritol-4-phosphate (MEP) pathway. The genes encoding terpene synthases (TPSs) are structurally related and constitute a medium-sized gene family occurring across the plant kingdom. For example, the Arabidopsis genome contains 32 genes encoding functional TPSs (Chen et al., 2011). By contrast, the *Vitis vinifera* genome contains 152 *TPS* genes (Martin et al., 2010), while the moss *Physcomitrella patens* contains only a single one (Holberger et al., 2015). Furthermore, transcription of *TPS* genes was reported to be upregulated by herbivory in various species.
including Arabidopsis (de Vos et al., 2005; Zhurov et al., 2014),
tomato (Kant et al., 2004; Martel et al., 2015), maize (Schnee et al., 2002) and 
legumes (Arimura et al., 2004).

Plant defences against biotic stressors can be affected by internal 
and external factors including light and the circadian clock. In Ara-
bidopsis, the expression of more than 40% of the genes induced by 
mechanical damage peaks at dusk and over 80% of the genes is sup-
pressed at dawn (Walley et al., 2007). Arabidopsis plants grown 
under a similar light : dark rhythm as the cabbage looper Trichoplus- 
ia ni, which has rhythmic feeding behaviour, had increased resis-
tance against this herbivore, while plants grown under an opposite 
light : dark rhythm as the insect were more susceptible (Goodspeed 
et al., 2012). Both the circadian clock and jasmonates were shown to 
be essential in maintaining this rhythmic defence.

Cucumber (Cucumis sativus), two-spotted spider mites (TSSM), emit a terpenoid- 
enriched volatile blend (Takahayashi et al., 1994; Mercke et al., 2004; 
Kappers et al., 2010) of which (E)-β-cismene and (E,E)-4,8,12-trimethyl-
1,3,7,11-tridecatetraene (TMTT) were shown to be essential for the attraction of 
Phytoseius persimilis, predators of TSSM (Dicke & Sabelis, 1988; Dicke et al., 1990a,b; 
Kappers et al., 2010, 2011). The cucumber TPS gene family has been 
described and partially characterized, although not in 
relation to herbivory (Wei et al., 2016). An earlier study demonstrated 
the induction of expression of the cucumber (E)-β-
OCIMENE/(E,E)-α-FARNESENE SYNTHASE by TSSM 
feeding (Mercke et al., 2004).

Two-spotted spider mites predominantly induce JA-related 
defences in Arabidopsis (Zhurov et al., 2014), tomato (Martel et al., 2015) and cucumber (He et al., 2020), 
while young nymphs induce salicylic acid (SA) in tomato (Liu et al., 2020) 
and TSSM induce SA in frijole (He et al., 2007), lima bean 
(Ozawa et al., 2000) and pepper (Zhang et al., 2020). Western 
flour thrips (Frankliniella Occidentalis) is another important gene-
est in many glasshouse crops, inducing JA-related defences 
(Shipp et al., 2000; Steenbergen et al., 2018; Sarde et al., 2019). By contrast, the generalist green peach aphid (Myzus persi-
cae) is a SA inducer, and the amounts of volatiles emitted by 
plants in response to phloem feeders such as aphids are generally low (Staadt et al., 2010) and sometimes even suppressed upon 
apsid herbivory (Pineda et al., 2013).

Here, we investigated the abundance and composition of the volatile 
blend of cucumber plants upon feeding by different types of 
herbivores and characterized the genes encoding the TPSs and 
their transcriptional regulation responsible for the specificity of 
the response to herbivores with different feeding guilds.

Materials and Methods

Plants and arthropods

Cucumis sativus plants (genotype ‘Corona’) were grown in potting 
soil in a glasshouse (16 h 22°C : 8 h 18 ± 2°C, light : dark) for 3 wk 
until five true leaves had developed. Arabidopsis thaliana Col-0 
(N1092) and p35S::LUC in Col-0 background (N9966) seeds were 
obtained from the Nottingham Arabidopsis Stock Centre (NASC) 
and grown in a climate chamber (12 h : 12 h, light : dark, 
150 µmol m−2 s−1, 22°C) for 4 wk. Female adult spider mites (T. 
urticae) were selected from a mass-rearing on lima beans. Aphids 
(Myzus persicae) were reared on radishes and wingless adults were 
used for experiments. Thrips (Frankliniella Occidentalis) were reared 
on pods of broad bean and 5-d-old larvae were used for experiments.

Assessment of leaf damage

Herbivory damage was ass after 3 d. For mite damage, visual 
observation of chlorotic spots was supported by trypan blue stain-
ing (Keogh et al., 1980). Quantification of TSSM and thrspi-
duced damage was performed using ImageJ software (imagej.
nih.gov/ij) as described by Visschers et al. (2018).

Identification of CsTPS genes

The cucumber genome (v.2 assembly; www.icugi.org) was screened for genes related to the terpenoid biosynthetic module 
using InterProScan (www.ebi.ac.uk/interpro) according to the 
method described by Hofberger et al. (2015). Genomic regions 
containing candidate genes and their flanking 4 kb sequence were 
extracted, re-annotated and confirmed by FGENESH (www.
softberry.com) and GENWISE (www.ebi.ac.uk/) according to the 
structure of previously reported TPS proteins (Chen et al., 2011). The TARGETP 1.1 server (Emanuelsson et al., 1999) was used for 
signal peptide prediction, and amino acid alignment of full-
length CsTPS enzymes was constructed using ClustalW (www.
genome.jp/tools-bin/clustalw) and MUSCLE (www.ebi.ac.uk/ 
Tools/msa/muscle/). A phylogenetic tree was constructed using 
the maximum-likelihood method in MEGAS (Tamura et al., 2011). Using the previously obtained RNA-Seq dataset comparing 
two genotypes that differ in TSSM susceptibility (He et al., 2020), reads of genes assigned to the terpenoid biosynthetic mod-
ule were mapped to assembled sequences to calculate read counts 
for each unigene. Differentially expressed genes (DEGs) between 
different experimental conditions were filtered using a Ben-
jamini–Hochberg false discovery rate of 0.05 and a threshold of 
log2-transformed fold-changes (treatment/control) > |1.5|.

Putative cis-element analysis

The 2000 bp intergenic sequences upstream from the initiation 
start of GTPS2, GTPS9 and GTPS19 were analysed for the presence of 
cis-acting elements using the PlantCARE database 
(http://bioinformatics.psb.ugent.be/webtools/plantcare/). 
Aligned motifs for each promoter were listed as their distances to 
the start codon of the gene.

Volatile collection and analysis

The second fully expanded leaves of 3-wk-old cucumber plants 
were infested with 50 adult TSSM, 10 thrips or 10 aphids, or 
were left uninfested. Volatile emissions of herbivore-infested and 
nontreated plants were collected on Tenax absorbent using 
dynamic headspace sampling as described by Zhang et al. (2020).
For semiquantification of volatiles, 1 µl of carvone in 10 µl MeOH was added to each Tenax liner before analysis and the areas under the curve (AUC) were normalized to that of the internal standard. For each condition, volatile emissions were collected from five independent plants.

RNA isolation and gene expression analysis
Total RNA from cucumber leaves was extracted and reverse-transcribed for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis as described previously (He et al., 2020). Expression levels were normalized to cucumber β-Actin (Csa6M484600) and α-Tubulin (Csa4G000580) using the ΔΔCt method (Livak & Schmittgen, 2001). Every measurement was performed with five biological replicates. Primer sequences are listed in Supporting Information Table S1.

Generation and expression of recombinant CsTPSs
Full-length CDNA sequences of CsTPS genes were cloned into the expression vector pACYCDuet (Novagen, Birmingham, UK) and transformed into the Escherichia coli strain BL21 (DE3). Primers used to obtain open reading frames are listed in Table S1. Production of heterologous protein was induced using 10 µM farnesyl pyrophosphate (FPP) or geranyl pyrophosphate (GPP) in a 1 ml vial as described previously (Mercke et al., 2004). As a negative control, raw protein extracts from E. coli expressing the empty pACYC2Duet vector with substrates (FPP, GPP or GGPP) were incubated as described earlier. To collect terpenoid products, a 10 mm polydimethylsiloxane (PDMS, film thickness 1 mm) stir bar (Gerstel, Mülheim, Germany) was enclosed in each assay vial for 60 min incubation at 30°C with 250 rpm shaking. Subsequently, the stir bar was briefly rinsed in water, dried under a stream of nitrogen and enclosed in a glass liner for GC-MS analysis. The PDMS stir bars in between the measurements were cleaned by heating them to 310°C for 40 min with a helium flow. The tentative identification of enzyme-derived compounds was based on the comparison of mass spectra with those in the NIST 2005, Adams (2007) and Wageningen Mass Spectral Database of Natural Products, as well as experimentally obtained linear retention indices (LRIs). Essential oil of basil (Ocimum basilicum) was used to characterize cadinol. For each TPS enzyme and substrate combinations, assays were repeated at least twice (n = 3) but most often three times (n = 4). For all replicates, the major products were similar and in the same order of relative magnitude. Terpenoids were semiquantified by calculating the area under the curve (AUC). To determine efficient mono- (sesqui-) TPS activity, the ratio of the sum of the AUC of all mono- (sesqui-) terpene products to that of all mono- (sesqui-) terpenes, including the nonspecific geraniol (farnesols), was set to be >50%.

Construction of cucumber promoter::reporter constructs in Arabidopsis
The 1000 bp intergenic regions upstream of CsTPS2 (Csa1G066560), CsTPS9 (Csa2M299880) and CsTPS19 (Csa3M095040) start codon were PCR-amplified using Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) and cloned into vector pMK-RQ (Thermo Fisher, Waltham, MA, USA). Agrobacterium tumefaciens (Agl0) harbouring the promoter::GUC/LUC3300 (Koo et al., 2007) fusion reporter constructs were transformed into Arabidopsis Col-0 plants by floral dipping (Logemann et al., 2006). Three independent homozygous T3 transgenic plants were selected for each reporter construct.

Induction and quantification of fflUC activity
Four-week-old Arabidopsis reporter plants were screened for temporal dynamic imaging of bioluminescence under a diurnal light regime with light ramping to mimic natural light conditions. Plants were acclimatized 24 h before imaging started. The first day of imaging was always under noninduced conditions. Plants were sprayed with 1 mM D-luciferin (Promega) twice a day. Thirty-minute interval imaging of firefly-LUCIFERASE (fflUC) activity and the determination of relative luminescence profiles were done as described by Van Hoogdalem (2020). Photon emission was depicted with false colour scales, with blue indicating low activity and red indicating high activity.

Transgenic Arabidopsis plants were monitored for reporter activity after various (a)biotic stresses, including mechanical damage (leaf puncturing using a 0.2-mm-diameter needle), JA, SA or abscisic acid (ABA) (all 5 µM 1 mM + 0.01% Tween-20), or individual TSSM, thrips or aphids. Leaves were visually checked for whether herbivores stayed on the leaf where they were introduced. After luminescence measurements were finished, plants were visually checked as to whether herbivores were alive. Experiments were performed with three independent lines per construct and five plants per experimental condition (n = 15). Arabidopsis p35S::fflUC reporter plants were used as controls.

Results
Cell-content feeding TSSM and thrips, and phloem-feeding aphids induce different terpenoid-enriched volatile profiles
After 3 d of TSSM feeding, damage as chlorotic spots was clearly visible by eye and total volatile emission increased 11-fold and 16-fold upon thrips feeding compared with the emission of noninfested plants (Fig. 1a). By contrast, after aphid feeding volatile emission increased less than two-fold. The volatile blend consisted of green leaf volatiles, benzoates, oximes and terpenoids, of which the latter comprised 30% of the total blend released by noninfested plants (Fig. 1b; Table S2). More than half of all terpenoids emitted by noninfested plants were sesquiterpenoids, of which (Z)-copaene was the most dominant. The presence and abundance of terpenoids changed depending on the herbivore. The contribution of terpenoids increased to 38% and 43% after 3 d of thrips and TSSM infestation, respectively (Fig. 1b). In both cases, (E,E)-farnesene was the dominant terpenoid followed by (Z)-β-ocimene, linalool, myrcene and (E)-4,8-dimethylona-1,3,7-triene (DMNT). Interestingly, infestation...
Fig. 1 Herbivore-induced volatile emission in Cucumis sativus. (a) Total volatile organic compound (VOC) emission by cucumber plants that were infested for 3 d with Tetranychus urticae spider mites (S), Franklinella occidentalis thrips (T) or Myzus persicae aphids (A) or were left uninfested (C); (b) proportion of terpenoids after 3 d of infestation in the total VOC blend, based on GC-MS areas under the curve, normalized to internal standard; (c) distribution of monoterpenes (mTP), monoterpane alcohols (mTP-OH), monoterpane aldehydes (mTP-Ald); sesquiterpenes (sTP), sesquiterpene alcohols (sTP-OH), sesquiterpene aldehyde (sTP-Ald) and homoterpenes (hTP); (d) percentage (% of total GC-MS signal) of individual compounds to the terpenoid blend. Data represent the means ± SD of five plants. Significance was tested using Mann–Witney U-test (*, P < 0.05; ***, P < 0.001). (E)-DMNT, (E,E)-4,8-dimethylnona-1,3,7-triene; (E,E)-TMITT, (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene.
by both cell-content feeders increased the contribution of terpene alcohols and aldehydes when compared to the blend of noninfested plants. While aphid infestation resulted in an increased contribution of terpenoids to the total volatile blend, the composition of the induced terpenoid blend differed from that of cell-content feeders with predominantly monoterpenes and monoterpenene alcohols but a lower proportion of sesquiterpenes compared with the blend of noninfested plants (Fig. 1c). Three days after the onset of feeding, limonene was the dominant terpeneoid in aphid-infested plants (Fig. 1d).

Identification of the terpene biosynthetic module

To study the regulation of terpene biosynthesis by herbivory, the cucumber genome was analysed for putative gene models associated with terpene biosynthesis (Fig. 2). Seventy genes could be assigned to one of the six functional modules in terpenoid biosynthesis, including eight prenyl-transferases, two IPP isomerases and 10 MEP and MVA pathway-associated genes (Table S3). Additionally, 12 triterpene synthases were detected, and 34 gene models were identified as putative TPS genes. From these, 24 full-length gene models encoding putative proteins with 313 to 813 amino acids and at least five exons were renamed as GTPS1-24 according to their chromosomal position, while three other ones that are too short to encode a functional TPS protein were renamed as GTPS25-27 (Table S4). The majority of GTPS genes were found in clusters located on chromosomes I, II and III, suggesting multiple duplication and neofunctionalization events on these chromosomes (Fig. S1). Phylogenetic analysis classified most cucumber TPSs as TPS-a (11 members), TPS-b (eight members) and TPS-g (three members) (Fig. S2). Chromosomes VI and VII both contain a single full-length TPS gene, GTPS23 and GTPS24 respectively, and the partial GTPS27 is located on chromosome VII. GTPS23 and GTPS24 are classified as TPS-c and TPS-e/f, respectively, and most likely encode COPALYL DIPHOSPHATE SYNTHASE and KAURENE SYNTHASE. Gene models associated with the MEP and MVA pathways or encoding IPP isomerases and prenyl-transferases were found to be located across all chromosomes and not specifically in the gene models associated with the MEP and MVA pathways or DIPHOSPHATE SYNTHASE and KAURENE SYNTHASE. respectively, and most likely encode COPALYL DIPHOSPHATE SYNTHASE (GPPS) while GERANYL DIPHOSPHATE SYNTHASE (GPPS) and FARNESYL DIPHOSPHATE SYNTHASE (FPS) were regulated similarly in both genotypes.

In control leaves, TPS5 showed the highest expression in both genotypes, but overall expression of the TPS genes was low, at 2.5–5.8% of the average overall gene expression (Table S5). TSSM feeding increased the expression of several TPSs (Fig. 2). Quantitative PCR analysis confirmed that TSSM induced GTPS9 and GTPS19, and to a lesser extent GTPS2-5 and GTPS21 (Figs 2, S3). Thrips feeding induced higher TPS expression than TSSM, with GTPS9, GTPS19 and GTPS21 as the most strongly induced genes. By contrast, aphid feeding resulted only in some induction of GTPS2 expression and, to a lesser extent, of GTPS-4, GTPS19 and GTPS21.

Characterization of TPS

Nineteen TPS, including all TSSM-induced TPSs, except for GTPS4, were successfully cloned and heterologously expressed in *E. coli* (Fig. 4; Table S6). All heterologous TPSs, except CsTPS13 and CsTPS18, accepted substrates GPP and FPP, resulting in the formation of various mono- and sesquiterpenes, respectively. CsTPS proteins with a predicted chloroplast-target peptide efficiently produced one or multiple monoterpenoids. These enzymes also catalysed the formation of minor amounts of sesquiterpenes from FPP.

CsTPS1-3 have predicted chloroplast-target peptides and produced predominantly linalool when GPP was supplied as substrate. Furthermore, CsTPS9 catalysed the formation of (B)-\(\beta\)-ocimene and myrcene, and small amounts of sabine and (Z)-\(\beta\)-ocimene from GPP. CsTPS11 and CsTPS15 both catalysed the formation of myrcene, limonene, (B)-\(\beta\)-ocimene, linalool and \(\alpha\)-terpineol from GPP in different amounts and/or ratios, and CsTPS11, in addition, catalysed the formation of an unidentified monoterpenone (LRI 1169). The product profile of CsTPS10 was distinct from the other chloroplast-targeted enzymes as it produced \(\alpha\)-pinene, \(\alpha\)-phellandrene, sabine, \(\beta\)-pinene, myrcene, linalool and \(\alpha\)-terpineol from GPP. Despite the presence of a
Fig. 2 Heat map of the differentially expressed terpenoid biosynthetic module genes in *Cucumis sativus* leaves infested with spider mites. (a) Genes encoding proteins related to 2-C-methylerythritol-4-phosphate (MEP), mevalonate (MVA) and isoprenoid submodules. (b) Genes encoding proteins related to prenyl transferase and terpene synthase submodules. Values are the log_2-fold changes compared with the average expression in noninfested leaves and represent (from left to right): genotype ‘Chinese Long’, infested for 1, 2 and 3 d; genotype ‘Corona’, infested for 1, 2 and 3 d. Pink indicates downregulation of gene expression levels and a strong downregulation is indicated in dark red. Light blue indicates upregulation and dark blue indicates strong upregulation of gene expression. Light grey indicates no changes in gene expression relative to noninfested control. RKPM values are presented in Supporting Information Table S5.
predicted chloroplast-target peptide, CsTPS1-3, CsTPS9-11 and CsTPS15 catalysed the formation of minor amounts of sesquiterpenes from FPP.

We were not able to clone CsTPS6-8, CsTPS16 and CsTPS20, all genes without a targeting sequence. CsTPS13 and CsTPS18 were not active in any of the assays that we performed. Other proteins without a predicted targeting peptide were CsTPS14 and CsTPS17, which all catalysed the formation of (E)-β-farnesene, and to a lesser extent (E,E)-α-farnesene and (E)-nerolidol from FPP. CsTPS19 predominantly catalysed the formation of (E,E)-α-farnesene from FPP, consistent with Mercke et al. (2004), but produced also traces of (E)-β-farnesene, (Z,E)-α-farnesene, bisabolene and an unknown sesquiterpene (LRI 1485). CsTPS21 catalysed the formation of (E)-caryophyllene and α-humulene from FPP, and the major products of CsTPS22 were (E)-nerolidol and cadinol. When cytosolic TPSs were supplemented with GPP, most of the enzymes produced small amounts of myrcene, limonene and linalool. An exception was CsTPS19 which efficiently catalysed the formation of (E)-β-ocimene, myrcene and linalool from GPP. CsTPS19 also accepted GGPP to produce the diterpenoid geranyl linalool (Fig. S4), confirming CsTPS19 to be an efficient mono- and sesquiterpene synthase. Both CsTPS23 and CsTPS24 were predicted to encode a diterpene synthase, but only CsTPS24 accepted GGPP to produce geranyl linalool. Both enzymes accepted GPP as a substrate to produce (E)-β-ocimene, linalool and myrcene in minor amounts. CsTPS24 produced a small amount of cadinol and both enzymes produced (E)-nerolidol from FPP.

Induction of herbivore-inducible TPS results in circadian enzymatic activity

*pGTPS2*, *pGTPS9* and *pGTPS19* were selected for further analysis of the regulation of terpene biosynthesis upon herbivory. Multiple cis-acting regulatory elements (CAREs) located in the 2000 bp sequences upstream of the initiation start of these genes, considered to represent the promoter (*pGTPS*), were identified as responsive to stress-related phytohormones JA, SA and ABA (Fig. 5a; Table S7). The number of these motifs in *pGTPS19* was about half of those of *pGTPS2* and *pGTPS9* (Table 1). Furthermore, the promoters contained multiple motifs related to light responsiveness and circadian rhythmicity (Table 1; Fig. 5a), suggesting that these *pGTPSi* may be regulated by photoperiod in addition to JA, SA and ABA.

The expression of *CsTPS2*, *CsTPS9* and *GTPS19* was very low in nonchallenged cucumber leaves (Table S5), and also in nonchallenged roots and flowers (Li et al., 2011). Indeed, nonchallenged transgenic Arabidopsis reporter plants harbouring *GTPS* promoter regions driving a dual β-GLUCURONIDASE (GUS) and ffLUC (*pGTPS-GUS/ffLUC*) showed no blue colour upon histochemical β-glucuronidase (GUS) staining in roots, leaves, flowers or siliques (Fig. S5).

The herbivore species used in our study all accept Arabidopsis as host (Zhurov et al., 2014; Kloth et al., 2015; Thoen et al., 2016). Visual damage caused by TSSM feeding could be observed after 2 d as white spots, mostly near the veins, and the occurrence of dead cells was confirmed by trypan blue staining (Fig. 5b). Thrips feeding resulted in silver damage as a result of collapsed cells, first visible at 2 d after the onset of feeding. Aphid infestation did not inflict visual damage but infestation was considered to be successful as offspring were present at 3 d after introduction of the aphids.

β-Glucuronidase staining of *pGTPS9::GUS/ffLUC* reporter plants showed that expression of the reporter gene was absent in noninfested plants, except for the cotyledons, which in some plants stained blue (Fig. S5). Upon TSSM feeding, leaves stained blue in a patchy pattern corresponding to the damage spots inflicted by the mites. Stained cells in these infested areas were mostly located in the mesophyll layer. Some of the younger leaves that were not damaged by TSSM showed minor staining in the petioles and the veins. By contrast, TSSM-infested *pGTPS19* reporter plants showed only blue colouring in local infested leaves, and no blue colouring was observed in TSSM-infested *pGTPS2* reporter plants. Thrips infestation resulted in a stronger response of *pGTPS9* and *pGTPS19* reporter plants compared with TSSM, but, similar to TSSM feeding, *pGTPS9* reporter...
Plants showed systemic induction of reporter activity while that of pCsTPS19 plants was mostly local. Minor blue colouring was observed in the small veins of pCsTPS2 plants after 96 h of aphid infestation, but not in aphid-infested pCsTPS9 and pCsTPS19 reporter plants.

To better visualize the dynamics of promoter activation, we used luminescence monitoring. Luminescence increased in pCsTPS9 reporter plants within 1 h after the introduction of a single thrips, and a 73-fold increase was observed at the end of the second light period (Fig. 5e). After recording luminescence, we observed that the originally infested leaf was seriously damaged by thrips and a number of other leaves showed silver damage spots as well. By contrast, aphids did not cause any detectable induction of luminescence in the pCsTPS9 reporter plants (Fig. 5e). Visual observation showed that aphids walked around for c. 2 h and then remained in the same position, implying they were probing/feeding (Kloth et al., 2015). After 96 h no damage was visible on the aphid-infested plants.

Luminescence in pCsTPS19 reporter plants increased upon TSSM and thrips feeding but not aphid feeding (Fig. 6), similar to pCsTPS9. Also, the greater damage inflicted by thrips infestation resulted in stronger luminescence than as a result of TSSM feeding. Damage-induced luminescence was only visible locally, at positions where thrips and TSSM had been feeding in pCsTPS19 reporter plants, while in pCsTPS9 some systemic luminescence was observed. By contrast, pCsTPS2 reporter plants only displayed a minor increase in luminescence upon thrips infestation, mechanical damage or JA treatment but were responsive to TSSM and aphid feeding and SA and ABA treatment (Fig. 6a).

**Discussion**

The cucumber TPS gene family is relatively small. The TPSs constitute a medium-sized gene family in plants (Chen et al., 2011). The GTPS gene family consists of 27 gene models.
Fig. 5  Activity of pCsTPS9::ffLUC Arabidopsis reporter plants. (a) Putative cis-acting regulatory elements identified in the upstream 2 kb sequence of CsTPS9. (b) Trypan blue staining indicates successful leaf infestation, 2 d after the introduction of spider mites. (c) Firefly-LUCIFERASE profile of 4-wk-old pCsTPS9::LUC reporter plants that were infested with spider mites or were left uninfested. Selected pictures that were taken 4, 28 and 80 h after the introduction of two adult spider mites on leaf 1. (d) Diurnal firefly-LUCIFERASE (ffLUC) activity profile of leaf 1, leaf 2 and leaf 3 as indicated in panel (c) during five subsequent days under 12 h : 12 h, light : dark cycles. Data are relative luminescence of individual leaves, measured every 20 min; luminescence of pCsTPS9/p35S was set to 0 at the start of the experiment; E, maximum increase in ffLUC activity of reporter plants (relative to similarly treated p35S:: ffLUC plants) upon treatment. Maximum ffLUC activity was observed after 24 h for abscisic acid, 24 h for salicylic acid, 24 h for jasmonic acid, 5 h after introduction of a single adult female thrips, 75 h after introduction of two adult female spider mites, 96 h after introduction of two wingless adult aphids and 4 h after repetitive puncturing for 5 min using a needle to inflict mechanical damage. Data are means ± SD of five biological replicates.
of which 19 encode complete TPS proteins, confirming the study of Wei et al. (2016), and hence form a relatively small TPS family compared with other flowering plant species such as Arabidopsis (40 putative TPS gene models; 32 putatively full length; Aubourg et al., 2002), tomato (44; 29; Falara et al., 2011), rice (57; 34; Chen et al., 2011) and grape (152; 69; Martin et al., 2010). Remarkably, the TPS family in apple consists of 55 gene models of which only 10 are functional (Nieuwenhuizen et al., 2013). The majority of the cucumber TPS genes are organized into four clusters located on three chromosomes, consistent with the clustering of TPS genes in other plant species, including Arabidopsis (Aubourg et al., 2002), tomato, (Falara et al., 2011) and grape (Martin et al., 2010). Clustering of metabolism-associated genes is relatively common, possibly ensuring co-inheritance to keep biosynthetic pathways complete (Nutzmann & Osbourn, 2014). Furthermore, clustered genes could share similar regulation mechanisms such as through chromatin modification (Wegel et al., 2009). TPSs were reported to frequently colocalize with P450 genes (Boutanaev et al., 2015). Remarkably, in cucumber only a single P450 gene and no members of other classes of genes such as glycosyl transferases were found located within or near any of the TPS clusters. Just as reported for other species, CsTPSs located within the same cluster in the genome were assigned to similar clades in the phylogenetic tree and are hence more homologous to each other, probably as a consequence of tandem duplication. Evolutionary analysis of terpenoid biosynthesis-related genes and supergene clusters of 17 genomes demonstrated that genes encoding TPSs are more enriched for tandem duplications than genes encoding enzymes involved in the upstream MVA pathway and IPP isomerases (Hofberger et al., 2015).

Like many TPSs characterized in other plant species, CsTPSs can accept different substrates. Most CsTPSs that were tested in vitro catalysed the formation of multiple terpenes from the same precursor, a common phenomenon in plant TPSs. For example, 10 different monoterpene were formed by a single Arabidopsis TPS (Chen et al., 2004). Most of the characterized tomato TPSs catalysed the formation of more than one terpene (Falara et al., 2011). At the same time, some of the terpenes we detected were synthesized by multiple CsTPSs. For example, linalool was the major product of TPS-g clade CsTPS1, CsTPS2 and CsTPS3, and also a minor product of most of the other CsTPSs. Linalool is a common floral and foliar volatile with two distinct enantiomers that have distinct roles in pollinator attraction and plant defence (Raguso, 2016; He et al., 2019). Linalool enantiospecific enzymes have been identified in, for example, Arabidopsis, producing (R)-(−) and (S)-(+)linalool, respectively, as their major products (Ginglinger et al., 2013). Whether cucumber leaves and flowers emit specific isomers is unknown and the present study did not allow us to distinguish between both enantiomers. Further studies might also investigate whether CsTPS1-3 contribute to enantiospecific-specific linalool formation, if any, and its specific role in plant–arthropod interactions.

Most cucumber TPSs convert GPP and FPP to acyclic monoterpenes while the formation of cyclic terpenes was catalysed by a limited number of CsTPSs only, including CsTPS10 which catalyses the formation of a pinyl cation en route

**Table 1** Percentage of cis-acting element motifs in the 2000 bp sequence upstream of the translational start of *Cucumis sativus* CsTPS2, CsTPS9 and CsTPS19 annotated to be involved in the indicated responsiveness.

| Keyword         | CsTPS2 | CsTPS9 | CsTPS19 |
|-----------------|--------|--------|---------|
| Percentage of motifs in promoter sequence |        |        |         |
| Key              |        |        |         |
| CsTPS2          | 100    | 100    | 100     |
| CsTPS9          | 100    | 100    | 100     |
| CsTPS19         | 100    | 100    | 100     |

**Table 6** Activity of pCsTPS2::fLUC (a) and pCsTPS19::fLUC (b) Arabidopsis reporter plants. Maximum increase in fLUC activity of reporter plants (relative to mock-treated p35S::fLUC plants). Time indicates the period after treatment until maximum activity was observed: abscisic acid (24 h), salicylic acid (24 h), jasmonic acid (24 h), single adult female thrips (5 h), two adult female spider mites (73 h), two wingless adult aphids (96 h), mechanical damage inflicted by repetitive puncturing for 5 min using a needle (4 h). Data are means ± SD of five biological replicates. Note the different scaling of the x-axis for both reporters. The dashed line indicates no increase (i.e. ‘1’).
to the formation of \( \beta \)-pinene, \( \alpha \)-pinene, sabinene and \( \alpha \)-phellandrene. The root-specific CsTPS11 was also demonstrated to use a pinyl cation as intermediate in the formation of cyclic terpenes (Wei et al., 2016). Other cyclic sesquiterpenes, including (E)-caryophyllene, \( \alpha \)-humulene and cadinol, were produced by just a few CsTPSs.

Depending on the presence of terpenoid precursors in different cell compartments, the product profile of CsTPSs in planta may differ from those in vitro. Previously, we demonstrated that targeting a nerolidol synthase from strawberry to different cell compartments in Arabidopsis determined the abundance and ratio of mono- and sesquiterpenoid products, confirming the importance of precursor availability for product formation (Aharoni et al., 2004; Kappers et al., 2020). The main components of these induced blends are terpenoids, including (E,E)-\( \alpha \)-ocimene and geranyl linalool, respectively, supporting its role as a genuine multiple-function TPS. Thus, the enzymatic activity of the CsTPSs in combination with their subcellular localization and their expression together determine which terpene compounds are produced in cucumber under which conditions.

Potential roles of CsTPSs in herbivore-induced volatile formation

The volatile blend of control cucumber leaves contained few terpenoids in low amounts, including limonene, (E)-\( \beta \)-ocimene and linalool, coinciding with expression of \( GTPS1 \)-3 and \( GTPS5 \) in these leaves. Upon TSSM and thrips feeding, the expression of \( GTPS2 \)-5, \( GTPS9 \), \( GTPS19 \) and \( GTPS21 \) increased, suggesting a role for these genes in the biosynthesis of volatile terpenoids induced by cell-content feeders, which are mainly associated with JA-related signalling (Zhurov et al., 2014; Steenbergen et al., 2018).

Previous studies documented the volatile blends emitted by different cucumber genotypes upon TSSM feeding (Takabayashi et al., 1994; Bouwmeester et al., 1999; Agrawal et al., 2002; Mercke et al., 2004; Kappers et al., 2010, 2011; He et al., 2020). The main components of these induced blends are terpenoids, including (E)-\( \beta \)-ocimene, linalool, \( E,E \)-\( \alpha \)-farnesene and TMTT, common constituents of many floral and herbivore-induced plant volatile bouquets with various functions in different plant–arthropod interactions, depending on the context (Dicke et al., 1999a,b; Tholl, 2006; He et al., 2019; Burdon et al., 2020).

Although thrips infestation resulted in more damage and a higher total volatile emission compared with that of TSSM, the composition of the terpenoid blend was comparable. By contrast, upon feeding by aphids, which mainly induces SA signalling (Moran & Thompson, 2001), the terpenoid blend differed both quantitatively and qualitatively from that of TSSM- and thrips-damaged plants.

Considering the multiple minor products that are produced by CsTPSs besides their major products, induction of these genes enables the plants to produce a wide spectrum of volatiles and fine-tune their volatile signature in response to herbivory. Most of the terpenoids emitted by noninfested and infested leaves correlated well with the product profiles of the CsTPSs and the expression of the corresponding genes. An exception was the increased emission of \( \alpha \)-pinene, \( \alpha \)-phellandrene and sabinene by leaves infested with cell-content feeding herbivores, while the gene that encodes the most likely corresponding terpene synthase (CsTPS10) was not upregulated. Genes associated with the biosynthesis of terpenoid precursors upstream of the TPSs were also found to be differentially regulated, and this might have implications for the availability of precursors for constitutively expressed TPSs in different cell organelles. Hence, the final terpenoid metabolite profile will be determined by TPSs that are induced upon herbivory as well as those that are constitutively expressed. Cucumber genotypes previously characterized for their herbivore-induced plant volatiles emit mostly similar compounds with different abundances that consequently affected the level of indirect defence (Kappers et al., 2010, 2011).

Further fine-tuning of the volatile signature in response to different herbivore feeding will have consequences for multitrophic interactions. Although we did not compare the different herbivore-induced volatile blends regarding the attractiveness of these odours towards natural enemies, natural enemies can distinguish different blends of terpenoid volatiles upon infestation by different herbivorous arthropods. For instance, lima bean plants emitted different volatile blends as a result of feeding by Spodoptera exigua and \( T. urticae \) and, consequently, \( P. persimilis \) predators were more attracted to plants infested by their prey, \( T. urticae \) (de Boer et al., 2004). When lima bean and cucumber plants were infested by both herbivores separately or together, the plants emitted different amounts of volatile compounds, including several terpenes, and the dual-infested plants were more attractive to predatory mites than those damaged by only a single herbivore species (De Boer et al., 2008).

Involvement of stress-related phytohormones in the response of CsTPS to herbivores with different feeding guilds

Two-spotted spider mites and thrips activated transgenic Arabidopsis \( pGTPS9 \) and \( pGTPS19 \) reporter plants, while aphids and TSSM activated \( pGTPS2 \) reporter plants, indicating that the promoters of \( GTPS9 \) and \( GTPS19 \) respond to cell-content feeders. As reporter activity of these plants was also activated by mechanical damage, a shared upregulation in the response to TSSM and thrips could be the result of the fact that both herbivores cause mechanical wounding. Indeed, thrips inflicted more damage than TSSM and, correspondingly, thrips induced stronger luminescence in reporter plants. Limited, one-time, mechanical damage quickly activated the promoter, which then decreased to the control level within 1 d. Repetitive mechanical damage of lima bean plants using an artificial caterpillar resulted in an induced volatile blend that was strikingly similar in quality to the blend induced by herbivore feeding (Mithofer et al., 2005), suggesting that repetitive mechanical damage inflicted by herbivory is sufficient to trigger the biosynthesis of herbivore-inducible volatiles in plants. Phloem-feeding aphids inflict less
damage, as they navigate their styles between the cell walls to reach phloem vessels with limited harming of cell integrity (Tjal nastęgi & Hogen Esch, 1993; Kloth et al., 2016). Comparison of the up- and downregulated genes in Arabidopsis infested by herbivores with different feeding habits showed that similar transcriptional responses were induced by chewing generalist species Plutella xylostella and Spodoptera litura, while generalist P. occidentalis and phloem-feeding generalists Bemisia tabaci and M. persicae caused more and different transcriptional changes compared with P. xylostella (Reymond et al., 2004; de Vos et al., 2005; Kempema et al., 2007; Kusnierczyk et al., 2007; Little et al., 2007; Ehling et al., 2008).

Both pCsTPS9 and pCsTPS19 reporter plants were responsive to JA but not to SA and ABA. The JA/ethylene pathway is activated in response to thrips feeding (Steenben et al., 2018) and TSSM infestation in multiple species, including lima bean (Dixie et al., 1999), tomato (Ament et al., 2004) and cotton (Miyazaki et al., 2014), although a recent study showed that, unlike adults, juvenile TSSM induce SA but not JA defences in tomato (Liu et al., 2020). Endogenous JA and SA increased within hours after the onset of TSSM infestation in Arabidopsis (Zhurov et al., 2014) and Capsicum (Zhang et al., 2020). In cucumber, JA induces a blend of volatiles that is qualitatively similar to the blend induced by TSSM (Kappers et al., 2010). Furthermore, methyl-SA was emitted upon TSSM herbivory by multiple plant species, including lima bean (Dixie et al., 1990a,b), tomato (Ament et al., 2004) and cucumber (Kappers et al., 2011). Neither SA nor ABA application triggered any response of the reporters driven by pCsTPS9 or pCsTPS19, and although the SA-regulation network may play a role, it appears that JA dominates the regulation of these TPSs that are part of the inducible defence to cell-content feeders.

Interestingly, the promoter activity of pGTPS2 reporter plants was triggered by aphids and TSSM, and by SA and ABA application, whereas JA only provoked minimal promoter activity in these plants. Aphids are known to induce formation of ABA, and ABA-regulated genes are over-represented among genes that are induced by M. persicae saliva infiltration into Arabidopsis leaves (Hillwig et al., 2016). Feeding by the carmine spider mite T. cinnabarinus altered ABA content in tomato plants (Gawrońska & Kielkiewicz, 1999). Furthermore, SA-regulated transcripts increase upon aphid feeding (Moran & Thompson, 2001), although this response is very local (de Vos et al., 2005). The induction of the GTPS2 promoter by TSSM and aphids could be explained via the presence of ABA- and SA-responsive elements in this promoter, suggesting that ABA and SA are important for regulation of GTPS2 and its contribution to the aphid feeding-induced volatile blend. Multiple CAREs present in the promoter sequences of GTPS2, GTPS9 and GTPS19 were identified as possibly involved in JA, SA or ABA responsiveness. For example, G-boxes (CACGTG), required for JA-mediated expression regulation (Kim et al., 1992; Endt et al., 2007), W-boxes (TTGACC) associated with responsiveness to SA (Li et al., 2006) and ABRE motifs, related to ABA responsiveness (Lenka et al., 2009), were present in all three promoters. The wound-responsive WUN motif was found in pGTPS9 and pGTPS19 but not in pGTPS2, and this might play an as-yet-unknown role in the different responses to the mechanical damage inflicted by herbivores from different feeding guilds. Whether motifs in these promoters really function as binding sites to potential transcription factors, and which conditions render specific CAREs indispensable for promoter activity are still unclear and were not the purpose of our study. However, the presence of these motifs probably allows promoters to be bound by transcription factors induced through JA, SA or ABA signalling, hence regulating the volatile blend resulting from hormonal crosstalk.

**Regulation of CsTPS by light and the circadian clock**

The observed rhythmical oscillation in luminescence might be the result of herbivore behaviour, as they often display rhythm feeding. For example, T. ni caterpillars show diurnal feeding behaviour (Goodspeed et al., 2012). However, regardless of whether feeding behaviour of the herbivores in this study was circadian, the nocturnal maximum activity of reporter plants upon JA treatment demonstrates that the expression of the studied CsTPSs displays circadian rhythmicity.

Rhythmic emission of volatiles and expression of genes involved in their biosynthesis have been reported in multiple species. Methyl-JA-induced emission of terpenes and methyl-SA from Norway spruce displayed a diurnal rhythm (Martin et al., 2003). Lima bean leaves mechanically damaged during the day emitted maximum amounts of (E)-β-ocimene and (Z)-3-hexenyl acetate in the late photo-phase, while nocturnally applied mechanical damage triggered nocturnal emission of (Z)-3-hexenyl acetate but only minor amounts of (E)-β-ocimene, which burst after onset of the photophase (Arimura et al., 2008). P. vulgaris released trace amounts of volatiles with no obvious rhythm, but upon infestations with Liriomyza eingebrensis larvae, plants released higher amounts of volatiles with a clear rhythm which peaked at the end of the day (Sufang et al., 2013). The expression of Artemisia annua QH6, encoding a pinene synthase is diurnally regulated (Lu et al., 2002) and luciferase activity driven by the QH6 promoter with a mutated G-box showed a rhythm lacking a peak in the early morning which was present when the intact G-box was present (Zhou et al., 2015). Multiple light-associated CAREs are present in the promoters we tested, including the light-responsive element box I (TTTCAA) (Yamada et al., 1994), and a circadian motif (CAANNNNATC, Piechulla et al., 1998). The G-box present in each of the promoter sequences could be essential for light regulation as well (Lopez-Ochoa et al., 2007).

Our results suggest that the promoters tested induce peak gene expression during the night, while the emission of the corresponding terpenes and green leaf volatiles occurs mainly during the light period (I. F. Kappers, unpublished). Possibly, high nocturnal expression of GTPS2, GTPS9 and GTPS19 results in the accumulation of active enzymes which are ‘ready to go’ when enough substrate becomes available at the onset of the day to fuel production of energy-costly secondary metabolites only during the photoperiod. This is in agreement with the burst of emission of (E)-β-ocimene upon the onset of light by lima bean plants which were damaged in the previous dark period (Arimura et al., 2008). In lima bean, the expression of β-O CIMENE SYNTHASE is regulated via JA accumulation at wounded sites and the
biosynthesis of (E)-β-ocimene is dependent on CO₂ fixation by photosynthesis in the chloroplasts (Arimura et al., 2008), where the MEP pathway synthesizing the terpenoid precursors GPP and GGPP is also located. The expression of the genes of the MEP pathway is light-dependent (Hemmerlin et al., 2012) and, indeed, expression of almost all MEP-pathway genes in Arabidopsis seedlings is repressed in darkness (Hsieh & Goodman, 2005). The expression of MEP-pathway genes encoding 1-DEOXY-D-XYLULOSE-5-PHOSPHATE SYNTHASE and two GPP SYNTHASES are upregulated in cucumber leaves upon TSSM infestation (He et al., 2020). Hence, it is not unlikely that the supply of precursors determines the diurnal emission of terpene volatiles in cucumber leaves triggered by herbivory. To verify this, the rhythmicity of expression of the genes encoding the precursor supply pathways should be evaluated.

In conclusion, we identified the cucumber TERPENE SYNTHASE (GTPS) gene family from the sequenced cucumber genome and characterized their role in the production of volatiles in leaves with and without herbivore feeding. We identified the GTPS genes that contribute to the volatile terpenoid blend of cucumber leaves upon feeding by important cucumber pest species with dissimilar feeding guilds and revealed the involvement of stress-related phytohormones and circadian rhythmicity in the regulation of the production of this terpenoid blend.

Acknowledgements

We thank Hong Gil Nam (DGIST, South Korea) for the GUS: LUC vector and Mariëlle Schreuder for technical assistance. This research was supported by the Netherlands Organisation for Scientific Research (NWO) (grant no. 834.13.001) and by the Dutch Technology Foundation STW which is part of NWO and partly funded by the Ministry of Economic Affairs (grant no. STW11151).

Author contributions

Conceptual design and funding, HJB, MD, IFK, experimental work, JH, FV, AJ, IFK, manuscript: all authors.

ORCID

Harjo R. Bouwmeester https://orcid.org/0000-0003-0907-2732
Marcel Dicke https://orcid.org/0000-0001-8565-8896
Jun He https://orcid.org/0000-0003-3733-0241
Iris F. Kappers https://orcid.org/0000-0003-3349-3473

Data availability

The data that support the findings of this study are available in the Supporting Information of this article.

References

Adams RP. 2007. Identification of essential oil components by Gas Chromatography/ Mass Spectrometry, 4th edn. Carol Stream, IL, USA: Allured Publishing Corp.

Agrawal AA, Janssen A, Bruin J, Posthumus MA, Sabelis MW. 2002. An ecological cost of plant defence: attractiveness of bitter cucumber plants to natural enemies of herbivores. Ecology Letters 5: 377–385.

Aharoni A, Giri AP, Verstappen FWA, Bertea CM, Sevener R, Sun ZK, Jongsm a MA, Schwab W, Bouwmeester HJ. 2004. Gain and loss of fruit flavou r compounds produced by wild and cultivated strawberry species. Plant Cell 16: 3110–3131.

Aharoni A, Jongsm a MA, Bouwmeester HJ. 2005. Volatile science? Metabolic engineering of terpenoids in plants. Trends in Plant Science 10: 594–602.

Ament K, Kant MR, Sabelis MW, Haring MA, Schuurink RC. 2004. Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. Plant Physiology 135: 2025–2037.

Arimura G, Huber DP, Bohlmann J. 2004. Forest tent caterpillars (Malacosoma disstria) induce local and systemic diurnal emissions of terpenoid volatiles in hybrid poplar (Populus trichocarpa × deltoides): cDNA cloning, functional characterization, and patterns of gene expression of (−)-germacrene D synthase, PdTPS1. The Plant Journal 37: 603–616.

Arimura G, Kopke S, Kunert M, Volpe V, David A, Brand P, Dabrowska P, Maffei ME, Boland W. 2008. Effects of feeding Spodoptera littoralis on lime bean leaves: IV. Diurnal and nocturnal damage differentially initiate plant volatile emission. Plant Physiology 146: 965–973.

Aubour g S, Lechany A, Bohlmann J. 2002. Genomic analysis of the terpenoid synthase (AtTPS) gene family of Arabidopsis thaliana. Molecular Genetics and Genomics 267: 739–745.

Balkema-Boomstra AG, Zijlstra S, Verstappen FW, Ingagner H, Mercke PE, Jongsm a MA, Bouwmeester HJ. 2003. Role of cicutacin C in resistance to spider mite (Tetranychus urticae) in cucumber (Cucumis sativus L.). Journal of Chemical Ecology 29: 225–235.

Bohlmann J, Martin D, Oldham NJ, Gershenzon J. 2000. Terpenoid secondary metabolism in Arabidopsis thaliana: cDNA cloning, characterization, and functional expression of a myrcene/(E)-beta-ocimene synthase. Archives of Biochemistry and Biophysics 375: 261–269.

Boutan aev AM, Moses T, Zi J, Nelson DR, Mugford ST, Peters RJ, Osbourn A. 2015. Investigation of terpenoid diversification across multiple sequenced plant genomes. Proceedings of the National Academy of Sciences, USA 112: E81–E88.

Bouwmeester HJ, Verstappen FW, Posthumus MA, Dicke M. 1999. Spider mite-induced (3S)-(−)-nerolidol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis. Plant Physiology 121: 173–180.

Burdon RCF, Raguso RA, Geegar RJ, Pierce EG, Kessler A, Parachnowitsch AL. 2020. Scented nectar and the challenge of measuring honest signals in pollination. Journal of Ecology 108: 2132–2144.

Cao R, Zhang Y, Mann FM, Huang C, Mukkamala D, Hudock MP, Mead ME, Prisic S, Wang K, Lin FY et al. 2010. Diterpene cyclases and the nature of the isoprene fold. Proteins 78: 2417–2432.

Chen F, Ro DK, Petri J, Gershenzon J, Bohlmann J, Pichersky E, Tholl D. 2004. Characterization of a root-specific Arabidopsis terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. Plant Physiology 135: 1956–1966.

Chen F, Tholl D, Bohlmann J, Pichersky E. 2011. The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. The Plant Journal 66: 212–229.

De Boer JG, Nordi jka CA, Posthumus MA, Dicke M. 2008. Prey and non-prey arthropods sharing a host plant: effects on induced volatile emission and predator attraction. Journal of Chemical Ecology 34: 281–290.

De Boer JG, Posthumus MA, Dicke M. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or nonprey herbivores by a predatory mite. Journal of Chemical Ecology 30: 2215–2230.

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Metraux JP, Van Loon LC, Dicke M et al. 2005. Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Molecular Plant–Microbe Interactions 18: 923–937.

Degenhardt J, Gershenzon J, Baldwin IT, Kessler A. 2003. Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. Current Opinion in Biotechnology 14: 169–176.
Dicke M, Gols R, Ludeking D, Posthumus MA. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *Journal of Chemical Ecology* 25: 1907–1922.

Dicke M, Sabelis MW. 1988. How plants obtain predatory mites as bodyguards. *Netherlands Journal of Zoology* 38: 148–165.

Dicke M, Sabelis MW, Takabayashi J, Bruin J, Posthumus MA. 1990. Plant strategies of manipulating predator-prey interactions through allelochemicals – prospects for application in pest-control. *Journal of Chemical Ecology* 16: 3091–3118.

Dicke M, Van Beek TA, Posthumus MA, Ben Dom N, Van Bokhoven H, De Groot A. 1990. Isolation and identification of volatile kairomone that affects acarine predator prey interactions. Involvement of host plant in its production. *Journal of Chemical Ecology* 16: 381–396.

Dudareva N, Pichersky E. eds. 2020. *Biological of plant volatiles*. Boca Raton, FL, USA: CRC Press.

Ehlting J, Chowira SG, Mattheus N, Aeschliman DS, Arimura G, Bohlmann J. 2019. *Variation in terpenoid biosynthesis pathways in the early steps of plant isoprenoid biosynthesis?*. In *Plant Cell Biology and Metabolism*, pp. 875–877.

Emanuelsson O, Nielsen H, von Heijne G. 1999. *CHLOROP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites.*

Ehlting J, Chowira SG, Mattheus N, Aeschliman DS, Arimura G, Bohlmann J. 2019. *Variation in terpenoid biosynthesis pathways in the early steps of plant isoprenoid biosynthesis?*. In *Plant Cell Biology and Metabolism*, pp. 875–877.

Emanuelsson O, Nielsen H, von Heijne G. 1999. *CHLOROP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites.*

Ehlting J, Chowira SG, Mattheus N, Aeschliman DS, Arimura G, Bohlmann J. 2019. *Variation in terpenoid biosynthesis pathways in the early steps of plant isoprenoid biosynthesis?*. In *Plant Cell Biology and Metabolism*, pp. 875–877.
Zhurov V, Navarro M, Bruinsma KA, Arbona V, Santamaria ME, Cazaux M, Wybouw N, Osborne EJ, Ens C, Rioja C et al. 2014. Reciprocal responses in the interaction between Arabidopsis and the cell-content-feeding chelicerate herbivore spider mite. *Plant Physiology* 164: 384–399.

**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Chromosomal position of CsTPS.

**Fig. S2** Phylogenetic tree of CsTPS.

**Fig. S3** Gene expression of selected genes in the terpenoid module.

**Fig. S4** Product profiles of heterologous CsTPS.

**Fig. S5** Histochemical β-glucuronidase staining.

**Table S1** Primers used in this study.

**Table S2** GC-MS analysis of volatile emissions.

**Table S3** Annotation of the terpenoid biosynthetic module genes.

**Table S4** Genomic information of GTPS genes.

**Table S5** RPKM values of genes in the terpenoid biosynthetic module.

**Table S6** Heterologous assays.

**Table S7** CARE motif analysis.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

---

**About New Phytologist**

- *New Phytologist* is an electronic (online-only) journal owned by the New Phytologist Foundation, a *not-for-profit organization* dedicated to the promotion of plant science, facilitating projects from symposia to free access for our Tansley reviews and Tansley insights.

- Regular papers, Letters, Viewpoints, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication ‘as ready’ via Early View – our average time to decision is <26 days. There are no page or colour charges and a PDF version will be provided for each article.

- The journal is available online at Wiley Online Library. Visit [www.newphytologist.com](http://www.newphytologist.com) to search the articles and register for table of contents email alerts.

- If you have any questions, do get in touch with Central Office (np-centraloffice@lancaster.ac.uk) or, if it is more convenient, our USA Office (np-usaoffice@lancaster.ac.uk)

- For submission instructions, subscription and all the latest information visit [www.newphytologist.com](http://www.newphytologist.com)