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Chapter 7

Engineering of Tomato Glandular Trichomes for the Production of Specialized Metabolites

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Abstract

Glandular trichomes are specialized tissues on the epidermis of many plant species. On tomato they synthesize, store, and emit a variety of metabolites such as terpenoids, which play a role in the interaction with insects. Glandular trichomes are excellent tissues for studying the biosynthesis of specialized plant metabolites and are especially suitable targets for metabolic engineering. Here we describe the strategy for engineering tomato glandular trichomes, first with a transient expression system to provide proof of trichome specificity of selected promoters. Using microparticle bombardment, the trichome specificity of a terpene-synthase promoter could be validated in a relatively fast way. Second, we describe a method for stable expression of genes of interest in trichomes. Trichome-specific expression of another terpene-synthase promoter driving the yellow-fluorescence protein-gene is presented. Finally, we describe a case of the overexpression of farnesyl diphosphate synthase (FPS), specifically in tomato glandular trichomes, providing an important precursor in the biosynthetic pathway of sesquiterpenoids. FPS was targeted to the plastid aiming to engineer sesquiterpenoid production, but interestingly leading to a loss of monoterpenoid production in the transgenic tomato trichomes. With this example we show that trichomes are amenable to engineering though, even with knowledge of a biochemical pathway, the result of such engineering can be unexpected.

Keywords
Glandular trichomes, Tomato, Genetic engineering, Terpenes, Farnesyl diphosphate synthase

Introduction

Trichomes are specialised structures of epidermal origin found as extrusions or appendages on plant surfaces, but they are also found on lichens and even algae (Engene et al., 2012). Trichomes are very diverse in appearance and function in stress resistance, including excessive light or temperature, and insect and pathogen defence. They are usually shaped as hair-like structures, but can also appear as scales, buds or papillae and range from very small unicellular to very big multicellular structures. On tomato the trichomes are shaped as hairs and are differentiated in eight types including four that are glandular (Glas et al., 2012). Glandular trichomes are typically multicellular with one or more glandular cells that can produce, store, emit, or exude specialised compounds into the environment (volatile) or onto the epidermal surface (nonvolatile) (Schilmiller, Last, & Pichersky, 2008; Wagner, 1991). Targeting of particular biosynthetic pathways to these specialised structures is thought to be highly regulated as not to interfere with the plants' development. Glandular trichomes are well-known sources of essential oils and resins that have a widespread use in agricultural, pharmaceutical, and cosmetic industries.

Specialised Metabolites and Application

In addition to the primary metabolites that most plants have in common, plants can produce a large variety of specialised metabolites. Many of these metabolites, including terpenoids, acylsugars, indoles, phenolic compounds, and methyl ketones have a role in defence against herbivores and pathogenic organisms. All these compounds have been described to be synthesized by or present in glandular trichomes (for a review, see Glas et al., 2012). Terpenoids are the largest group of specialized metabolites with more than 30,000 terpenoids known to date to be produced by plants. Also tomato glandular trichomes produce a wide array of terpenoids, which are mostly specialized metabolites with roles in defence against pests and in attracting beneficial insects such as natural enemies of herbivores (Kappers et al., 2005; Simmons & Gurr, 2005; Weinhold & Baldwin, 2011).

Production of Specialised Metabolites in Tomato Trichomes

Terpenoids are synthesized via distinct metabolic pathways inside tomato trichome head cell(s). They are derived from rather simple universal C5 isoprene building blocks but can result in a wide variety of decorated $(C5)_n$ molecules. Two main pathways were identified for the production of these building blocks. Both these pathways result in the production of the intermediate C5-molecules isopentenyl diphosphate (IPP) and its double bond isomer dimethylallyl diphosphate (DMAPP) but in different cellular compartments (Fig. 1). Both C5 compounds are required in specific ratios by the prenyltransferases that subsequently form terpene precursors. The cytosolic mevalonate (MVA) pathway starts from acetyl-CoA and proceeds through the intermediate MVA into IPP and DMAPP and results in the biosynthesis of C15-sesquiterpenes. The MVA pathway is present in all eukaryotes, but plants have an alternative biosynthetic pathway toward IPP and DMAPP called the MEP pathway (methyl-erythritol phosphate pathway) that is localized in the plastid. In tomato trichome plastids, IPP and DMAPP are then converted to C10-, C15-, and C20-precursors that are further converted into mono-, sesqui-, and diterpenes, respectively (Fig. 1). There are indications that some intermediate molecules are able to cross the plastid membrane (Dudareva et al., 2005). The glandular

trichomes of tomato are rather special in the sense that, besides canonical trans-precursors (GPP, geranyl diphosphate; FPP, farnesyl diphosphate; and GGPP, geranylgeranyl diphosphate) they can also convert IPP and DMAPP to *cis*-precursors in plastids. Neryl diphosphate is the *cis*-precursor for many monoterpenes in *Solanum lycopersicum* (Schilmiller et al., 2009) and in the wild tomato *Solanum habrochaites* plastidial sesquiterpenes are produced from *cis*-FPP (Sallaud et al., 2009).

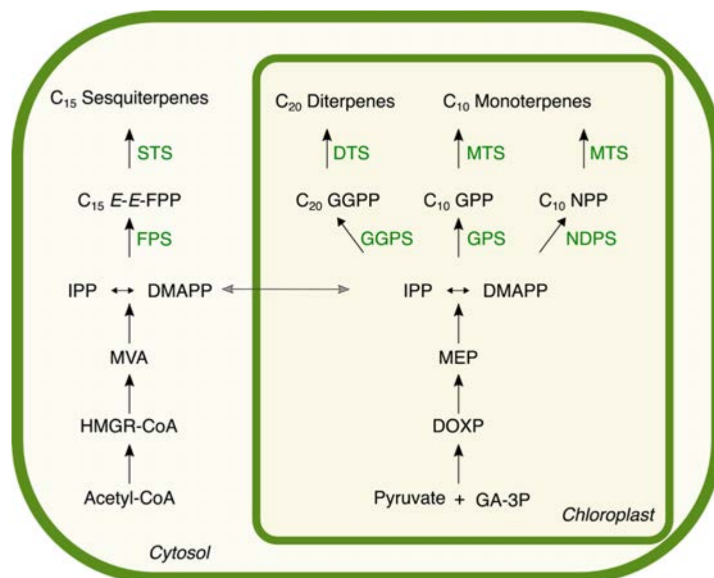

Figure 1. Schematic overview of terpenoid biosynthesis in trichomes of cultivated tomato. The synthesis of terpenoids takes place in the cytosol through the mevalonate (MVA) pathway, in the plastid via the methyl-erythritol phosphate (MEP) pathway but also (partly) in the mitochondria, ER and/or peroxisomes (not depicted). Abbreviations: Acetyl-CoA, acetoacetyl-coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; MVA, mevalonate; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; *E*,*E*-FPP, trans-farnesyl diphosphate; FPS, farnesyl diphosphate synthase; STS, sesquiterpene synthase; GA-3P, glyceraldehyde-3-phosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, geranylgeranyl diphosphate synthase; DPS, diterpene synthase; GPP, geranyl diphosphate; GPS, geranyl diphosphate synthase; NDP, neryl diphosphate; NDPS, neryl diphosphate synthase; MTS, monoterpene synthase.

Modifying Terpene Production

The initial attempts to increase terpene production in plants were not done in glandular trichomes. Aharoni et al. (2003) showed that ectopic expression of a terpene synthase (FaNES, a strawberry linalool/nerolidol synthase) in Arabidopsis thaliana leaves was more successful when targeted to plastids than in the cytosol but did lead to growth retardation upon high expression levels. Targeting a terpene synthase to a specific cellular compartment for generating higher levels of terpenes was also pursued by Wu et al. (2006) and Huang et al. (2010). Wu et al. (2006) showed that sesquiterpene production in Nicotiana tabacum leaves was elevated as a consequence of directing a naturally cytosolic prenyltransferase (farnesyl diphosphate synthase, FPS) plus a sesquiterpene synthase to the plastid. It was reasoned that this increase in

sesquiterpene production in the plastid was caused by the compartmentalized production. In addition to the plastid, IPP isomerase as well as FPS are also present in mitochondria (Cunillera, Boronat, & Ferrer, 1997; Phillips, D'Auria, Gershenzon, & Pichersky, 2008), providing an alternative engineering route. When FaNES was targeted to the mitochondria nerolidol levels were greatly increased compared to control, but also compared to plastid-targeted FaNES, due to the ample availability of FPP in the mitochondria (Kappers et al., 2005). Terpenoid biosynthetic pathways are under regulation of feedback inhibition. It is known that the levels of IPP and DMAPP, but also downstream metabolites such as FPP can influence enzyme activity of upstream precursors (Banerjee et al., 2013; Closa et al., 2010; Tholl & Lee, 2011).

Glandular trichomes are regarded excellent tissues for studying the biosynthesis of specialized plant metabolites, and as especially suitable targets for metabolic engineering (Ennajdaoui et al., 2010; Schilmiller et al., 2008). Using trichome-specific promoters rather than strong ubiquitous promoters has the advantage that possibly toxic products are not produced outside of these specialized tissues and that perturbation of metabolic pathways in the rest of the plant is avoided (Tissier, 2012). However, unexpected negative effects of trichome-targeted expression have been observed, though it is speculated this might be due to the use of an enhancer element in the expression cassette causing ectopic expression in meristematic or progenitor cells (Wu et al., 2012; Zhang et al., 2015).

Despite its potential, relatively few cases of trichome engineering have been reported (Tissier, 2012). Examples of the successful use of trichome-specific promoters include the targeted expression of a taxadiene synthase in tobacco under control of the cembatrienol synthase promoter (Rontein et al., 2008) and a beta-ketothiolase under control of two different cotton trichome promoters (John & Keller, 1996). Two genes from tomato, essential for the production of santalene and bergamotene, were successfully expressed in the glandular trichomes of tobacco (Sallaud et al., 2009). *Z*-abienol production was successfully engineered in the glandular trichomes of Nicotiana sylvestris, which normally does not produce this compound (Sallaud et al., 2012). In tomato, methylketone production was slightly increased by expressing two methylketone synthases under control of a trichome-specific promoter, while the same genes expressed under control of the strong and ubiquitous cauliflower mosaic virus 35S promoter showed lesions in their leaves (Yu & Pichersky, 2014). Two genes from wild tomato *S. habrochaites*, necessary for the production of 7-epizingiberene were introduced into cultivated tomato under different trichome-specific promoters (ShMKS1 and SlTPS5, respectively) leading to production of 7-epizingiberene (Bleeker et al., 2012). Although this was sufficient to validate insect resistance, compared to the wild tomato from which these genes originate, only very low levels of this sesquiterpene were produced. This despite the fact that both the precursor and biosynthetic gene are plastid localized with the less stringently regulated MEP pathway (Bleeker et al., 2012; Sallaud et al., 2009). These examples indicate that optimizing metabolic engineering in trichomes might require additional factors, such as the upstream component of the precursor pathway (Lange et al., 2011).

Focus of this chapter

Various promoters of genes expressed specifically in glandular trichomes have been identified (Table 1) which can be used to drive the expression of genes of interest. Here we focus on the use of (a number of) tomato trichome-specific promoters to achieve targeted expression. In this method, we describe the engineering of tomato glandular trichomes, first with a transient expression system to get proof of trichome specificity of promoters, and second a stable expression system for production of specialized compounds. As proof of concept, we describe a case of the latter with the specific over-expression of an FPS. Like Wu et al. (2006), we opted for avian FPS to avoid (post)transcriptional regulation and feedback inhibition, and as the cytosolic MVA flux appears to be much more regulated (Wu et al., 2012), we decided to target the expression of FPS to the plastids of glandular trichome secretory cells mediated by a transit peptide signal sequence.

Methods

Transient Transformation of Tomato Trichomes by Microparticle Bombardment

Transient-plant transformation is a broadly applicable and relatively rapid way of checking the expression of designed plant-expression vectors in vivo. For this purpose, promoter activity is often visualized driving GUS (beta-glucuronidase) or fluorescent markers. The Agrobacterium-mediated transient-transformation assay (ATTA) is a well-known technique with high transformation efficiency (Kapila, De Rycke, Van Montagu, & Angenon, 1997; Wroblewski, Tomczak, & Michelmore, 2005). However, construct delivery to glandular trichomes by Agrobacterium is difficult, possibly due to the trichome barrier cells that warrant specificity of transport to the stalk and head cells (Dell & McComb, 1978; Werker, 2000). Previous promoter studies in glandular trichomes have been done in stably transformed plants (Table 1), which is more time-consuming compared to transient transformation. Here we present a step-wise optimized method to transiently transform glandular trichomes on tomato stems using a microparticle bombardment system. The presented method is exemplified by transforming a GUS–sYFP marker gene driven by the *S. lycopersicum* Terpene Synthase 5 (SlTPS5) promoter. SlTPS5 has previously been described and demonstrated to encode a linalool synthase with specific expression in type VI tomato trichomes (Falara et al., 2011; van Schie, Haring, & Schuurink, 2007).

Table 1. List of trichome-specific promoters.

Gene	Protein Description	Species	Reporter	Trichome Type	References
AaADS	Amorpha-4,11-diene synthase	*Artemisia annua*	GUS	Glandular trichomes	Wang et al. (2011)
CYP71AV1	Cytochrome P450	*Artemisia annua*	GUS	Glandular and nonglandular trichomes	Wang, Han, Kanagarajan, Lundgren, and Brodelius (2013)
AaDBR2	Artemisinic aldehyde Δ11(13) reductase	*Artemisia annua*	GUS	Glandular secretory trichomes	Jiang et al. (2013)
AaGL2	Transcription factor	*Artemisia annua*	GUS	Glandular and T-shaped trichomes	Jindal, Longchar, Singh, and Gupta (2015)
AaMIXTA-Like1	Transcription factor	*Artemisia annua*	GUS	Glandular and T-shaped trichomes	Jindal et al. (2015)
SQAPI	Squash aspartic protease inhibitor	*Cucurbita maxima*	GUS	Long glandular trichomes in mature plant	Anandan, Gatehouse, Marshall, Murray, and Christeller (2009)
NsCBTS-2a	Cembratrien-ol synthase	*Nicotiana sylvestris*	GUS	Tall glandular trichomes	Ennajdaoui et al. (2010)
CYP71D16	Cytochrome P450	*Nicotiana tabacum*	GUS	Glandular and nonglandular trichomes	Wang (2002)
T-phylloplanin	Surface-localized protein	*Nicotiana tabacum*	GFP, GUS	Short, procumbent glandular trichomes	Shepherd, Bass, Houtz, and Wagner (2005)
NtLTP1	Lipid transfer protein	*Nicotiana tabacum*	GFP, GUS	Long glandular trichomes	Choi et al. (2012)

Table 1. List of trichome-specific promoters - continued

Gene	Protein Description	Species	Reporter	Trichome Type	References
*Nt*CPS2	Copalyl diphosphate synthase	*Nicotiana tabacum*	GUS	Glandular-secreting trichomes	Sallaud et al., 2012
*Sh*MKS1	Methylketone synthase	*Solanum habrochaites*	GFP	Type VI glandular trichomes	Akhtar et al., 2013
*Sh*MKS2	Methylketone synthase	*Solanum habrochaites*	GFP	Strong in type VI, weak in other types	Yu and Pichersky, 2014
*Sl*ASAT4	Acylsucrose acyltransferase	*Solanum lycopersicum*	GFP	Type I and IV glandular trichomes	Schilmiller et al., 2012
*Sl*EOT1	Transcription factor	*Solanum lycopersicum*	GUS–sYFP1	Type VI glandular trichomes	Spyropoulou et al., 2014
*Sl*TPS5	Linalool synthase	*Solanum lycopersicum*	GUS–sYFP1	Type VI glandular trichomes	Spyropoulou et al., 2014
*Sl*ASAT3	Acylsucrose acyltransferase	*Solanum lycopersicum*	GFP	Type I and IV glandular trichomes	Schilmiller et al., 2015
*Sl*IPMS3	Isopropylmalate synthase	*Solanum lycopersicum*	GFP, GUS	Type I and IV glandular trichomes	Ning et al., 2015

Abbreviations: GUS, β-glucuronidase; GFP, green fluorescent protein; sYFP1, yellow fluorescent protein.

Preparation of the pSlTPS5: GUS–sYFP1 Construct

A 1254-bp genomic sequence from *S. lycopersicum* cv. Moneymaker upstream of the start codon of TPS5 (AY840091) was cloned between restriction sites SacI (50) and XbaI, replacing the 35S promoter of vector pJVII, a pMON999-based vector (Monsanto, St. Louis, MO). The SlTPS5 promoter was cloned upstream of the start codon of uidA (GUS) fused to a yellow fluorescent protein (sYFP1) (Kremers, Goedhart, van Munster, & Gadella, 2006), followed by the Nos terminator (tNos) (Fig. 2A). The final construct was sequenced and then transformed to Escherichia coli DH5α cells, grown overnight at 37°C in Luria Broth containing the appropriate antibiotic, after which the plasmid was isolated using a Midi Plasmid Purification Kit (Qiagen).

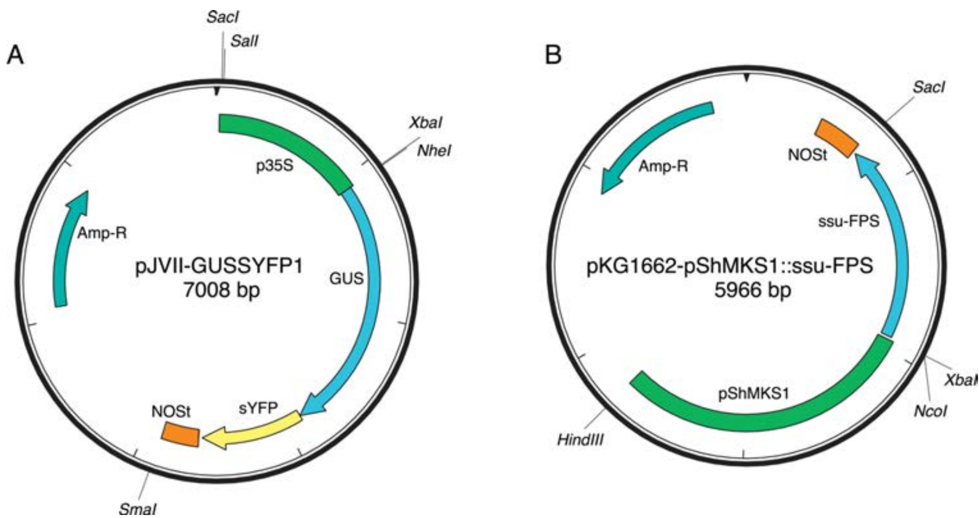

Figure 2. Maps of plasmids used for engineering. (**A**) Map of the pJVII–GUS–sYFP plasmid used for transient and stable tomato transformation with trichome-specific promoters. For transient transformation by particle bombardment, the *S. lycopersicum* terpene synthase 5 (SlTPS5) promoter was used to drive GUS–sYFP. For stable transformation, the promoter of *S. lycopersicum* terpene synthase 9 (SlTPS9) was used. (**B**) pKG11662-pShMKS1:ssu-FPS construct used in the engineering of specialized metabolism in tomato trichomes. Abbreviations: GUS, β-glucuronidase; sYFP1, yellow fluorescent protein; NOSt, nopaline synthase terminator; Amp-R, β-lactamase ampicillin-resistance gene.

Particle Coating with DNA

Thirty-five microliter of gold particle solution (74 mg Au in 1.2 mL 100% ethanol) was placed in a 1.5-mL tube and spun shortly at maximum speed. The ethanol was carefully removed and without disturbing the pellet 1 mL of deionized water MQ was added to the tube. After spinning at 2000 rpm for 2 min, water was removed as much as possible. Next, the appropriate amount of plasmid (25 μg) was added in 250 μL of ice-cold MQ water. A quick vortexing step was followed by addition of 250 μL of ice-cold 2.5 M $CaCl_2$ and 50 μL of ice-cold freshly prepared 1.4 mM spermidine solution. Spermidine (05292 Sigma-Aldrich) was melted at 65°C and aliquoted under argon and stored at 80°C prior to use. The final mixture was vortexed for 10 min at 4°C and pelleted by spinning for 10 min at 500 rpm. After removal of the supernatant, the pellet was washed six times with 600 μL of 100% ethanol, by pipetting up and down to resuspend the pellet

and spinning 1 min at maximum speed. Washing steps were repeated until a homogeneous suspension was obtained without visible gold clumps. Finally, 36 µL of 100% ethanol was added and the pellet was resuspended by vortexing. This amount of DNA-coated microparticles suspension was sufficient to perform three particle bombardments (10 µL each).

Plant Material and Growing Conditions

Tomato plants (S. lycopersicum cv. Moneymaker) were grown in soil in a greenhouse with a day/night temperature of 23/18°C and a 16/8 h light/ dark regime for 4 weeks. For the transient transformation of trichomes, 3–4 cm long stem pieces were collected and distributed in the center of three Petri dishes containing 1% MS medium immediately before the bombardment.

Bombardment Conditions

Ten microliter of the DNA-coated gold particles (microcarrier) suspension was loaded onto the center of a macrocarrier, desiccated, and delivered according to the procedure described for the Biolistic PDS-1000/He system (https://www.bio-rad.com). Specific conditions used were rupture disks of 1100 PSI accelerating pressure, 9 cm target distance and 28 in. Hg vacuum pressure. The Petri dishes were sealed with Parafilm and bombarded stems were incubated in a growth chamber at 26°C for 48h.

GUS Expression Assay

Forty-eight hours after bombardment, stem pieces were transferred to 15 mL tubes and submerged in 5-bromo-4-chloro-1H-indol-3yl-b-D-glucopyranosiduronic acid (X-Gluc) buffer containing 1 mM X-Gluc, 100 mM sodium phosphate buffer, pH 7.0, and 0.1% Triton X-100 (v/v). After 16 h of incubation at 37°C in the staining buffer, the stem pieces were washed three times with 50 mM sodium phosphate buffer (pH 7.2) and finally resuspended and preserved in the same buffer. Blue staining was assessed using a binocular microscope.

Analysis of Promoter Trichome Specificity

Here examination of the bombarded tomato stems showed that, even though the whole stem was hit by the DNA-coated particles, only trichomes type VI expressed the GUS gene (Fig. 3A). A closer view on the stained trichomes showed that the blue coloring was not restricted to the four head cells but was also present in the basal stem of the type VI trichome (Fig. 3B1 and B2). This was likely due to diffusion of chloro-bromoindigo, to which GUS converts X-Gluc, from the head to adjacent cells as the staining was always found in a diffused pattern in the basal cells. However, it is also possible that the GUS protein itself has diffused (Mascarenhas & Hamilton, 1992). Taken together, transient transformation by microparticle bombardment as presented here can be a quick and powerful way to assay the specificity of promoters in glandular trichomes. Importantly, trichomes can remain intact after delivery of the plasmid and are still able to synthesize the reporter protein. We therefore conclude this is a proper method to validate new trichome-specific promoters.

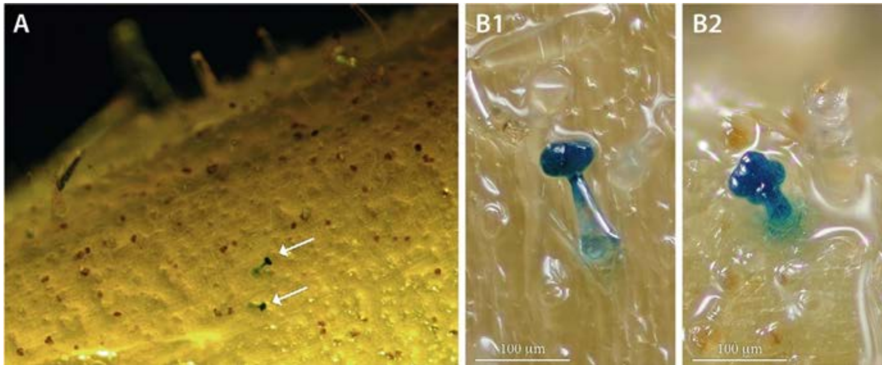

Figure 3. GUS activity shows trichome-specific activity of the SlTPS5 promoter. β-Glucuronidase (GUS) enzymatic activity in *S. lycopersicum* cv. Moneymaker stem trichomes transformed, by particle bombardment, with the pJVII-pSlTPS5:GUS construct containing GUS gene driven by the *S. lycopersicum* terpene synthase 5 (SlTPS5) promoter. Panoramic view (**A**) and close up (**B1,2**) of type VI glandular trichomes expressing SlTPS5: GUS. GUS activity was determined by incubation with X-Gluc (16 h). *Photographs by Jan van Arkel, IBED, University of Amsterdam.*

Stable Transformation of Tomato Trichomes

Although particle bombardment can indicate trichome specificity, the number of trichomes hit might not be very high and the procedure is destructive. Alternatively, for a more in-depth study, one can make stable transgenic plants expressing a promoter–reporter construct. In this section, a construct with the SlTPS9 promoter driving a GUS–sYFP1 fusion was introducedinto *S. lycopersicum* cv. Moneymaker to verify the promoter activity of SlTPS9 in stably transformed plants.

Preparation of Construct pSlTPS9: GUS–sYFP1

A 1557-bp fragment of genomic sequence upstream from the start codon of SlTPS9 was cloned in vector pJVII between restriction sites SacI (50) and NheI as described in Section 2.1.1 (Fig. 1B). The construct was verified by sequencing and then the expression cassette was transferred to the MCS of the binary vector pBINplus (van Engelen et al., 1995) using the SacI and SmaI restriction sites. The final construct was transformed to Agrobacterium tumefaciens GV3101 (pMP90).

Stable Tomato Transformation

Tomato (S. lycopersicum cv. Moneymaker) seeds were surface sterilized in 70% ethanol for 2 min followed by 20 min in 25% hypochlorite. After rinsing five times in sterile water they were placed on germination medium, which consists of 2.5 g L^{-1} Murashige and Skoog medium-including Gamborg B5 vitamins (MS+Vit B5), 10 g L^{-1} sucrose, and 0.5 g L^{-1} MES, pH 5.8 (Cortina & Culianez-Macia, 2004). Seedlings were grown at 25°C and 70% relative humidity for 10 days (90 µmol m-2 s-1; 8 h dark, 16 h light). Cotyledons of sterile tomato seedlings were cut off, and the tips were removed and sectioned transversely with a scalpel in two fragments. Cotyledon cuts were placed adaxial-side down in 90 x 15 mm Petri dishes containing coculture medium (COM) and incubated for 1 day. The COM medium was composed of 4.5 g L^{-1} MS+Vit B5, 30 g L^{-1} sucrose, 0.5 g L^{-1} MES, 2 mg L^{-1} zeatin, 0.1 mg L^{-1} indole-3-acetic acid (IAA), 0.05 mg L-1 2,4-

dichlorophenoxyacetic acid (2,4-D), and 200 µM acetosyringone, pH 5.8. *A. tumefaciens* strain GV3101 (pMP90) harboring the construct, which also contains the selection gene neomycin phosphotransferase (NPTII), was grown overnight to OD600 of 0.6–0.8 in modified Luria Bertani medium (1% Bacto Trypton, 0.5% yeast extract, and 0.25% NaCl, pH 7.0). Prior to cocultivation the culture was centrifuged (15 min at 3000 rcf) and the pellet was resuspended in liquid medium consisting of 4.5 g L-1 MS+Vit B5, 30 g L-1 sucrose, and 0.5 g L-1 MES, pH 5.8. Tomato cotyledon explants were removed from the COM plates and transferred to the bacterial suspension for 5 min. Next, they were placed on fresh COM plates after shortly drying on sterile filter paper. After 2 days of cultivation on COM, the explants were placed on postculture medium consisting of 4.5 g L^{-1} MS+Vit B5, 30 g L^{-1} sucrose, 0.5 g L^{-1} MES, 2 mg L^{-1} zeatin, 0.1 mg L^{-1} IAA, 200 mg L^{-1} cefotaxime, and 50 mg L^{-1} vancomycin, pH 5.8. Another 3 days later, the explants were transferred to shoot-inducing medium (SIM) composed of 4.5 g L^{-1} MS+Vit B5, 10 g L^{-1} glucose, 0.5 g L^{-1} MES, 2 mg L^{-1} zeatin, 0.1 mg L^{-1} IAA, 100 mg L^{-1} kanamycin, and 500 mg L^{-1} carbenicilin, pH 5.8. The plates were incubated at 25°C, 70% RH under fluorescent light (90 µmol m^{-2} s^{-1}; 8 h dark, 16 h light). Explants were transferred to fresh SIM every 2 weeks. Calli were removed from explants when they grew over 0.5 cm in width and transferred to fresh SIM. Emerging shoots from these calli were harvested and placed in sterile plant containers (68 x 66 mm) containing rootinducing medium (RIM). RIM consisted of 4.5 g L^{-1} MS+Vit B5,10 g L^{-1} sucrose, 0.5 g L^{-1} MES, 0.25 mg L^{-1} indole-3-butyric acid (IBA), and 200 mg L^{-1} cefotaxime, pH 5.8. After root formation, the plants were gently removed from the containers and potted in soil.

Transgene expression

Five independent transgenic primary (T0) lines were obtained after PCR verification of the presence of the YFP gene on genomic DNA isolated from leaves. Based on YFP expression in T0 stems and leaves, one transgenic line was selected for further analysis. Transgene expression was further confirmed by observing YFP fluorescence using an EVOSfl inverted microscope (http://www.thermofisher.com) in stems and leaves collected from the transgenic T0 plants. The selected transgenic line exhibited strong YFP fluorescence in glandular stem and leaf trichomes (Fig. 4B and D). Fig. 4 shows that fluorescence was specific to the four glandular head cells of the type VI trichomes. This result confirms the trichome-specific expression of SlTPS9 as found by Bleeker, Diergaarde, et al. (2011) and Bleeker, Spyropoulou, et al. (2011). It furthermore shows that the method presented here is suitable to drive transgene expression specifically in the glandular trichomes of stably transformed tomatoes.

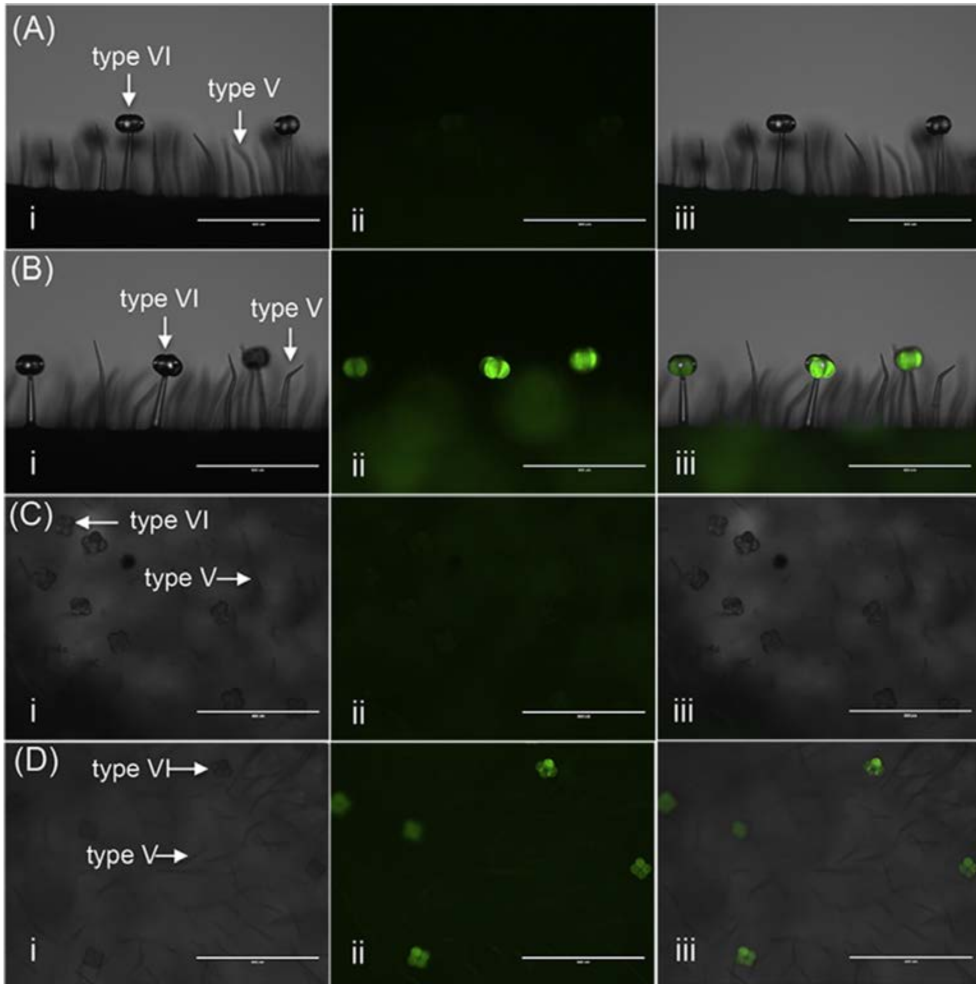

Figure 4. The SlTPS9 promoter drives sYFP expression specifically in type VI glandular trichomes of stable transgenic tomato plants. (**A**) Stem trichomes of untransformed plant. (**B**) Stem trichomes of tomato transformed with the SlTPS9:GUS–sYFP construct. (**C**) Leaf trichomes of untransformed plants. (**D**) Leaf trichomes of tomato transformed with the SlTPS9:GUS–sYFP construct. Images: (**i**) Normal light (brightfield), (**ii**) GFP filter, and (**iii**) merged image of (i) and (ii). Images were taken using the EVOSfl inverted microscope (http://www.thermofisher.com). Arrows indicate type VI glandular trichomes and type V nonglandular trichomes. Scale bars: 400 μm.

Proof of concept: Targeted expression of a terpene precursor gene in tomato glandular trichomes

Stable Trichome-Specific Expression of FPS

Preparation of pMKS1:ssu-SlFPS and pMKS1:ssu-GgFPS Transgenic Tomatoes

The ShMKS1 promoter sequence was amplified from genomic DNA of the wild tomato S. habrochaites accession PI126449. The 1733-bp fragment was cloned in front of GUS in the pKG1662 vector (patent US2011/0113512A1), of which the 35S promoter was removed using *Hind*III and *Nco*I. The signal peptide of the Rubisco-small subunit (ssu) (168 bp) was amplified from S. lycopersicum with primers creating *Nco*I (30) and modified *Nco*I (50) ends. Gallus gallus FPS (GgFPS) was amplified from chicken liver cDNA and digested with *Nco*I and *Sac*I. The two gene fragments were ligated into the ShMKS1 promoter containing pGK1662 vector using the *Nco*I and *Sac*I restriction sites, replacing the GUS gene. This pMKS1:ssu- GgFPS:35ST cassette was subsequently cut out using *Hind*III/*Sac*I and ligated into pBINplus (van Engelen et al., 1995). Similar reactions were done to create the ssu-SlFPS constructs with the exception that FPS was amplified from *S. lycopersicum* cDNA. All constructs were checked by sequencing and then transformed to *A. tumefaciens* GV3101 and used to transform tomato *S. lycopersicum* var. Moneymaker as described above.

7

After stable plant transformation, successfully transformed plants growing on selective medium were checked for the presence of the transgene by PCR. Amplification was done on gDNA from leaf material using a MKS1 promoter-specific primer and a gene-specific primer. In this way, at least three independent lines, containing either the ssu-SlFPS (ssu-SlFPS lines) or the ssu-GgFPS (ssu-GgFPS lines) transgene were selected for further analysis.

Expression Analysis of the Different FPS Transgenes

To confirm transgene expression, FPS transcript levels in the trichomes of three independently transformed lines were measured by quantitative realtime PCR (qRT-PCR) and compared to trichomes of untransformed plants. Glandular trichomes can contain large amounts of etheric oils and saccharides, which can make it hard to isolate RNA. Commercially available kits often suffice to obtain high quality RNA out of trichomes. However, for high RNA yield, we used an 80°C phenol extraction buffer followed by chloroform:isoamylalcohol phase separation and precipitation in 2 M LiCl. For a full method description, see Verwoerd, Dekker, and Hoekema (1989). After RNA isolation, DNase treatment and cDNA synthesis were done using commercially available kits from Ambion and Fermentas, respectively (www.thermofisher.com). To quantify the FPS transcripts in the ssu-SlFPS and ssu-GgFPS lines, we used a FPS-specific primer pair to amplify the cDNA and normalized their levels to those of Rubisco conjugating enzyme 1 (RCE1). As shown in Fig. 5, FPS levels in the trichomes of three independent ssu-SlFPS lines are only moderately elevated compared to untransformed tomato (n=3). It must be noted that these also include basal expression levels of the native, cytosolic, FPS. Therefore transgene ssu-SlFPS transgene expression is expected to be lower than represented. Interestingly, transgene GgFPS expression of the three independent ssu-GgFPS lines showed to be much higher compared to the ssu-SlFPS lines (Fig. 6). From these results it appears that SlFPS levels are under

(post)transcriptional regulation in tomato trichomes. The ShMKS1 promoter has been shown to be highly active in transgenic tomato trichomes when driving GFP (Akhtar et al., 2013). However, we found only a moderate increase in SlFPS transcript levels. Only driving FPS by the trichome-specific and constitutive ShMKS1 promoter seems not suitable to get much higher expression levels. To boost transcript levels, using a nonplant ortholog of FPS, like GgFPS, can be a way to circumvent the regulatory mechanism.

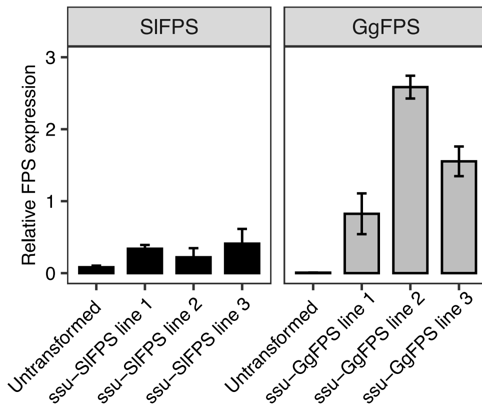

Figure 5. Higher transcript levels of SlIFPS and GgFPS in transgenic tomato plants. Transcript levels of FPS in three independent transgenic T2 lines were analyzed by quantitative real-time PCR (qRT-PCR). Transcript levels of ssu-SlFPS lines were analyzed using SlFPS-specific primers (black bars) and transcript levels of ssu-GgFPS lines were analyzed using GgFPS specific primers (gray bars) with untransformed tomato plants as control. Transcript levels were normalized to Rubisco conjugating enzyme 1 (RCE1). Bars represent mean expression ± SE (n=3). Primers used: SlFPS; 5'-acagatgattctcgtaaatggg-3' (F) and 5'-agatagtcctcggttcagcttc-3'(R). GgFPS; 5'-ggagaggtacaaagccatcg-3'(F) and 5'-ccttactgtcgatcccaacc-3'(R). RCE1; 5'-gattctctctcatcaatcattcg-3'(F) and 5'-tttggggacatcttcggatgaa-3'(R).

Functional Validation of Trichome Engineering

Redirecting FPS to the Plastid Alters Terpenoid Production in Glandular Trichomes

Successful metabolic engineering largely depends on available precursor molecules to be utilized. Limited availability of precursor molecules makes it hard to produce an altered metabolic trait in transgenic plants. Also, metabolic fluxes between cell compartments in plants are only poorly understood (Rodriguez-Concepcion & Boronat, 2015). To see if the enhanced expression levels of FPS also result in an increase of the FPP pool, we measured FPP levels from trichomes of 4-week-old ssu-GgFPS (line 3) plants. The trichome content was extracted using methanol:water (1:3, v/v). Next, the extracts were purified on a CHROMABOND HR-XA column and quantified on LC–MS/MS. For a full description of the method, we refer to Nagel, Gershenzon, and Schmidt (2012). Fig. 6A shows the relative amounts of FPP detected in the trichomes of untransformed plants and ssu-GgFPS transgenics (n ¼ 3). In the transgenic lines, the relative amount of FPP increased by ~3.5-fold compared to untransformed. So, targeting GgFPS to the plastid did not only lead to elevated transcripts but also resulted in increased production of FPP.

We further analyzed the trichomes content of ssu-GgFPS transgenic plants by gas chromatography–mass spectrometry (GC–MS). In short, we isolated trichomes from stems of 4 weeks old tomato plants by shortly vortexing stem pieces frozen in liquid nitrogen in a 50-mL Greiner tube. Volatiles were extracted from frozen isolated trichomes with hexane spiked with 5 ng mL-1 benzyl acetate. Compounds were separated on a capillary DB-5

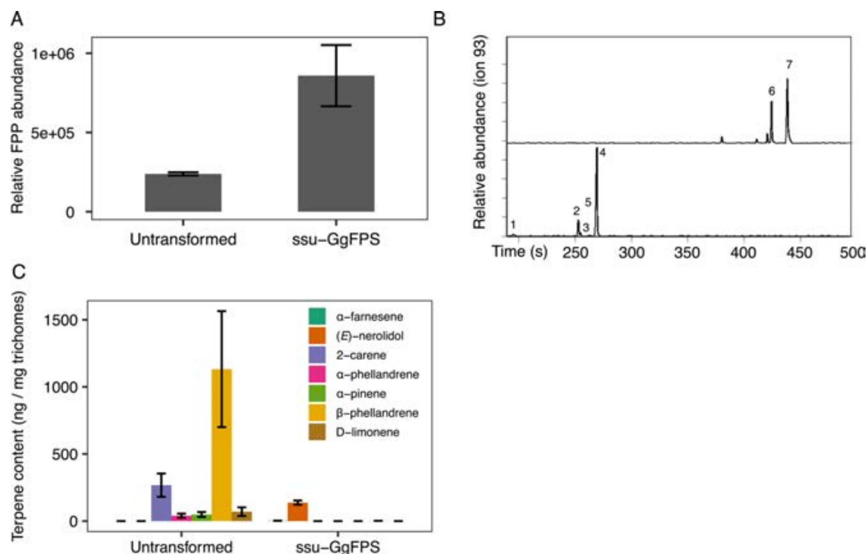

Figure 6. Modified terpenoid metabolism in the trichomes of ssu-GgFPS transformed plants. (**A**) Measurements of sesquiterpene precursor farnesyl diphosphate (FPP). Bars represent mean values ± SE (n=3). (**B**) GC-MS chromatograms of trichome extracts of untransformed (lower chromatogram) and ssu-GgFPS plants (upper chromatogram). Peaks: (1) α-pinene, (2) 2-carene, (3) α-phellandrene, (4) β-phellandrene, (5) D-limonene, (6) α-farnesene, and (7) (E)-nerolidol. (**C**) Quantified amounts of terpenes per mg trichomes. Bars represent mean values ± SE (n=3).

column (10 mx 180 µm, film thickness 0.18 µm; Hewlett-Packard) and mass spectra of eluting compounds were collected on a time-of-flight mass spectrometer (Leco Pegasus III). For a detailed description of the method, see Bleeker et al. (2009). We quantified the peak areas using the available terpene standards and normalized this to the fresh trichome weight.

Strikingly, we observed that terpene production shifted from mainly high levels of monoterpenes in untransformed trichomes, to only low levels of sesquiterpenes in trichomes of ssu-GgFPS transgenic lines (Fig. 6B). Compounds detected in untransformed plant samples were identified as α-pinene (1), 2-carene (2), α-phellandrene (3), β-phellandrene (4), and D-limonene (5). In the transgenic lines, two additional terpenes were detected in the trichome extract: α-farnesene (6) and (*E*)-nerolidol (7). Quantification showed that the total terpene levels dropped dramatically in ssu-GgFPS lines. As shown in Fig. 6C, only trace amounts of monoterpenes were detected in the extracts of transgenic plants. Among these, the most abundant monoterpene was β-phellandrene (2.48 ng mg^{-1} trichomes, SE ± 0.27, n=3) which is a massive decrease compared to trichomes of untransformed plants (1131.69 ng mg^{-1} trichomes, SE ± 431.93, n=3). While we

could not detect any sesquiterpenes in the extracts of untransformed trichomes, ssu-GgFPS lines produced α-farnesene (2.17 ng mg^{-1} trichomes, SE ± 0.61) and (*E*)-nerolidol (137.42 ng mg^{-1} trichomes, SE ± 16.46) as main products. Although we did not introduce a sesquiterpene synthase in the plastid, additional sesquiterpenes were observed in the transgenic lines. We therefore assume there is a plastidial terpene synthase that can utilize FPP. In tomato, the plastidial MTS1 (TPS5) normally synthesizes the monoterpene (R)-linalool from GPP. However, in vitro experiments showed that MTS1 can utilize FPP resulting in the production of (*E*)-nerolidol (van Schie et al., 2007), providing an explanation for the production of (*E*)-nerolidol in the transgenic plants with FPS targeted to the plastid. The drastic depletion of monoterpene synthesis in the transgenic plants remains unexplained.

Biological Relevance of Changing Specialized Metabolites in Trichomes

Plant volatiles can function as a cue in insects' host preference. The whitefly *Bemisia tabaci* is an invasive generalist that constitutes a global problem in vegetable agriculture, as it serves as a vector for the geminiviruses causing crop damages recorded up to 95% (Polston & Anderson, 1997). Changing the volatile blend of commercial tomato, by the introduction of a novel sesquiterpene synthase from a wild species, has shown to improve repellence to *B. tabaci* (Bleeker et al., 2009, 2012). Here it was investigated if the observed change in terpenoid production of the ssu-GgFPS lines would also lead to improved repellence. The preference response of *B. tabaci* towards wild-type tomatoes and the transgenic line with an altered volatile blend was assessed in a free-choice bioassay. In this assay, we placed three untransformed, and one transgenic plant of the same age and size in the corners of a 1 x 1 m^2 (Fig. 7A). A total of 150 whiteflies were released in the middle of the setup and recaptured from the plants after 20 min. In repetitions (n=4) plants were rotated to avoid positional effects. The experiment was performed a total of four times with different ssu-GgFPS plants. *B. tabaci* displayed significantly less preference for ssu-GgFPS plants compared to untransformed plants (Fig. 7B). There was no difference in the percentage of recaptured flies between untransformed lines (26%, 29%, and 32%, $p > 0.05$). In contrast, significantly fewer whiteflies were recaptured from ssu-GgFPS transgenic plants (14%, $p < 0.01$). Thus, altering the production of terpenes in the trichomes can affect the plant's attractiveness to whiteflies. Whiteflies and other insects use, among other cues, semiochemical signals to detect their host plant (Birkett et al., 2004; Bleeker et al., 2009). The major loss of monoterpene production observed in ssu-GgFPS plants therefore might have resulted in a loss of cues for *B. tabaci* to select its host plant. On the other hand, ssu-GgFPS lines produce (a low amount of) (*E*)-nerolidol that has been previously identified to play a role in the indirect defence of multiple plant species against herbivores (Bouwmeester et al., 1999; Degenhardt & Gershenzon, 2000; Kant et al., 2004; Kappers et al., 2005; Tholl et al., 2011). Also, the emission of various sesquiterpenes including 7-epizingiberene, (*R*)-curcumene, and (*E*, *E*)-α-farnesene have been shown to act as a repellent to insects and small herbivores (Bhatia, Maisnam, Jain, Sharma, & Bhattacharya, 2015; Bleeker, Diergaarde, et al., 2011; Bleeker, Spyropoulou, et al., 2011; Lopez et al., 2013). Despite the fact that nerolidol levels in the transgenic lines are low, we cannot exclude that emission of this novel compound played a role in the decreased attractiveness of plants expressing FPS in the plastid.

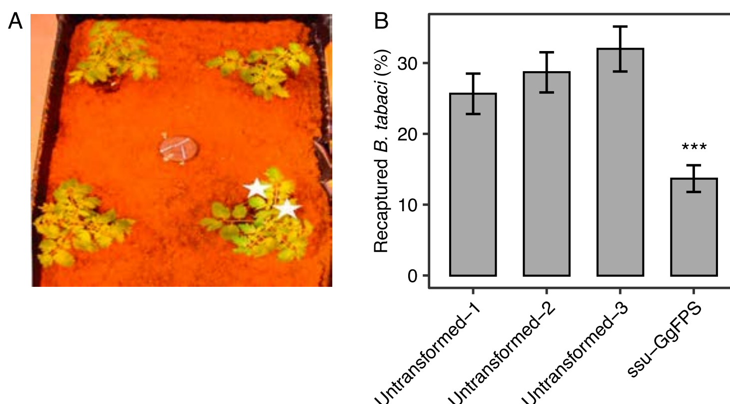

Figure 7. ssu-GgFPS tomato plants are less attractive to whiteflies. (**A**) Experimental setup: 150 whiteflies (*Bemisia tabaci*) were released in the middle of a 1 1 m2 with in three corners untransformed plants and in the fourth corner a ssu-GgFPS transgenic plant (indicated with stars). After 20 min, the whiteflies were recaptured from the plants and counted. After each assay, plants were rotated to exclude any positional effect. (**B**) Percentage of whiteflies that were recaptured on each plant. Significantly less whiteflies were recaptured on ssu-GgFPS transgenic plants (ANOVA: $p < 0.01$). Bars represent mean percentage of recaptured whiteflies ± SE ($n = 12$).

Summary

In this chapter, we described a generic outline for metabolic engineering of glandular trichomes of plants by presenting a case study of tomato glandular trichomes. What is first and foremost needed for the plant of choice are one or more glandular trichome-specific promoters as any specific engineering of the glandular trichome will, in most cases, not affect normal plant development. Table 1 presents trichome-specific promoters that are readily available but screening of promoters can be rapidly done by particle bombardment of the trichomes of choice. Trichome databases (e.g. www.planttrichome.org/trichomedb) are accessible and since the costs for RNAseq are rapidly coming down, transcriptomes of the trichomes of any plant are now within reach. Upon selecting putative trichome-specific genes, using for instance qRT-PCR on different tissues and organs, the specificity of the corresponding promoters still needs to be tested. Particle bombardment is a very fast and effective tool for this since expression of the reporter genes can be observed within days. In particular, as depicted in Fig. 3, GUS activity can be visualized with X-Gluc as substrate in trichomes. Since the gold particles not only hit the trichomes but also the epidermal cells and the mesophyll cells below, any activity of the promoters in these cells would directly be visible. In addition, one can also use other tissues and organs for particle bombardment to determine if expression is absent using a constitutive promoter as a positive control. Still transgenic plants with the promoter driving the reporter gene (Fig. 4) are necessary to know if the promoter is also expressed in other cells, if any, beside the glandular trichomes. It is not necessary to mention that knowledge of the metabolism to be engineered is paramount. Trichomes are amenable to engineering, but even with extensive knowledge of the terpenoid biosynthesis pathways, the outcome may be unpredictable. By simply locating FPS in the plastid, we almost completely eliminated monoterpene biosynthesis

(Fig. 6B and C). The mechanisms involved remain to be resolved. We did measure an increase in FPP levels (Fig. 6A) in trichomes expressing FPS in their plastids, but we do not know how this influenced, for instance, NDP levels, the substrate for the most abundant monoterpene, β-phellandrene. Our results suggest that high FPP levels inhibited NDPS, similarly as NPP inhibits GGPPS (Gutensohn et al., 2014). In the cytosol, FPP was thought to serve as a regulator of carbon flux in the cytosolic MVA pathway via feedback inhibition of HMGR (Closa et al., 2010). In addition, the plastidial linalool synthase that, in vitro, also accepts FPP as a substrate now produced the sesquiterpene nerolidol. This indicates that additional targeting of a sesquiterpene synthase to the plastid would result in production of higher levels of the desired sesquiterpenes. Modifiers of these sesquiterpenes, such as P450s, could then be
added as well, under control of trichome-specific promoters.

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Author contributions to the article

RS and PB conceived the study. RK, PB and RS wrote the manuscript. RK and PB did transcript analysis, GC-MS analysis and whitefly bioassays. PB, PD, ES performed cloning of the constructs. JX and AR performed stable transformation and particle bombardment of the reporter lines. IO performed the stable transformation of the ssu-FPS lines. RN and AS did the IPP measurements. MB revised the manuscript.